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# Induction of rat facial nerve regeneration by functional collagen scaffolds

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ABSTRACT

Nerve conduit provides a promising strategy for nerve regeneration, and the proper microenvironment in the lumen could improve the regeneration. Our previous work had demonstrated that linear ordered collagen scaffold (LOCS) could effectively guide the oriented growth of axons. Laminin is known as an important nerve growth promoting factor and can facilitate the growth cone formation. In addition, ciliary neurotrophic factor (CNTF) and brain-derived neurotrophic factor (BDNF) can effectively improve the nerve regeneration after nerve injuries. However, in practice, diffusion caused by the body fluids is the major obstacle in their applications. To retain CNTF or BDNF on the scaffolds, we produced collagen binding CNTF (CBD-CNTF), collagen binding BDNF (CBD-BDNF) and laminin binding CNTF (LBD-CNTF), laminin binding BDNF (LBD-BDNF) respectively. In this work, we developed laminin modified LOCS fibers (L × LOCS) by chemical cross-linking LOCS fibers with laminin. Collagen binding or laminin binding neurotrophic factors were combined with LOCS or L × LOCS, and then filled them into the collagen nerve conduit. They were found to guide the ordered growth of axons, and improve the nerve functional recovery in the rat facial nerve transection model. The combination of CNTF and BDNF greatly enhanced the facial nerve regeneration and functional recovery.


## 1. Introduction

Facial nerve injuries affect many people and the functional recovery remains clinically challenging. To restore the motor and sensory functions, the transected nerve fibers need to regenerate across and beyond the injury site and form connections with the target tissue. For extensive nerve lesions, a nerve graft is needed for bridging the gap. Autologous nerve graft has been considered as the golden standard [1,2]. However, donor tissue availability, extra incisions, sacrifice of the donor nerve and danger of neuroma forming are still the major concerning factors [3].

Biomaterials provide promising alternative for nerve injury repair [4–6]. An ideal biomaterial should possess the following functionalities: firstly, it should have good tissue compatibility and possess sufficient mechanical strength for sustaining nerve regeneration [7]; secondly, it should give the oriented guidance for the regenerated nerve fibers [7–9]; thirdly, the biomaterial should have bio-activities that can efficiently improve the regeneration [7,10,11].

Nerve conduits are commonly used to bridge the transected nerve stumps and sustain nerve regeneration [12–18]. Collagen has been widely utilized for its favorable biocompatibility, biodegradability and weak immunogenic reactions [19]. In this study, a collagen nerve conduit was produced to provide a "regenerated room" for the injured nerve. Collagen and laminin are two main extracellular matrixes in nerve system [20,21], the linear ordered collagen scaffolds (LOCS) and the laminin modified linear ordered scaffolds (L × LOCS) were both proper guiding materials for nerve regeneration [22–24]. To guide the regeneration of the nerve fibers, LOCS and L × LOCS were applied in the lumen of the tube respectively.

Neurotrophic factors play important roles in nerve regeneration [25–33]. However their clinical applications had been limited by their diffusion in the body. Therefore, it requires periodic injections. To solve these problems, a specific collagen or laminin binding domain was fused to the growth factor [24,34–38]. CNTF and BDNF are two important factors in nerve regeneration. It had been reported that BDNF had strong effects on neuronal survival while CNTF was effective in stimulating neurite outgrowth [39]. After the nerve injury, axons will be destroyed and the neurons will undergo apoptosis. Thus, co-delivery of CNTF and BDNF to the injury site may provide better effect for the regeneration.

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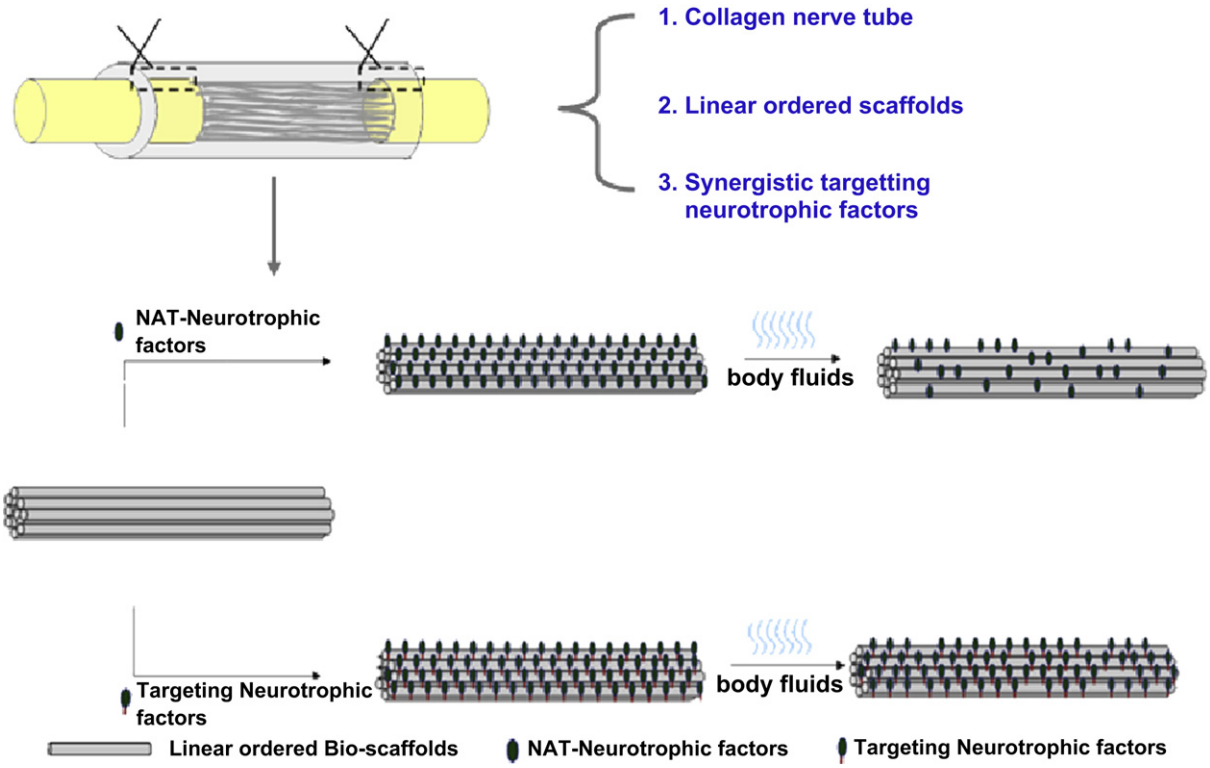


**Fig. 1.** Functional biomaterials designed for nerve regeneration. The functional biomaterials consisted of the nerve conduit, linear ordered scaffolds, and the collagen or laminin binding neurotrophic factors. The nerve conduit provided an independent chamber for nerve regeneration, linear ordered scaffolds guide axonal growth, and the collagen or laminin binding neurotrophic factors could specifically bind to the scaffolds, promoting nerve regeneration.

In the present study, a nerve repair device was designed: (1) the collagen nerve conduit was used to bridge the transected nerve stumps, providing a "regeneration room" for the injured nerve; (2) the liner ordered biomaterials LOCS or L × LOCS in the conduit lumen would guide the axonal regeneration; (3) the recombinant collagen or laminin binding factors CBD-CNTF, CBD-BDNF, LBD-CNTF and LBD-BDNF can bind to the relative scaffolds. And the synergetic effect of CNTF and BDNF may significantly enhance the

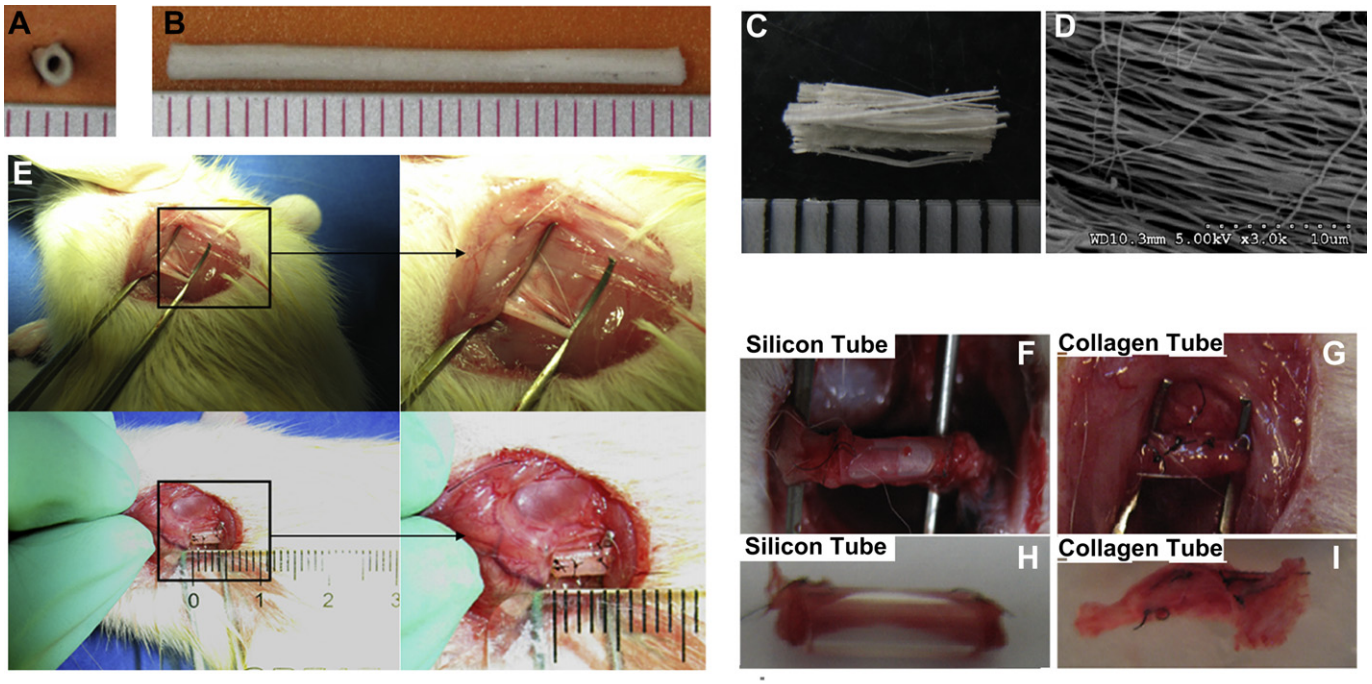


**Fig. 2.** The biomaterials, facial nerve injury model and the regenerated nerves after different treatment (A, B) The transverse and the longitudinal photo of the collagen nerve tube. (C) Photo of the linear ordered scaffolds. (D) The microstructure of linear ordered scaffolds exhibited by the SEM image.(E)Photos of the surgery procedures. The truck of the facial nerve was exposed. Then the transected nerve was bridged by the functional biomaterials.(F-I) Photos of the nerves regenerated in the collagen tube and the silicon tube.

nerve function recovery (Fig. 1). The devices were evaluated in a 4 mm rat facial nerve trunk gap model.

## 2. Materials and Methods

### 2.1. Preparation of collagen nerve conduit

Collagen membrane and collagen solution were used to make up collagen nerve conduit. The collagen membrane was made from bovine collagen by freeze-drying. Collagen solution (15 mg/ml, in 0.5 M acetic acid) was evenly placed into the scrolled collagen membrane and air-dried. The collagen conduit was then treated with cross-linking solution (30 mM EDC and 10 mM NHS in 50 mM MES solution, pH 5.5) overnight. After washing with $NaH_2PO_4$ (0.1 M) and distilled water, molds were removed from the collagen conduits after freeze-drying (Fig. 2A,B).

### 2.2. Preparation of LOCS and laminin coated LOCS (L × LOCS)

LOCS was prepared from bovine aponeurosis as described previously [40]. Briefly, fresh white aponeurosis were separated from muscles and cleaned with cold distilled water. After removing the adjunctive tissues, including the residual muscles, connective tissues and fats, the aponeurosis were treated with 1% tri (n-butyl) phosphate (TnBP) (Aldrich, Munich, Germany) in 50 mM Tris–Cl buffer (pH 8.0) for 48 h at 4 °C to remove the cellular components. Then the soluble proteins and cellular elements were further extracted in 50 mM Tris–Cl buffer (pH 8.0, 1 M NaCl) at 4 °C for 48 h. Subsequently, the samples were soaked in distilled water and rinsed repeatedly to remove the residual agents completely. The collagen filaments (LOCS) were separated from processed aponeurosis and freeze-dried (Fig. 2C). The microstructure of LOCS was observed by scanning electron microscope (Fig. 2D).

L × LOCS was prepared as described before [24]. Briefly, a bunch of LOCS fibers (4 mm × 1 mm × 1 mm) was soaked in 50 mM MES solution (pH 5.5) with NHS (1.2 mg/ml; Sigma Aldrich) and EDC (2 mg/ml; Sigma–Aldrich), incubated at 37 °C for 15 min, then loaded with 10 μL laminin (100 μg/ml) (Sigma Aldrich), and incubated at 37 °C for 3 h.

### 2.3. Production of CBD-CNTF, LBD-CNTF, CBD-BDNF and LBD-BDNF

The recombinant proteins CBD-CNTF, LBD-CNTF, CBD-BDNF and LBD-BDNF were expressed, purified, refolded and identified as described previously [22,24].

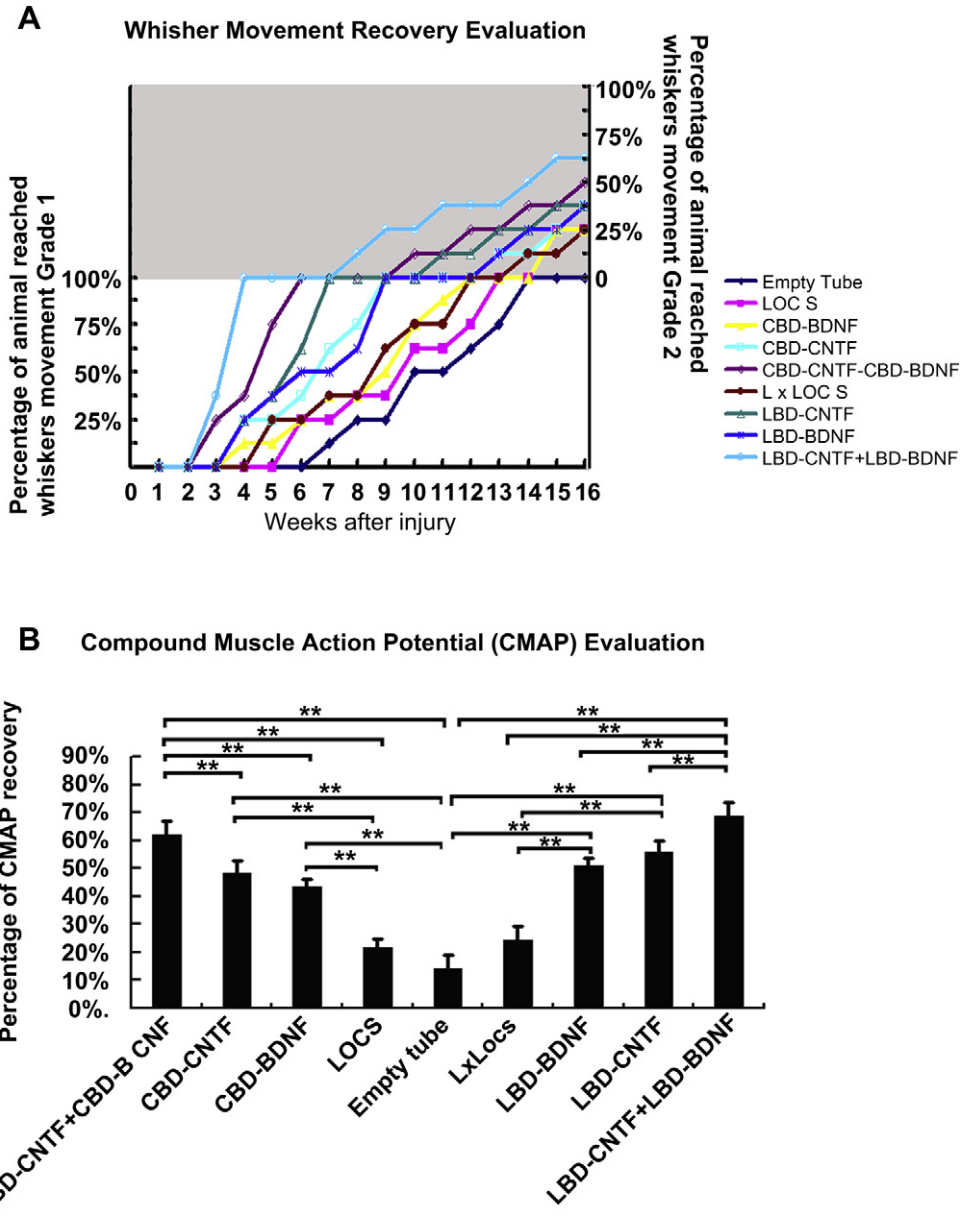


**Fig. 3.** Functional recovery of the facial nerve. (A) The whisker movement recovery was evaluated at different time points after injury. (B) Electrophysiological evaluation. The compound muscle action potential was measured at 16 weeks after nerve transaction. $n = 8$, $^{*}P < 0.05$, $^{**}P < 0.01$.

### 2.4. Surgical procedures of facial nerve transection and grouping

Experiments were performed in accordance with Guide for the Care and Use of Laboratory Animals from National Institutes of Health and approved by the Animal Care and Use Committee of Nanjing University.

Female Sprague Dawley rats (200–230 g) were anesthetized by an intraperitoneal injection of sodium pentobarbital (40 mg/kg body weight), after sterilization, an incision was made subcutaneously to expose the trunk of the right facial nerve. A segment of 3 mm nerve was removed by sharp microsurgery scissors. The proximal and distal nerve stumps were bridged by a 5 mm long collagen nerve conduit, leaving 4 mm gap between the two ends. Two 8/0 monofilament nylon interrupted sutures were applied at each side of the anastomosis. Then the muscle layers and the skin were sutured separately. The procedure of the surgery was showed in Fig. 2E. All the animals were divided into 9 groups with different treatments. 1, the group of empty tube: the transected nerve was bridged by an empty collagen nerve conduit; 2, the group of LOCS: the transected nerve was bridged by the collagen nerve conduit filled with a bunch of LOCS fibers (4 mm long and 1 mm diameter); 3, the group of CBD-CNTF: the bridged collagen nerve conduit were filled with LOCS fibers (4 mm long and 1 mm diameter) loaded with 0.25 nmol CBD-CNTF; 4, the group of CBD-BDNF: the bridged collagen nerve conduit were filled with LOCS fibers (4 mm long and 1 mm diameter) loaded with 0.25 nmol CBD-BDNF; 5, the group of CBD-CNTF + CBD-BDNF: the bridged collagen nerve conduit were filled with LOCS fibers (4 mm long and 1 mm diameter) loaded with 0.125 nmol CBD-CNTF and 0.125 nmol CBD-BDNF; 6, the group of LOCS × L: the bridged collagen nerve conduit were filled with L × LOCS fibers (4 mm long and 1 mm diameter); 7, the group of LBD-CNTF: the bridged tubes were filled with L × LOCS fibers (4 mm long and 1 mm diameter) carrying 0.25 nmol LBD-CNTF; 8, the group of LBD-BDNF: the bridged collagen nerve conduit were filled with L × LOCS fibers (4 mm long and 1 mm diameter) carrying 0.25 nmol LBD-BDNF; 9, the group of LBD-CNTF + LBD-BDNF: the bridged collagen nerve conduit were filled with L × LOCS fibers (4 mm long and 1 mm diameter) carrying 0.125 nmol LBD-CNTF +0.125 nmol LBD-BDNF. All the animals were kept under standardized laboratory conditions in an air-conditioned room with free access to food and water. The rats were sacrificed by cervical dislocation at different time points,

### 2.5. Nerve functional assessment

Vibrissal whisking evaluation and electrophysiology examination were performed to evaluate the facial nerve function.

As previously described, whisker movements were divided into four different categories with scores ranging from 0 to 3 (0, no whisker movement; 1, slight whisker movement; 2, slow movement; 3, rapid movement undistinguishable from the contralateral uninjured side) [41]. The whisker movement evaluation was performed in every week after the injury. To avoid subjective bias, double-blind assay was performed.

As for electrophysiological tests, the regenerated facial nerve was re-exposed under general anesthesia at Week 16 post-surgery. The electrophysiological test

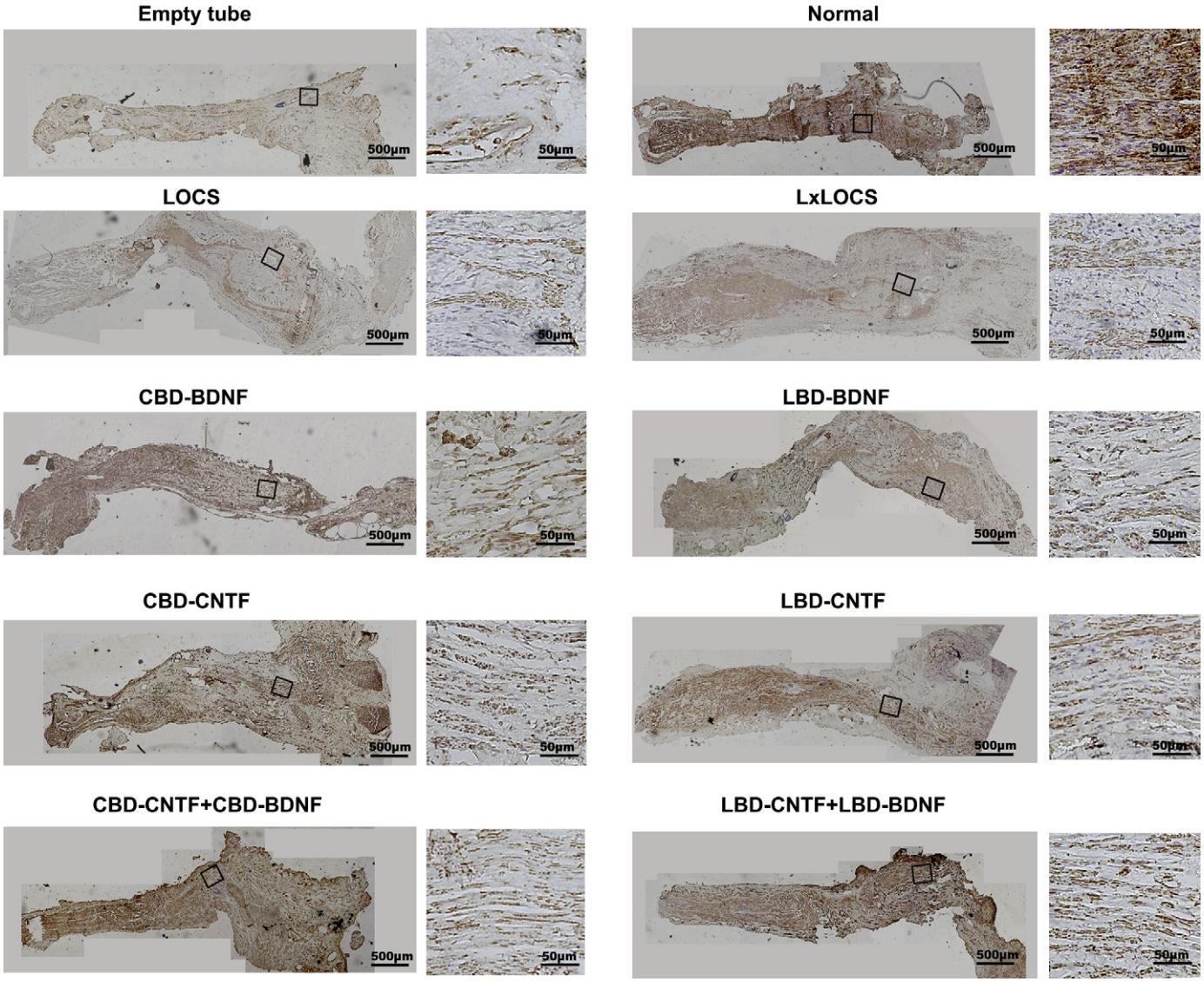


**Fig. 4.** Longitudinal sections of the regenerated nerve stained with NF antibody. The magnification of a selected area in the distal end of the newborn nerve was showed in the right panel.

was evaluated by an electromyography system (RM6240, Chengdu, China). The stimulating electrode was placed in the proximal end of the facial nerve trunk, the record electrode was insert into the ipsilateral orbicularis oris muscle, the reference electrode was insert into the ipsilateral fore limb subcutaneously. The compound muscle action potentials (CMAPs) in both sides were measured. The ratio of CMAPs in the injured sides compared with normal sides was used to evaluate the facial nerve functional recovery.

### 2.6. Histological analysis

The histological assessments were performed at week 6 and week 16 after operation. The regenerated nerves were isolated and fixed them in 4% (vol/vol) formaldehyde for 48 h. Then the segments were embedded in paraffin. 5 μm sections were cut from each segment and examined by immunohistochemistry using antibodies to neurofilament (anti-NF, 1:1000 dilution; Abcam) and S100 (1:2000

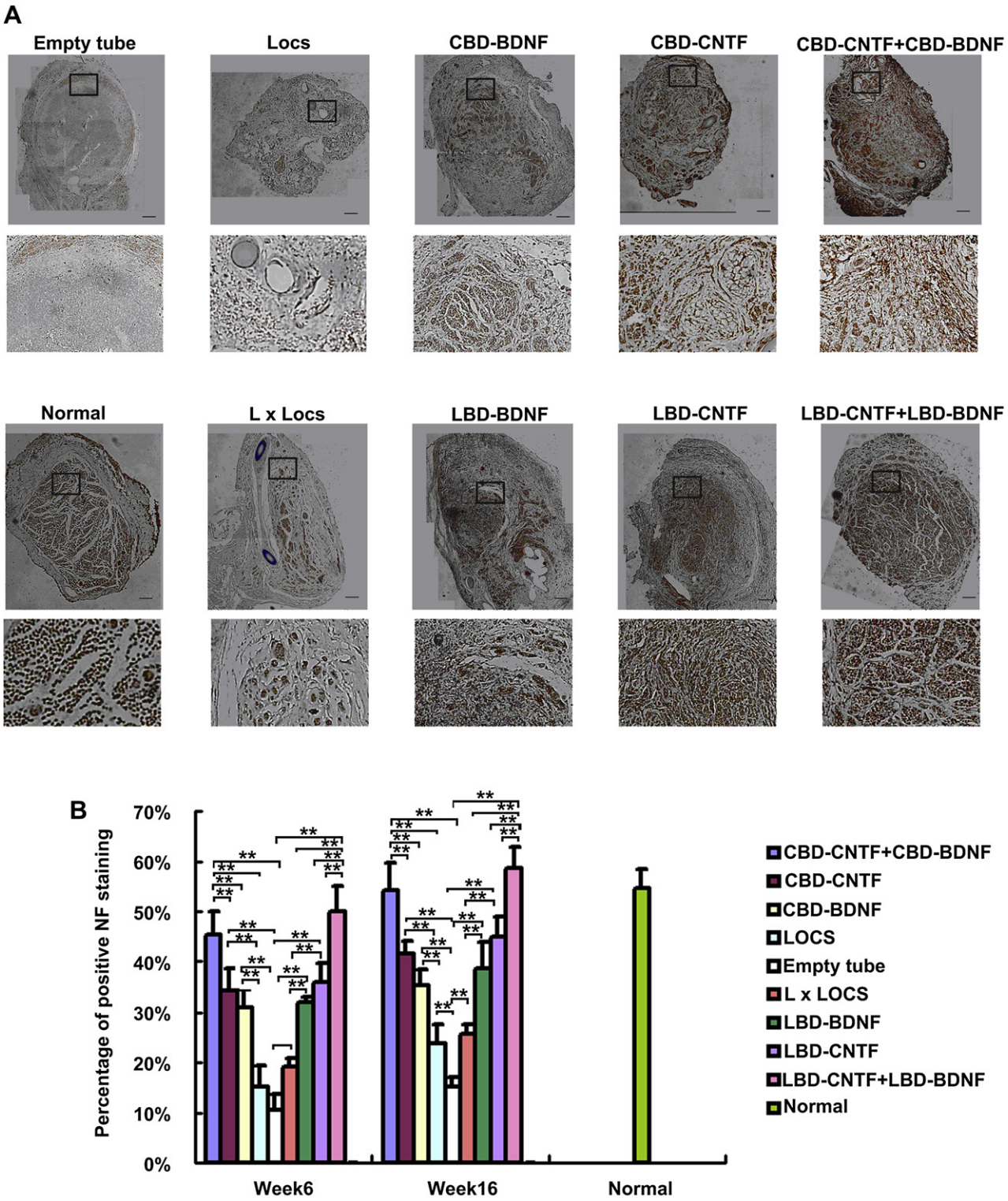


**Fig. 5.** Transverse sections of the distal end of the regenerated nerve stained with NF antibody. (A) Immunostaining with anti-NF antibody of the regenerated nerves 16 weeks after injury. A magnification of a selected area was showed below. (B) The statistical analysis of neurofilament-positive area in transverse section of each group. $n = 8$, $^{*}P < 0.05$, $^{**}P < 0.01$.

dilution; Sigma) respectively. Using Image-Pro Plus software (Media Cybernetics), we quantified the percentage of neurofilament-positive and S100-positive area (positive staining area/total area) by selecting at least 3 randomly fields per sample at 200× magnification. The remyelination of the regenerated nerves was observed by transmission electron microscope. The number, diameter and the thickness of the newborn myelin sheath were evaluated by Image-Pro Plus software (Media Cybernetics).

#### 2.6.1. Statistical analysis

The SPSS 13.0 was used for all statistical analysis. Multiple comparisons were performed with one-way ANOVA (S-NK). *P* values <0.05 was considered significant.

## 3. Results

### 3.1. Collagen nerve conduit testing

The collagen tube was tested in an extreme facial nerve injury model as described. Fig. 2F–I showed the nerve tissues regenerated in the silicon tube and the collagen tube. Compared with the silicon tube, the collagen tube also could successfully sustain the nerve regeneration without collapsing. Moreover, the collagen nerve tube was degradable and showed better biocompatibility in vivo.

### 3.2. Functional recovery of the injured facial nerve

Facial nerve controls the movements of the facial muscles. The most obvious phenotype is the paralysis of whisker movements after transaction of the facial nerve trunk. The recovery of the whisker movements was used to reflect the reconstruction of the nerve conduction function. As showed in Fig. 3A, all the animals showed complete facial paralyzed at the injury side immediately after the surgery. 3 weeks after the injury, there were about 25%–40% animals in LBD-CNTF + LBD-BDNF group and CBD-CNTF + CBD-BDNF group showed slight whisker movement recovery (Grade 1). At Week 16, the ratio of the animals that have reached Grade 2 had been accessing to 70% and 50% in the two groups respectively. In the groups of CBD-CNTF, CBD-BDNF, LBD-CNTF and LBD-BDNF, there were about 10%–25% animals began to showed Grade 1 recovery at Week 4, and about 30%–40% animals reached Grade 2 recovery at Week 16. While in the groups of LOCS and L × LOCS, 10–25% animals began to showed slight recovery (Grade1) at 5–6 weeks after injury, and only 25% animals reached Grade 2 recovery at Week 16. The recovery in the empty tube group was slower and limited, only about 10% animal began to show slight recovery at Week 7, and no animal reached Grade 2 recovery till Week 16.

The electrophysiological index compound muscle action potentials (CMAPs) at both the injury side and the healthy side were measured at Week 16. The ratio of CMAPs in the injured sides compared with normal sides was used to evaluate the facial nerve functional recovery (Fig. 3B). Compared with the empty tube group, CMAP was significantly restored in the groups of LBD-CNTF + LBD-BDNF, CBD-CNTF + CBD + BDNF, LBD-CNTF, LBD-BDNF, CBD-CNTF, CBD-BDNF ($n = 8$, $p < 0.01$). CMAP restoration of group LBD-CNTF + LBD-BDNF and CBD-CNTF + CBD + BDNF were up to 67 ± 5.1% and 60 ± 4.8% respectively, which were significantly higher than other groups.

### 3.3. Histological analysis

The longitudinal and transversal sections of the newborn nerve in each group were stained with anti-NF antibody for evaluating the axon regeneration (Figs. 4 and 5). The results showed that, compared with the other groups, the density of regenerated axons in the distal end of the newborn nerve was significant higher in the groups of LBD-CNTF + LBD-BDNF, CBD-CNTF + CBD + BDNF, ($n = 8$, $p < 0.01$), and the axonal arrangement in these two groups were

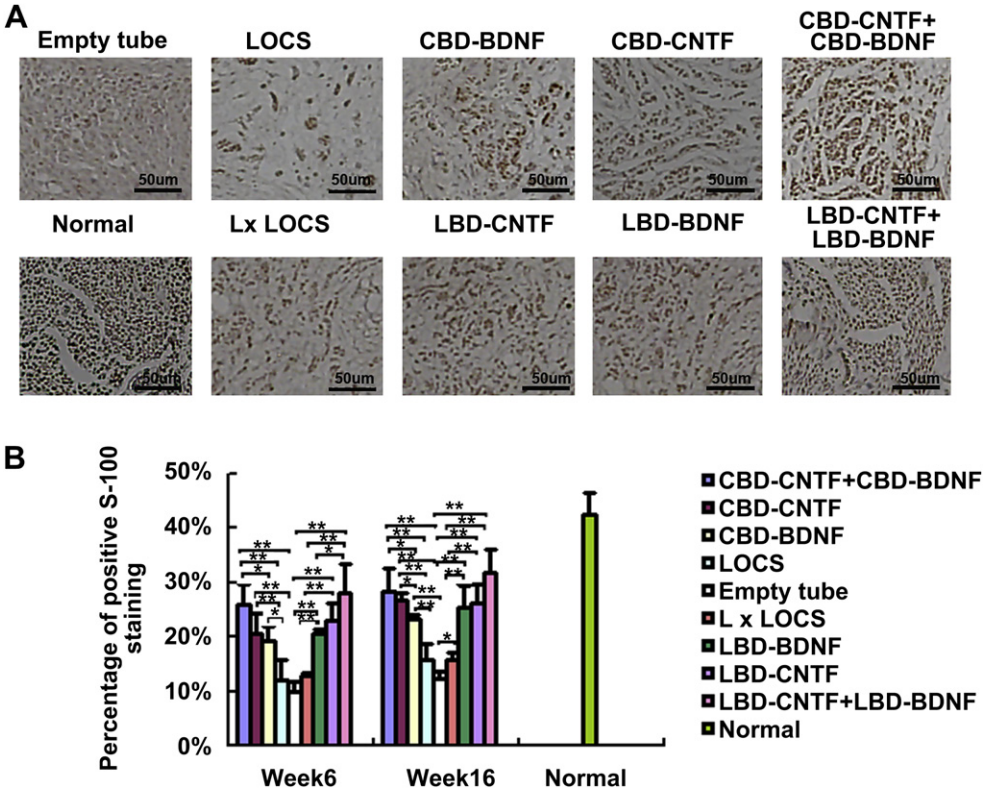


**Fig. 6.** Schwann cell regeneration of the regenerated nerve. (A) Immunostaining of the Schwann cell marker S100 in the transverse sections of regenerated nerve in each group. (B) The statistical analysis of S100-positive area in transverse section of each group. $n = 8$, $^{*}P < 0.05$, $^{**}P < 0.01$.

more similar with that of the normal nerve (Fig. 4). However, only a few nerve fibers grew along the wall of the conduit and the arrangement of the regenerated nerve fiber was much more disordered in the empty tube.

The regeneration and migration of Schwann cells played key roles for the successful axonal regeneration over the extreme transected nerve gap. As it showed in Fig. 6, the groups of LBD-CNTF + LBD-BDNF and CBD-CNTF + CBD + BDNF exhibited the best regeneration of the Schwann cell by showing their S-100 positive staining area percentage, which were closed to normal level at Week 16 after injury.

### 3.4. Remyelination analysis

Remyelination was another important process of nerve functional recovery. The regenerated myelin sheaths were observed by transmission electron microscope (TEM), and the number, diameter and the thickness of the regenerated myelin sheaths were evaluated (Fig. 7).

The regenerated myelin sheaths are smaller and thinner in all the groups compared with the normal myelin sheaths. The quantity, diameter and thickness of the myelin sheaths in the empty tube group were much lower than those in other groups. The groups

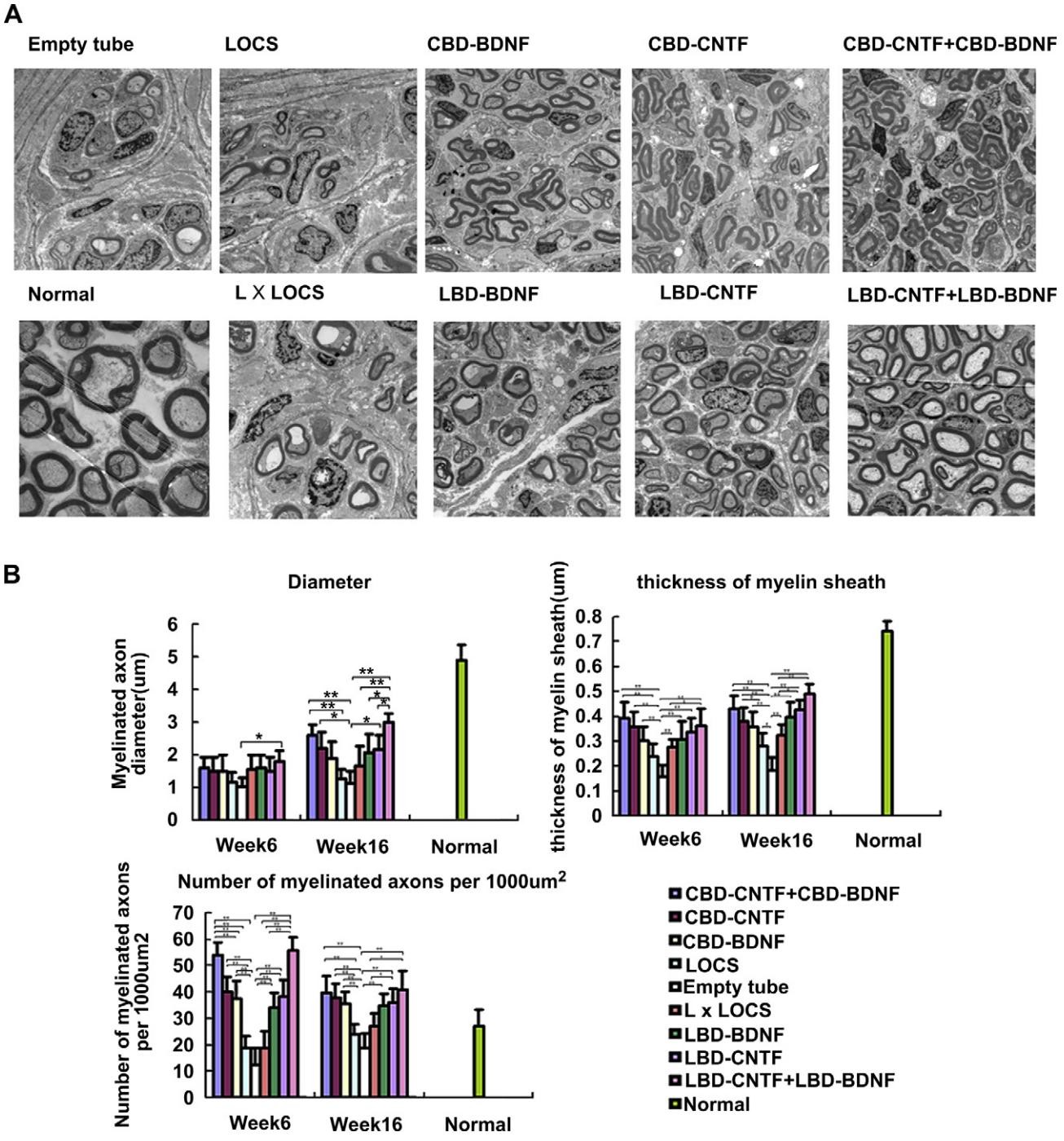


**Fig. 7.** Remyelination of the regenerated nerve. (A) Photos of transmission electron micrographs of regenerated nerve at 16 weeks after injury. (B) The statistical analysis of the myelinated axons diameter, myelinated axons number and thickness of myelin sheath. $n = 8$, $^{*}P < 0.05$, $^{**}P < 0.01$.

of LCOS and L × LOCS also showed limited remyelination. But in the groups of LBD-CNTF + LBD-BDNF, CBD-CNTF + CBD + BDNF, LBD-CNTF, LBD-BDNF, CBD-CNTF, CBD-BDNF, the regeneration of myelin sheaths had been significantly improved. Especially in the groups of LBD-CNTF + LBD-BDNF and CBD-CNTF + CBD + BDNF, the newborn nerves showed the best remyelination, the quantity and quality of the regenerated myelin sheaths were significantly higher than those in other groups (Fig. 7, $n = 8$, $p < 0.05$).

## 4. Discussion

In the present study, we had produced a biodegradable collagen nerve conduit and performed the synergistic application of the recombinant collagen or laminin binding CNTF and BDNF in the facial nerve repair. The collagen or laminin binding proteins presented higher binding ability and sustained release kinetics at the injury site. Though BDNF and CNTF works through different signal mechanism, "cross-talk" exists in the downstream of the pathways induced by the two factors. For example, CNTF can activate STAT3, Ras/Raf/MAP/ERK Kinase/ERK/p90RSK, and PI3K/Akt pathways [42–44], while the downstream of BDNF signaling includs Ras/MAPK and PI3K/Akt [45,46]. The "overlapping" and "differences" in the mechanisms of CNTF and BDNF pathway may amplify the therapeutic efficiency. This may explain why the nerve regeneration and functional recovery were significantly improved in the groups of CBD-CNTF + CBD-BDNF and LBD-CNTF + LBD-BDNF in our experiments.

After the injury, the groups of CBD-CNTF + CBD-BDNF and LBD-CNTF + LBD-BDNF exhibited the earliest locomotion recovery, and most of the rats in the two groups had achieved Grade 2 recovery at Week 16 after the injury. The electrophysiology examination also showed that the nerve conduct restoration were significantly higher in the double-factor treated groups than that in the relative single factor control groups. Consisting with the results of the functional recovery, animals of the two double-factor treated groups exhibited the best regeneration of axons, Schwann cells, and myelin sheaths.

Though the nerve regeneration showed no significant differences between the groups of the laminin modified linear ordered scaffolds (L × LOCS) and the groups of collagen linear ordered scaffolds (LOCS), the average values of some tested indexes were higher in the LBD-CNTF + LBD-BDNF group than the CBD-CNTF + CBD-BDNF group. In addition, the regenerated myelin sheaths in the groups treated with laminin modified scaffolds were more close to the normal control (Fig. 7A). Laminin has been shown to support the nerve regeneration, promoting neurite growth and facilitating the growth cone formation. This may explain why L × LOCS trended to be superior to LOCS. In the extreme nerve injury model (the removal nearly the total length of the facial nerve trunk), simple laminin and linear ordered scaffolds treatment were not enough to sustain the nerve functional recovery. The application of neurotrophic factors played more important roles for the nerve regeneration, and the combination of CNTF and BDNF showed the significant regeneration effect compared with single factors. Thus the co-application of CNTF and BDNF is a more efficient therapy strategy for the extreme nerve injuries.

## 5. Conclusion

We have produced a biodegradable collagen conduit together with linear order scaffolds LOCS or L × LOCS to guide the ordered regeneration of nerve fibers. Collagen or laminin binding CNTF and BDNF were co-delivered to exert synergistic effect on nerve regeneration. Both the collagen and laminin biomaterials could guide the ordered growth of axons, and improve the nerve functional recovery effectively in the extreme facial nerve injury mode, especially with the co-application of collagen or laminin binding CNTF and BDNF.

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