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Chitosan conduits combined with nerve growth factor microspheres repair facial nerve defects

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Research Highlights

- (1) Microspheres for sustained release of nerve growth factor were prepared by compound method, and the sustained *in vitro* release of active nerve growth factor lasted for at least 90 days.
- (2) Microspheres were implanted into chitosan conduits to repair 10-mm defects on the buccal branches of the facial nerve in rabbits. Results showed that the muscular atrophy induced by facial nerve defects was attenuated, and the nerve conduction velocity and amplitude were significantly increased.
- (3) Microspheres for sustained release of nerve growth factor in combination with chitosan conduits can improve axon and myelin sheath regeneration of injured facial nerve.
- (4) The combination of nerve growth factor-releasing microspheres and chitosan conduits exhibits superior effects in repairing facial nerve injury compared with nerve growth factor alone.

Abstract

Microspheres containing nerve growth factor for sustained release were prepared by a compound method, and implanted into chitosan conduits to repair 10-mm defects on the right buccal branches of the facial nerve in rabbits. In addition, chitosan conduits combined with nerve growth factor or normal saline, as well as autologous nerve, were used as controls. At 90 days post-surgery, the muscular atrophy on the right upper lip was more evident in the nerve growth factor and normal saline groups than in the nerve growth factor-microspheres and autologous nerve groups. physiological analysis revealed that the nerve conduction velocity and amplitude were significantly higher in the nerve growth factor-microspheres and autologous nerve groups than in the nerve growth factor and normal saline groups. Moreover, histological observation illustrated that the diameter, number, alignment and myelin sheath thickness of myelinated nerves derived from rabbits were higher in the nerve growth factor-microspheres and autologous nerve groups than in the nerve growth factor and normal saline groups. These findings indicate that chitosan nerve conduits bined with microspheres for sustained release of nerve growth factor can significantly improve facial nerve defect repair in rabbits.

Key Words

neural regeneration; peripheral nerve injury; tissue engineering; nerve growth factor; microsphere; facial nerve defect; chitosan; nerve conduit; grants-supported paper; neuroregeneration

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INTRODUCTION

The repair of facial nerve defects is difficult. At present, because end-to-end anastomosis is unable to repair facial nerve defects, autologous nerve grafting remains the gold standard^[1]. However, nerve autografts exhibit some drawbacks, such as the finiteness and denervation of the donor^[2].

For peripheral nerve repair, much effort has been devoted to developing artificial nerve grafts to replace traditional autograft techniques^[3-5]. However, peripheral nerves do have the potential to regenerate after injury. Current strategies to repair damaged axonal pathways in the peripheral nerve system focus on developing bridging scaffolds that guide axonal regeneration across the lesion site. For example, guidance conduits are used to direct regenerating axons towards their targets, minimize the migration of connective tissue into the conduits, and inhibit the formation of axonal growth-inhibiting scar tissue and neuroma^[6-8]. Different synthetic materials, such as silicone, collagen, and chitosan, have been used as nerve guides to bridge nerve gaps^[9-10]. These materials are mainly divided into two groups: non-absorbable and absorbable artificial nerve grafts^[11]. Non-absorbable materials can lead to scarring and fibrosis, and can cause nerve dysfunction if they are maintained at the injury site for a long time; therefore, a second surgical procedure is needed to remove them^[12]. In contrast, absorbable artificial nerve grafts potentially prevent these problems^[13].

Chitosan nerve conduits are absorbable and have some distinct properties when compared with other absorbable conduits, such as low cost, natural abundance, biocompatibility and low antigenicity^[14]. In addition, the degradation velocity of chitosan conduits can vary with molecular weight and the degree of deacetylation^[15]. The chitosan molecule has free amino, hydroxyl and polylysine or gelatinum, and can promote nerve cell adherence and growth along the surface of the material^[14]. It can enhance the adherence and in-

flux of Schwann cells, thus encouraging the growth of axons^[16]. However, physical nerve guidance by a nerve conduit may not be sufficient to foster optimal recovery^[17]. Nerve growth factor (NGF) promotes the survival of cell bodies and supports the regeneration of axons towards specific target organs^[18]. However, NGF levels decrease rapidly as it degrades in aqueous media^[19]. To combat this, polymeric microspheres have been developed to encapsulate NGF for local sustained delivery^[20].

This study aimed to develop a chitosan nerve conduit loaded with microspheres that provide sustained release of bioactive NGF and to evaluate whether the incorporation of this sustained release system into nerve conduits can improve the regeneration of facial nerves.

RESULTS

Quantitative analysis of experimental animals

Thirty-six rabbits were used to establish models of 10-mm defects in the right buccal branch of the facial nerve. The model rabbits were equally and randomly assigned to four groups as follows: NGF-microspheres group, chitosan conduits combined with NGF microspheres; NGF group, chitosan conduits combined with NGF alone; normal saline group, chitosan conduits combined with normal saline; autologous nerve group, with nerve autografting performed. All 36 rabbits were involved in the final analysis.

Morphology, *in vitro* NGF release and bioactivity of microspheres

Scanning electron microscopy showed that the microspheres had uniform size and smooth surface. At 90 days of *in vitro* release, a large number of pores appeared on the surface of the microspheres and they had partially degraded (Figure 1).

NGF release from the microspheres extended over the entire 90-day period of testing, and the accumulated release ratio achieved $91.2 \pm 2.2\%$. The microspheres

showed an almost constant release after a minimal burst during the first day of incubation. The release curve was recorded (Figure 2).

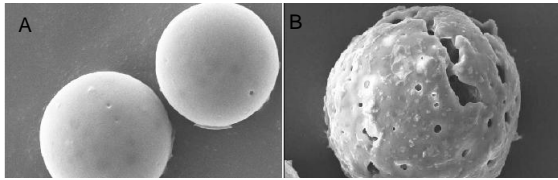


Figure 1 Electron micrographs of nerve growth factor-releasing microspheres ($\times 2\,000$).

(A) Before releasing nerve growth factor *in vitro*, the microspheres present uniform size and smooth surface.

(B) After releasing nerve growth factor *in vitro*, a large number of pores appeared on the surface of the microspheres and the microspheres partially degraded.

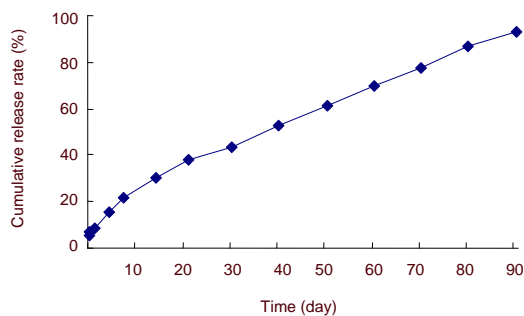


Figure 2 Accumulative release rate of nerve growth factor *in vitro*.

The microspheres were cultured in PBS (pH 7.4, 37°C). Data are expressed as mean ($n = 3$).

When PC12 cells were co-incubated with bioactive NGF, the cells differentiated and extended neurite outgrowth. At 90 days of co-incubation with the microspheres, almost 50% of the cells extended neurites, and some cells grew over each other so that their individual morphology became indistinguishable. This suggests that bioactive NGF was continuously released over the period of incubation.

NGF-releasing microspheres improved recovery of neurological function in rats with facial nerve defects

All rabbits presented partial facial paralysis on the right side after surgery and had difficulty in moving their upper lips. Beards on their lower jaw were sunken, and their wink reflection was weakened. At 2 weeks post-surgery, all of the rabbits displayed light muscular atrophy on the right upper lip, which was still evident at 1 month post-surgery. Facial paralysis decreased with time, but muscle movement remained worse than the

healthy side. In addition, the muscular atrophy in the NGF and normal saline groups was more evident than the NGF-microspheres and autologous nerve groups. Meanwhile, the recovery of upper lip movement in the NGF and normal saline groups occurred later than in the NGF-microspheres and autologous nerve groups. The wounds in all of the rabbits healed well without infection.

Gross pathology indicated there was no tissue inflammation. At 90 days after surgery, in the NGF-microspheres, NGF and normal saline groups, nerve defects were repaired by regenerated nerve; chitosan conduits became thinner and were partially absorbed; and the conduit wall was not intact and easy to fracture. In addition, there was no neuroma formation in the proximal end of regenerative nerve, and the appearance of nerves was similar to normal. In the autologous nerve group, the nerve anastomosis also recovered well.

NGF-releasing microspheres improved facial nerve conduction velocity

In electromyography, the prolongation of time-latency, increase of current threshold and decrease of voltage amplitude to stimuli may be attributed to an impairment of neuromuscular function after injury^[21]. When nerve injury is recovering, a decrease of time-latency and threshold and increase of amplitude should be observed. At 90 days post-surgery, latency period, amplitude and conduction of velocity in each group were recorded with threshold stimulation. The latency period was significantly shortened, conduction of velocity significantly faster and amplitude significantly greater in the NGF-microspheres and autologous nerve groups than in the NGF and normal saline groups ($P < 0.01$), but there was no statistical difference between the NGF-microspheres and autologous nerve groups ($P > 0.05$; Table 1).

NGF-releasing microspheres improved facial nerve regeneration

Specimens were stained with hematoxylin-eosin and anti-S-100 antibody for observation by light microscopy. Normal nerves were composed of one to five fascicles enveloped by epineuria with spindle shaped cells, and axons were regularly arranged and had similar diameters. Myelin was dense, with no deterioration. Schwann cells were well distributed, with similarly sized and shaped cell nuclei. The perineurium was a condensation of loose connective tissue. At 90 days post-surgery, hematoxylin-eosin staining results indicated rich vessels in the epineurium, bundled regenerative nerve

fibers, and many neovessels in the nerve fiber bundles in the NGF-microspheres group; fewer regenerative axons with uneven distribution and poor development, and a lot of fibrous connective tissue in the NGF and normal saline groups; and nerve bundles with uniform size and tissue similar to normal nerve in the autologous nerve group (Figure 3).

Table 1 Latency period (ms) and amplitude (mV) of neuromuscular action potentials and conduction of velocity (m/s) of the facial nerve in each group 90 days post-surgery.

Group	Latency period	Amplitude	Conduction of velocity
NGF-microspheres	2.23±0.14 ^a	2.17±0.32 ^a	23.86±1.97 ^a
Autologous nerve	2.29±0.15 ^a	2.42±0.18 ^a	25.74±2.39 ^a
NGF	3.29±0.24	1.03±0.11	17.45±1.45
Normal saline	3.35±0.19	0.97±0.08	16.69±0.33

Data are expressed as mean ± SD of nine rabbits from each group. ^a $P < 0.01$, vs. nerve growth factor (NGF) and normal saline groups. Differences between groups were compared using one-way analysis of variance, with least significant difference test pairwise comparisons applied as appropriate.

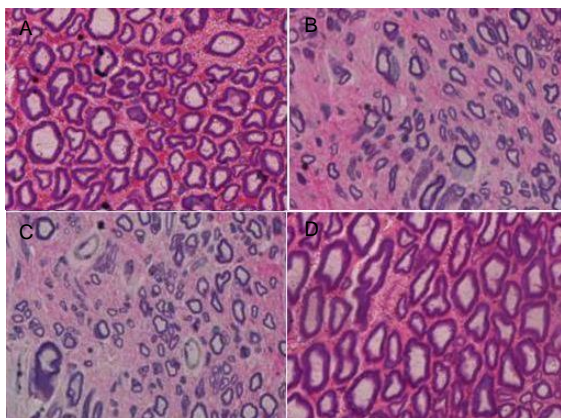


Figure 3 Influence of nerve growth factor (NGF)-microspheres on the morphology of regenerated nerve fibers of cross-sectioned injured facial nerve in rabbits at 90 days (hematoxylin-eosin staining, × 400)

(A) NGF-microspheres group; (B) NGF group; (C) normal saline group; (D) autologous nerve group.

The myelinated nerve fibers were uniform and larger in groups A and D than in groups B and C.

S-100 antibody immunohistochemistry confirms the presence of nerve fibers^[5]. At 90 days post-surgery, S-100 staining revealed that nerve fibers were present in all groups. A brown area represented positive expression, and dark brown indicated more developed nerve fibers. Nerve fibers were more dense in the NGF-microspheres and autologous nerve groups than

in the NGF and normal saline groups (Figure 4).

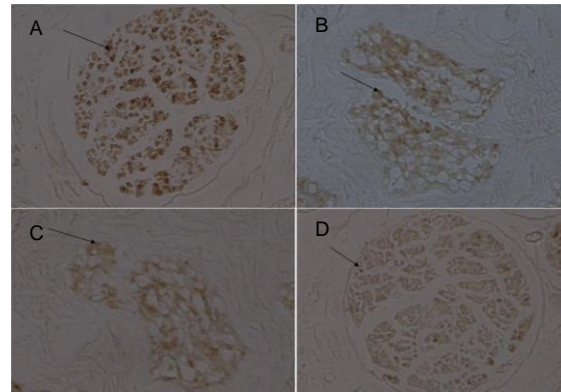


Figure 4 Effect of nerve growth factor (NGF)-microspheres on S-100 expression in the regenerated facial nerves at 90 days post-surgery (immunohistochemical staining, × 100).

In the NGF-microspheres group (A) and autologous nerve group (D), there was higher S-100 positive expression than in the NGF group (B) and normal saline group (C). Arrows indicate S-100 positive expression.

Influence of NGF-microspheres on the ultrastructure of regenerated facial nerves

Scanning electron microscopy revealed that the cross section of the conduits became looser and porous compared with the normal conduits at 90 days post-surgery (Figure 5).

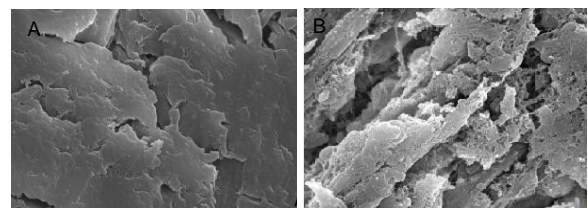


Figure 5 Electron micrographs of the cross section of chitosan conduits (scanning electron microscope, × 5 000).

(A) Normal conduit before *in vivo* implantation: the cross section of the conduits was smooth and there were only a few pores.

(B) Conduit implantation for 90 days: the cross section of the conduits became looser and porous.

Transmission electron microscopy revealed many myelinated nerve fibers with clear myelin layer structure, clearly visible Schwann cells, and rich organelles in the axoplasm of the NGF-microspheres and autologous nerve groups, while dysplasia of the myelin sheath, disordered myelin layer structure and many fibrous connective tissues were observed in the NGF and

normal saline groups (Figure 6).

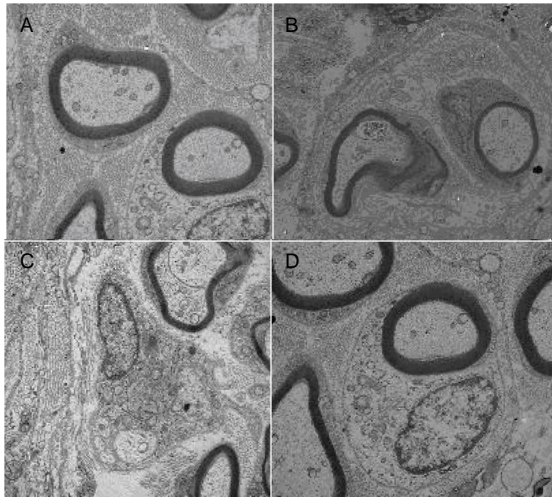


Figure 6 Ultrathin sections of regenerated nerve stained with toluidine blue at 90 days post-surgery (transmission electron microscope, $\times 15\ 000$)

(A) Nerve growth factor (NGF)-microspheres group; (B) NGF group, (C) normal saline group; (D) autologous nerve group.

In A and D, myelinated nerve fibers had clear myelin layer structure and uniform size; in B and C, myelinated nerve fibers had disordered myelin layer structure and a lot of fibrous connective tissue.

In line with light microscopy, transmission electron microscopy illustrated the diameter, alignment and myelin sheath thickness of myelinated nerve derived from rabbits were similar in the NGF-microspheres and autologous nerve groups ($P > 0.05$), significantly greater than in the NGF and normal saline groups ($P < 0.05$; Table 2).

Table 2 Number (n) and diameter (μm) of myelinated nerve fibers, and thickness of myelin sheath (μm) of facial nerve at 90 days post-surgery.

Group	Number of myelinated nerve fibers	Diameter of nerve fibers	Thickness of myelin sheath
NGF-microspheres	2 739 \pm 356 ^a	3.97 \pm 0.32 ^a	0.92 \pm 0.07 ^a
NGF	2 032 \pm 205	3.30 \pm 0.27	0.70 \pm 0.06
Normal saline	2 120 \pm 339	3.18 \pm 0.23	0.77 \pm 0.06
Autologous nerve	2 941 \pm 432 ^a	4.15 \pm 0.45 ^a	1.01 \pm 0.08 ^a

Data are expressed as mean \pm SD of nine rabbits from each group. ^a $P < 0.05$, vs. nerve growth factor (NGF) and normal saline groups. Differences between groups were compared using one-way analysis of variance, with least significant difference test applied as appropriate.

DISCUSSION

Previous studies using various nerve conduits in combination with a single administration of NGF in different animal models demonstrated that NGF may promote sensory and motor nerve regeneration between 3 and 7 weeks. After that period, no improvement in overall histological appearance could be seen, probably because of a failure in maintaining sufficient NGF concentrations over the long duration of nerve regeneration^[18]. This study aimed to integrate a microsphere NGF delivery system into chitosan conduits to supply sustained release of NGF. The major result of this investigation was improved nerve regeneration for 90 days after applying this new tissue engineering approach. Understanding the features associated with our method and its application may help develop more effective therapeutic approaches that promote functional recovery of injured nerves.

Artificial nerve grafts have beneficial biocompatibility, hydrophilic activity, non-immunogenic property, and inertness in mediating scarring and fibrosis^[22-23]. Chitosan, a predominant absorbable material, has received much attention because of its physicochemical and mechanical properties. In a previous study, chitosan conduits were shown to be effective in inducing nerve tissue regeneration^[24]. Chitosan conduits create a barrier between the regenerating axons and the surrounding environment. This environment promotes axon extension from the proximal stump across a 10-mm gap where it directly contacted with the distal stump. Chitosan conduits provide directional guidance to regenerating axons, leading to an increase in axon diameter^[25].

However, chitosan conduits do not function as well as nerve autografts in most cases^[26]. Just as in our experiment, the use of normal saline within chitosan conduits could not achieve the same effect in facial nerve regeneration as autografting. Several tissue engineering approaches have been developed to improve the performance of conduits^[27-29], including the delivery of neurotropic factors within these hollow tubes.

NGF is an important neurotrophic factor that can provide a beneficial microenvironment to promote nerve regeneration. NGF delivered within conduits may significantly increase the morphological and/or functional recovery of transected and repaired nerves^[30-31]. Furthermore, it was found that the duration of NGF administration subsequently dictates the degree of behavioral recovery following peripheral nerve injury^[32]. However, while the delivery of NGF promoted nerve regeneration when administered at the early stage, the promoting effect did not

last very long^[33]. NGF has a short half-life and degrades easily in aqueous media at 37°C^[34]. Therefore, methods for achieving sustained protein release and protection against degradation are more appealing for implantation than applying naked proteins. To this end, polymeric microspheres have been developed for the encapsulation of NGF^[35]. In this study, we fabricated NGF microspheres by oil-oil emulsion/solvent evaporation combined with polymer-alloys. NGF was analyzed by enzyme linked immunosorbent assay for sensitivity and convenience as concentrations in the nanogram range had to be quantified according to the intended and clinically relevant release rate. The loading level achieved for NGF was 0.578 µg/mg, and bioactive NGF release from the microspheres extended over the entire 90-day period of testing, as demonstrated by neurite outgrowth assay of PC12 cells. Furthermore, in the *in vivo* experiment, chitosan conduits combined with NGF-microspheres, which supply local sustained delivery of NGF, improved the regeneration of facial nerves. This was in accordance with another study^[17] where NGF enhanced sensory and motor nerve regeneration only between 3 and 7 weeks, while applying a new tissue engineering approach incorporating a microsphere NGF delivery system into nerve conduits to greatly enhance peripheral nerve regeneration.

At present, the evaluation methods used in artificial nerve research include qualitative histology, immunohistochemistry, quantitative histology (density and diameter of axons, thickness of myelin sheath, and number and diameter of nerve fibers), electrophysiology, and functional assessment of the terminal organ connection^[36].

At 90 days post-surgery, the myelin sheath thickness, number of myelinated fibers and conduction motor nerve velocity in the NGF-microspheres and autologous nerve groups were significantly higher than in the NGF and normal saline groups, but there was no significant difference between the NGF-microspheres and autologous nerve groups, and the NGF and normal saline groups. This illustrates that chitosan conduits alone are unable to achieve the same effects as autografts in facial nerve regeneration. Furthermore, NGF assures the survival of cell bodies and supports the regeneration of axons toward specific target organs. NGF microspheres were also superior to NGF alone in promoting facial nerve regeneration. This result illustrates that the duration of NGF release is critical for enhanced regeneration.

In conclusion, this study proves the feasibility of chitosan conduits loaded with NGF-microspheres for promoting nerve regeneration, and raises new possibilities of al-

ternatives to autografting for nerve repair.

MATERIALS AND METHODS

Design

A randomized, controlled, animal study.

Time and setting

The experiments were conducted in the Chinese PLA General Hospital, China from March to September 2010.

Materials

Thirty-six male healthy New Zealand rabbits, aged 6 months, weighing 2.5–3.0 kg, were provided by the Experimental Animal Laboratories of Chinese PLA General Hospital (license No. SCXK (Jing) 2007-0003). Animal procedures were conducted in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China^[37].

Methods

Preparation of NGF-microspheres

NGF (Xiamen Bioway Biotech Co., Ltd., Xiamen, Fujian Province, China) microspheres, 256.7 mg, were prepared by oil-oil emulsion/solvent evaporation combined with polymer-alloys^[38], and stored in a 4°C desiccative environment. The method involved the use of two phases: (1) the micronization of NGF: 300 µg depurated NGF solution, mixed with 0.5 mL bovine serum albumin solution, containing 10 mg bovine serum albumin. The mixture was freeze-dried to obtain NGF medicine powder that was nicely distributed in bovine serum albumin base material; (2) microencapsulation of NGF: the copolymer of polylactic acid and polylactic acid-glycolic acid, 300 mg (polylactic acid: polylactic acid-glycolic acid = 1:3; Shandong Medical Equipment Institute, Shandong Province, China), was mixed with freeze-dried NGF powder and 2 mL dichloromethane (CH₂Cl₂) to obtain an oil phase; the aqueous phase was 10 mL polyvinyl alcohol solution (2%). This water phase was mixed into the oil phase at high speed for 30 seconds to form an oil-oil emulsion/solvent evaporation emulsion. The emulsion was added to deionized water (400 mL) containing NaCl (10% w/v) to extract the organic solvent. This obtained the NGF-microspheres.

Characteristics of NGF-microspheres

The outline form of the microspheres was observed by scanning electron microscopy (Shimadzu ss-550, Tokyo, Japan). The size of microspheres ranged from 15 µm to 50 µm, and the mean diameter was 22.47 µm. The

loading level achieved for NGF was 0.578 $\mu\text{g}/\text{mg}$. *In vitro* release of NGF was measured by dissolving NGF microspheres in PBS (pH 7.4) buffer solution. The samples were incubated at 37°C in an overhead shaker, and the dilution was exchanged by buffer solution at 6 and 12 hours, as well as 1, 4, 7, 14, 21, 30, 40, 50, 60, 70, 80, and 90 days. NGF samples were stored at -20°C until further analysis by enzyme linked immunosorbent assay (R&D Systems, Minneapolis, MN, USA). PC12 cells (Institute of Biochemistry and Cell Biology, CAS, Shanghai, China) differentiated in response to NGF into a neuronal phenotype observed by neurite extension. The ratio of positive PC12 cells reflected the bioactivity of released NGF. For quantification, cells were considered fully differentiated when the outgrowth of the exposed neurites exceeded double the size of their cellular body.

Preparation of chitosan nerve conduits

Chitosan conduits were prepared by Beijing Yierkang Biological Engineering Development Center, China according to previously described method^[15]. The chitosan conduits was 1.8 mm in inside diameter, 2 mm in outside diameter, and 0.1 mm in wall thickness. The conduits were irradiated with ⁶⁰Co for sterilization and sealed in a package. The conduits were soaked in 75% ethanol for 30 minutes prior to implantation.

Model establishment and repair treatment

Surgery was conducted on the four groups of rabbits under general anesthesia with intramuscular injection of sodium pentobarbital (50 mg/kg) in sterile conditions. A horizontal incision was made to expose the main stem of the right facial nerve. The upper buccal branch was dissected from surrounding tissue and transected 8 mm away from its origin, and then a nerve gap defect of approximately 10 mm was made after contraction. A conduit of 12 mm in length was implanted into the defect in the NGF-microspheres, NGF and normal saline groups. The two ends of the conduits were sutured to the epineurium of the facial nerve using 9-0 nylon stitching. Each nerve end was sutured into the conduits about 1 mm. In the NGF-microspheres group, 20 μL normal saline suspension of microspheres containing 10 mg NGF-microspheres (5 μg NGF) was injected into the chitosan conduits with a micropipette. In the NGF group, 20 μL of normal saline solution with 5 μg NGF was injected into the chitosan conduits with a micropipette. In the normal saline group, 20 μL of normal saline was injected into the chitosan conduits with a micropipette. In the autologous nerve group, the removed facial nerve was reversed, and sutured back in place *via* a single 9-0 nylon suture. The muscle layers and the skin were

closed with 4-0 silk sutures. Animals were housed in isolator cages without any immunosuppression.

Gross observation

Beard movement and muscle atrophy on the right face was observed and compared with the left side after implantation. During the period, we also observed the weight, appetite and the wound recovery of the rabbits.

Neuroelectrophysiological examination

At 90 days post-surgery, the rabbits were anesthetized and the previous incision was cut to re-expose the chitosan conduits and facial nerve. The normal facial nerve on the left side was also exposed. The neuromuscular action potentials were examined using an electrophysiological tester (Keypoint, Frederiksberg, Denmark). A stimulating needle was placed on the facial nerve proximal to the conduits. A recording needle was placed in the orbicularis oris. Compound muscle action potential was measured and the latency period and peak amplitude were recorded on the normal and regenerated nerve. The stimulating intensity was 2.0 mA, the stimulating frequency was 1 Hz, the sensitivity was 5 ms/D, the scanner speed was 5 ms/D and the stimulating time was 0.1 ms.

Histological observation

After electrophysiological examination, the regenerative facial nerves with chitosan conduits and the control nerves were removed. The rabbits were sacrificed by an overdose of pentobarbital. The chitosan conduits and the regeneration nerves were examined.

A systematic random sampling procedure was used for histomorphometric analysis. The total nerve section area was divided into three sampling fields from the distal end to proximal end to prevent over- or under-estimation of data values. Each field was divided into two subfields. One subfield of each field was randomly selected for examination by light microscopy, while the other was used for electron microscopy.

For the light microscopy examination, the nerve sections were fixed in 10% buffered formalin for 24 hours, dehydrated, and embedded in paraffin blocks. Five-micron sections in each subfield were cut across the transverse axis. The sections were de-waxed and stained with hematoxylin and eosin or anti-S-100 alpha 6 ([CACY-100]; Abcam, Cambridge, MA, USA) immunohistochemistry. The stained sections were examined *via* a light microscope (H-7650; Hitachi, Tokyo, Japan) at 100 \times magnification. The observer was blinded to the identity of the

groups.

For the electron microscopy examination, the chitosan conduits were separated from the regenerated facial nerves and were fixed in glutaraldehyde (2.5%) buffered in cacodylate (0.025 mol/L) overnight, and then the specimens were dried and gold-coated. A scanning electron microscope was used to observe the microstructure of the cross section of the chitosan conduits. The nerve sections were fixed in glutaraldehyde (2.5%) buffered in cacodylate (0.025 mol/L) overnight, washed, and then stored in cacodylate buffer (0.15 mol/L). Samples were subsequently fixed in osmium tetroxide (2%), washed in a graded alcohol series, and then embedded in Epon. Sections of 100 nm in each subfield were cut across the transverse axis. The sections were stained with toluidine blue (Yuanye Biological, Shanghai, China) and used for observation on a transmission electron microscope (Olympus, Tokyo, Japan). Five randomly selected fields of view at 3 600 × magnification were used to calculate the total number, fiber diameter, and myelin sheath thickness.

Statistical analysis

Data were expressed as mean ± SD, and one-way analysis of variance followed by least significant difference test was performed. A level of $P < 0.05$ was considered statistically significant. Statistical analysis and treatment were performed with SPSS software (version 10.0; SPSS, Chicago, IL, USA).

REFERENCES

- [1] Pfister LA, Alther E, Papaloizos M, et al. Controlled nerve growth factor release from multiply alginate/chitosan-based nerve conduits. *Eur J Pharm Biopharm.* 2008;69(2):563-572.
- [2] Fansa H, Keilhoff G. Comparison of different biogenic matrices seeded with cultured Schwann cells for bridging peripheral nerve defects. *Neurol Res.* 2004;26(2):167-173.
- [3] de Boer R, Borntraeger A, Knight AM, et al. Short- and long-term peripheral nerve regeneration using a poly-lactic-co-glycolic-acid scaffold containing nerve growth factor and glial cell line-derived neurotrophic factor releasing microspheres. *J Biomed Mater Res A.* 2012;100(8):2139-2146.
- [4] Ren Z, Wang Y, Peng J, et al. Role of stem cells in the regeneration and repair of peripheral nerves. *Rev Neurosci.* 2012;23(2):135-143.
- [5] Hu M, Zhang L, Niu Y, et al. Repair of whole rabbit facial nerve defects using facial nerve allografts. *J Oral Maxillofac Surg.* 2010;68(9):2196-2206.
- [6] Zhou L, Du HD, Tian HB, et al. Experimental study on repair of the facial nerve with Schwann cells transfected with GDNF genes and PLGA conduits. *Acta Otolaryngol.* 2008;128(11):1266-1272.
- [7] Liu JJ, Wang CY, Wang JG, et al. Peripheral nerve regeneration using composite poly(lactic acid-caprolactone)/nerve growth factor conduits prepared by coaxial electrospinning. *J Biomed Mater Res A.* 2011;96(1):13-20.
- [8] Wang X, Hu W, Cao Y, et al. Dog sciatic nerve regeneration across a 30-mm defect bridged by a chitosan/PGA artificial nerve graft. *Brain.* 2005;128(Pt 8):1897-1910.
- [9] Lundborg G. A 25-year perspective of peripheral nerve surgery: evolving neuroscientific concepts and clinical significance. *J Hand Surg Am.* 2000;25(3):391-414.
- [10] Dahlin LB, Anagnostaki L, Lundborg G. Tissue response to silicone tubes used to repair human median and ulnar nerves. *Scand J Plast Reconstr Surg Hand Surg.* 2001;35(1):29-34.
- [11] Zhang H, Wei YT, Tsang KS, et al. Implantation of neural stem cells embedded in hyaluronic acid and collagen composite conduit promotes regeneration in a rabbit facial nerve injury model. *J Transl Med.* 2008;6(67):1-11.
- [12] Pfister LA, Papaloizos M, Merkle HP, et al. Nerve conduits and growth factor delivery in peripheral nerve repair. *J Peripher Nerv Syst.* 2007;12(2):65-82.
- [13] de Ruitter GC, Malessy MJ, Yaszemski MJ, et al. Designing ideal conduits for peripheral nerve repair. *Neurosurg Focus.* 2009;26(2):E5.
- [14] Kumar MN, Muzzarelli RA, Muzzarelli C, et al. Chitosan chemistry and pharmaceutical perspectives. *Chem Rev.* 2004;104(12):6017-6084.
- [15] Wang A, Ao Q, Wei Y, et al. Physical properties and biocompatibility of a porous chitosan-based fiber-reinforced conduit for nerve regeneration. *Biotechnol Lett.* 2007;29(11):1697-1702.
- [16] Zhu N, Li MG, Guan YJ, et al. Effects of laminin blended with chitosan on axon guidance on patterned substrates. *Biofabrication.* 2010;2(4):045002.
- [17] Mohanna PN, Young RC, Wiberg M, et al. A composite poly-hydroxybutyrate-gial growth factor conduit for long nerve gap repairs. *J Anat.* 2003;203(6):553-565.
- [18] Xu X, Yee WC, Hwang PY, et al. Peripheral nerve regeneration with sustained release of poly(phosphoester) microencapsulated nerve growth factor within nerve guide conduits. *Biomaterials.* 2003;24(13):2405-2412.
- [19] Derby A, Engleman VW, Friedrich GE, et al. Nerve growth factor facilitates regeneration across nerve gaps: morphological and behavioral studies in rat sciatic nerve. *Exp Neurol.* 1993;119(2):176-191.
- [20] Ao Q, Wang A, Cao W, et al. Manufacture of multimicrotubule chitosan nerve conduits with novel molds and characterization in vitro. *J Biomed Mater Res A.* 2006;77(1):11-18.
- [21] García-Losarcos N, González-Hidalgo M, Franco-Carcedo C, et al. Electrical stimulation of the facial nerve

- with a prognostic function in parotid surgery. *Rev Neurol*. 2009;49(3):119-122.
- [22] Brännvall K, Bergman K, Wallenquist U, et al. Enhanced neuronal differentiation in a three-dimensional collagen-hyaluronan matrix. *J Neurosci Res*. 2007;85(10):2138-2146.
- [23] Tian WM, Hou SP, Ma J, et al. Hyaluronic acid-poly-D-lysine-based three-dimensional hydrogel for traumatic brain injury. *Tissue Eng*. 2005;11(3-4):513-525.
- [24] Yang Y, Gu X, Tan R, et al. Fabrication and properties of a porous chitin/chitosan conduit for nerve regeneration. *Biotechnol Lett*. 2004;26(23):1793-1797.
- [25] Patel M, Vandevord PJ, Matthew H, et al. Video-gait analysis of functional recovery of nerve repaired with chitosan nerve guides. *Tissue Eng*. 2006;12(11):3189-3199.
- [26] Guo BF, Dong MM. Application of neural stem cells in tissue-engineered artificial nerve. *Otolaryngol Head Neck Surg*. 2009;140(2):159-164.
- [27] Yang Y, Zhao W, He J, et al. Nerve conduits based on immobilization of nerve growth factor onto modified chitosan by using genipin as a crosslinking agent. *Eur J Pharm Biopharm*. 2011;79(3):519-525.
- [28] Xue C, Hu N, Gu Y, et al. Joint use of a chitosan/PLGA scaffold and MSCs to bridge an extra large gap in dog sciatic nerve. *Neurorehabil Neural Repair*. 2012;26(1):96-106.
- [29] Hsieh SC, Tang CM, Huang WT, et al. Comparison between two different methods of immobilizing NGF in poly(DL-lactic acid-co-glycolic acid) conduit for peripheral nerve regeneration by EDC/NHS/MES and genipin. *J Biomed Mater Res A*. 2011;99(4):576-585.
- [30] Bryan DJ, Holway AH, Wang KK, et al. Influence of glial growth factor and Schwann cells in a bioresorbable guidance channel on peripheral nerve regeneration. *Tissue Eng*. 2000;6(2):129-138.
- [31] Yildiz M, Karlidag T, Yalcin S, et al. Efficacy of glial growth factor and nerve growth factor on the recovery of traumatic facial paralysis. *Eur Arch Otorhinolaryngol*. 2011;268(8):1127-1133.
- [32] Kemp SW, Webb AA, Dhaliwal S, et al. Dose and duration of nerve growth factor (NGF) administration determine the extent of behavioral recovery following peripheral nerve injury in the rat. *Exp Neurol*. 2011;229(2):460-470.
- [33] Wang L, Zhao Y, Cheng X, et al. Effects of locally applied nerve growth factor to the inferior alveolar nerve histology in a rabbit model of mandibular distraction osteogenesis. *Int J Oral Maxillofac Surg*. 2009;38(1):64-69.
- [34] Uebersax L, Mattotti M, Papaloizos M, et al. Silk fibroin matrices for the controlled release of nerve growth factor (NGF). *Biomaterials*. 2007;28(30):4449-4460.
- [35] Johnson PJ, Skornia SL, Stabenfeldt SE, et al. Maintaining bioactivity of NGF for controlled release from PLGA using PEG. *J Biomed Mater Res A*. 2008;86(2):420-427.
- [36] Clements IP, Kim YT, English AW, et al. Thin-film enhanced nerve guidance channels for peripheral nerve repair. *Biomaterials*. 2009;30(23-24):3834-3846.
- [37] The Ministry of Science and Technology of the People's Republic of China. *Guidance Suggestions for the Care and Use of Laboratory Animals*. 2006-09-30.
- [38] Gu H, Long D, Song C, et al. Recombinant human NGF-loaded microspheres promote survival of basal forebrain cholinergic neurons and improve memory impairments of spatial learning in the rat model of Alzheimer's disease with fimbria-fornix lesion. *Neurosci Lett*. 2009;453(3):204-209.
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