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PDLLA/*β*-TCP/HA/CHS/NGF Sustained-release Conduits for Peripheral Nerve Regeneration

YAN Xiumei[1], WANG Jing[2], HE Qundi[3], XU Haixing[1*], TAO Junyan[4], KORAL Kelly[4], LI Kebi[1], XU Jingyi[1], WEN Jing[1], HUANG Zhijun[1], XU Peihu[1*]

(1. Department of Pharmaceutical Engineering, School of Chemistry, Chemical Engineering and Life Science, Wuhan University of Technology, Wuhan 430070, China; 2. China Resources Sanjiu Medical & Pharmaceutical Co., Ltd., Shenzhen 518029, China; 3. Wuhan Mafangshan Middle School, Wuhan 430070, China; 4. Department of Pathology, University of Pittsburgh, Pittsburgh, PA, USA)

Abstract: Using nerve guide conduits (NGCs) to promote the regeneration of PNI is a feasible alternative to autograft. Compared with NGCs made of single material, composite NGCs have a greater development prospect. Our previous research has confirmed that poly(D, L-lactic acid)/*β*-tricalcium phosphate/hyaluronic acid/chitosan/nerve growth factor (PDLLA/*β*-TCP/HA/CHS/NGF) NGCs have excellent physical and chemical properties, which can slowly release NGF and support cell adhesion and proliferation. In this study, PDLLA/*β*-TCP/HA/CHS/NGF NGCs were prepared and used to bridge a 10 mm sciatic nerve defect in 200-250 g Sprague-Dawley (SD) rat to verify the performance of the NGCs *in vivo*. Substantial improvements in nerve regeneration were observed after using the PDLLA/*β*-TCP/HA/CHS/NGF NGCs based on gross post-operation observation, triceps wet weight analysis and nerve histological assessment. *In vivo* studies illustrate that the PDLLA/*β*-TCP/HA/CHS/NGF sustained-release NGCs can effectively promote peripheral nerve regeneration, and the effect is similar to that of autograft.

Key words: sustained-release; composite nerve conduits; peripheral nerve regeneration

1 Introduction

Peripheral nerve injury (PNI) is the loss of peripheral nerve structure and/or function caused by accident, trauma and other reasons, which will lead to partial or complete loss of sensory, motor and autonomic nerve functions and neuropathic pain[1]. Although human peripheral nerves can regenerate after injury, due to the limited ability of regeneration, this spontaneous process is not enough to achieve normal functional recovery[2]. Hence a considerable number of PNI patients will have sequelae, such as chronic pain, sensory or motor dysfunction and muscle atrophy[3]. Historically, PNI has been poorly repaired, especially in cases of delayed treatment and/or severe injury[4].

At present, autograft is regarded as the gold standard for PNI[5,6], but the success rate of it is only 50%[7]. At the same time, autograft can lead to sensory loss, scar and neuroma formation in the donor site[8]. In addition to donor complications, the disadvantages of autograft include limited supply, second operation, mismatch of nerve size, *etc*[5,9].

In order to overcome the shortcomings of autograft, nerve guide conduits (NGCs) have received increasing attention as an alternative treatment method[2]. NGCs can overcome many limitations of nerve transplantation[10]. In the past few decades, researchers have used a variety of biomaterials to synthesize NGCs, including natural materials and synthetic polymers. The requirements of ideal NGCs include bionic structure, structural characteristics of regenerated axons arranged longitudinally, sufficient mechanical properties, good permeability, conductivity, flexibility and biodegradability[5]. A single material can not meet the above-mentioned requirements. So, the preparation of composite NGCs by mixing natural polymer and synthetic polymer is the focus of current research[2]. The composite NGCs is expected to achieve

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YAN Xiumei(闫秀美): E-mail: 1124621854@qq.com

*Corresponding authors: XU Haixing(徐海星): Ph D; Prof.; E-mail: xhx040328@whut.edu.cn; XU Peihu(许沛虎): Ph D; E-mail: whutxph68@163.com

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or even exceed the repair effect of autograft.

Poly(D, L-lactic acid) (PDLLA) has good biocompatibility and biodegradability, and has been approved by FDA for biomedical application[11]. The PDLLA/TA conductive NGCs prepared by Guo xinglei *et al*[12] have good biocompatibility which have application values in tissue projects. However, the degradation rate of PDLLA based materials is slow, and the degradation period may exceed 10 months[13,14]. As a kind of bioactive ceramics, *β*-tricalcium phosphate (*β*-TCP) has good biocompatibility, bone conductivity and biodegradability[15]. Hyaluronic acid (HA) is the main component of extracellular matrix[16], which can affect the mechanical properties of materials and regulate cell processes[17]. Chitosan (CHS) is widely used in regenerative medicine because of its biocompatibility, biodegradability and structural similarity to glycosaminoglycan[18].

What's more, combination of neurotrophic factors and NGCs can provide opportunities to repair large nerve defects[19]. As a widely studied neurotrophic factor, nerve growth factor (NGF) can promote the growth and survival of sensory neurons[20], actively regulate the formation of myelin sheath of Schwann cells (SCs) and promote the development of axons in the process of nerve regeneration[19].

However, previous studies have shown that hyperphysiological doses of NGF can cause axons and SCs to be wrapped and hinder nerve regeneration[21], while single dose administration of NGF, due to its short half-life in vivo, is only 2-4 minutes[22], greatly limiting its biological application[23,24]. The ideal route of administration should be to minimize the dosage and to prolong the biological activity of NGF through continuous administration in physiological related time periods[25,26].

The PDLLA/*β*-TCP/HA/CHS/NGF NGCs was designed and fabricated with LBL-ESA and cross-linking method in our previous experimental[27]. The results of ATR-FTIR and SEM showed the conduit was prepared uniformly and had an excellent interconnected pore structure, which could provide an optimal microenvironment for cell adhesion and proliferation in nerve repair. The good mechanical and hydrophilic properties are also more consistent with the requirements of nerve tissue engineering. The degradation rate of the conduit could match the regeneration rate of nerve growth. Furthermore, cell evaluation showed that it can promote cell growth and proliferation. The present study aims to explore the feasibility of PDLLA/*β*-TCP/HA/CHS/NGF NGCs as a peripheral nerve repair material *in vivo*.

2 Experimental

2.1 Materials

Sodium pentobarbital, medical alcohol (75%), saline and iodide was purchased from China National Pharmaceutical Group Corporation (Shanghai, China). Surgical blades, hemostatic forceps, eye scissors and needle holders were from Wuhan Boyide Co. Ltd (Wuhan, China). The surgical suture needles and sutures were from Ningbo Medical Needle Co. Ltd (Ningbo, China). Adult male Sprague-Dawley (SD) rats weighing 200-250 g were from Hubei Center for Disease Control and Prevention (Wuhan, China). All SD rats were placed separately and provided with sufficient food and water.

2.2 Experimental animals and groups

Eighty SD rats were randomly divided into 4 groups: PDLLA group (n=20), PDLLA/*β*-TCP/HA/CHS group (n=20), PDLLA/*β*-TCP/HA/CHS group (n=20) and autograft group (n=20), respectively. The autograft group was the positive control and the PDLLA group was the negative control. Ten rats from each group were examined at one month and the other ten rats in each group were examined at three months post-surgery, respectively.

2.3 Preparation of experimental materials

The preparation method of experimental materials has been explained in the previous published article[27]. In this experiment, the PDLLA film with a thickness about 0.15 mm was cut into a size of 10 mm × 10 mm. Then in the ultra-clean workbench, the film was placed under the UV lamp for 30 min on both sides to sterilize and prepare the PDLLA NGCs. The 20-layer PDLLA/*β*-TCP/HA/CHS NGCs and PDLLA/*β*-TCP/HA/CHS/NGF NGCs were selected for UV-irradiation sterilization, and then placed in a dryer for backup.

2.4 Surgical procedures

Prior to implantation, the rats were anesthetized by intraperitoneal injection of 0.4% pentobarbital sodium in accordance with the specifications of 1 mL/100 g. The right hind legs were shaved, and 75% medical alcohol was used to disinfect them. Then, the skin was cut with the surgical blade, and the sciatic nerve was exposed through layer by layer of blunt separation. A 10 mm nerve segment was cut off. Subsequently, the nerve gap was bridged by PDLLA NGCs, PDLLA/*β*-TCP/HA/CHS NGCs, PDLLA/

β-TCP/HA/CHS/NGF NGCs or autografts. Finally, the muscle and skin incisions were sutured with 4-0 chromic gut sutures and 2-0 silk sutures, respectively. One month and three months after surgery, the nerve regeneration was measured and compared with each group.

2.5 Gross post-operation observation

Postoperative wound healing and walking of rats in each group was observed. The degradation of the NGCs, growth of the regeneration nerve, and adhesion of the wound and tissue were also observed.

2.6 Triceps wet weight analysis

Triceps are muscles supplied by the sciatic nerve, and muscle cells atrophy after sciatic nerve surgical injury. With the promotion of NGCs, the injured sciatic nerve gradually recovers its ruling function, and the weight of the triceps also increases slowly. Therefore, this can indirectly reflect the degree of sciatic nerve recovery.

First, the rats were euthanized via intracardiac perfusion of fixatives at specified experimental time points (1 month or 3 months) after surgery. Then the intact triceps on the operated and un-operated were harvested. The blood on the surface was washed with normal saline, and the surface adhesion of the tissue was removed. The wet-weight of the triceps was measured using an electronic microbalance. Finally, the weight ratio of muscles was calculated based on the following formula. The relative weights were presented as percentages:

$$\text{Triceps weight}(\%) = \frac{\text{Tricpes weight of the operated leg}}{\text{Triceps weight of leg the unoperated}} \times 100\%$$

A statistically significant difference was defined as the probability (p) value less than 0.05 ($p < 0.05$).

2.7 Nerve histological assessment

One month and three months after surgery, the distal 4-6 mm segment of regenerated nerves in rats were taken, respectively. Then, the nerves were fixed with 4% glutaraldehyde and 1% osmium acid. The nerves were embedded with epoxy resin and sliced. The nerve sections were stained with methylene blue, hematoxylin and eosin (HE), NGF and S-100 for immunohistochemistry, and then were observed under an optical microscope. Finally, the nerve sections were observed and photographed by a transmission electron microscope (TEM) after staining with lead citrate and uranylacetate.

3 Results and discussion

3.1 Gross post-operation observation

A small number of postoperative rats showed redness and swelling at the suture site, which healed quickly after anti-inflammatory treatment. Most of the remaining rats healed well (n=74). Within one month after surgery, the rats' appetite and reaction to external stimuli were normal, but there were obvious difficulties in movement. Within 3 months after surgery, the muscles of the PDLLA/*β*-TCP/HA/CHS/NGF group and the autograft group recovered faster than those of the other two groups. The two other groups exhibited a significant pain response (p<0.05) in comparison to the PDLLA/*β*-TCP/HA/CHS/NGF group and the autograft group. However, there was no significant difference between the two experimental groups PDLLA/*β*-TCP/HA/CHS/NGF conduit versus the autograft in terms of pain response.

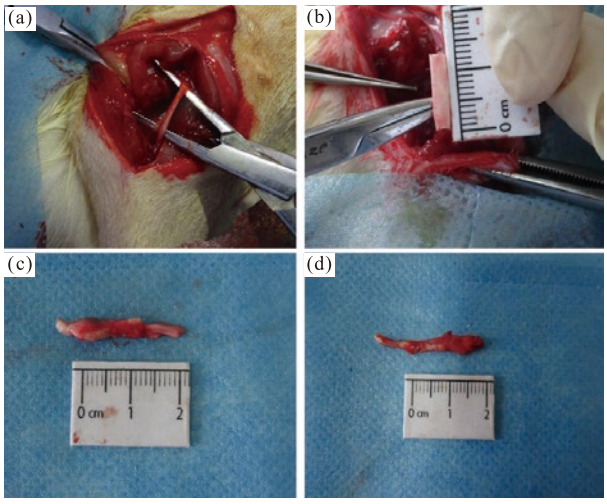

Fig.1 (a) The sciatic nerve of right hind leg in rats; (b) The 10 mm sciatic nerve defect was bridged with NGCs; The regenerated sciatic nerves 3 months after implantation with (c) PDLLA/*β*-TCP/HA/NGF NGCs and (d) Autograft

One month after surgery, examination of the conduit revealed that the materials of the NGCs did not bond with the surrounding tissue and were partially degraded. Three months after surgery, the materials of the NGCs had undergone extensive degradation and part of the conduits were thinner and fragmented. Compared with the other two groups, the regenerated nerve was thicker, and no obvious scar was seen in the PDLLA/*β*-TCP/CHS/NGF group and the autograft group (Fig. 1).

3.2 Triceps wet weight analysis

The triceps wet weight ratio reflects the degree of recovery of the sciatic nerve. Fig.2 illustrates that

the triceps wet weight ratio of the PDLLA/*β*-TCP/HA/CHS/NGF group and autograft groups was significantly higher than that of PDLLA group ($p<0.05$), while the triceps wet weight ratio of PDLLA/*β*-TCP/HA/CHS/NGF group was not significantly different from that of the autograft group ($p>0.05$). The data indicates that the PDLLA/*β*-TCP/HA/CHS/NGF NGCs materials significantly improved the recovery of the target muscle with a stronger repair capability for the sciatic nerve in comparison to the PDLLA control. Besides, the PDLLA/*β*-TCP/HA/CHS/NGF conduit performed similar to the autograft in terms of triceps wet weight ratio.

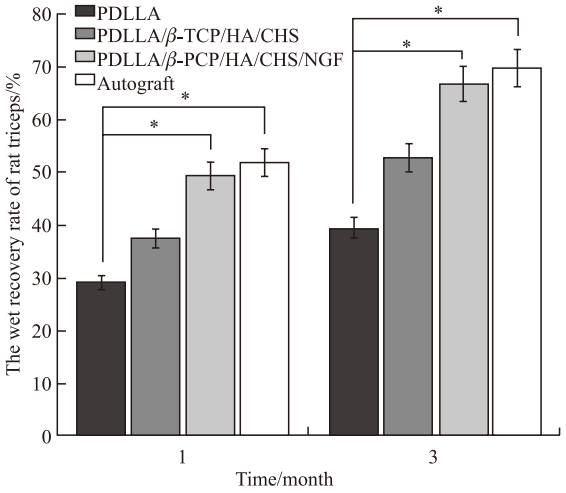

Fig.2 Recovery rate of triceps wet weight at 1st month and 3rd month post-surgery (n=5, *p < 0.05)

3.3 Nerve histological assessment

3.3.1 Methylene blue staining evaluation

Methylene blue staining on the harvested regenerated nerve samples was used to evaluate the number and diameter of regenerated axons in all of the groups. Among all of the groups, PDLLA/*β*-TCP/HA/CHS/NGF and autograft groups had a larger number of myelin sheaths with a uniform size in comparison to the single material PDLLA and the PDLLA/*β*-TCP/HA/CHS conduits (Fig.3). Three months after surgery, the nerve fibers were more mature than those collected one month after surgery. Compared with those of the PDLLA and PDLLA/*β*-TCP/HA/CHS groups, the rats with the PDLLA/*β*-TCP/HA/CHS/NGF conduit implanted had more fibers, thicker myelin sheaths and a more uniform arrangement, which was similar to the autograft group.

3.3.2 Hematoxylin and eosin (HE) staining

To evaluate the extent of connective tissue growth to the wall of the conduits, HE staining was utilized. These images show the extent of degradation in all

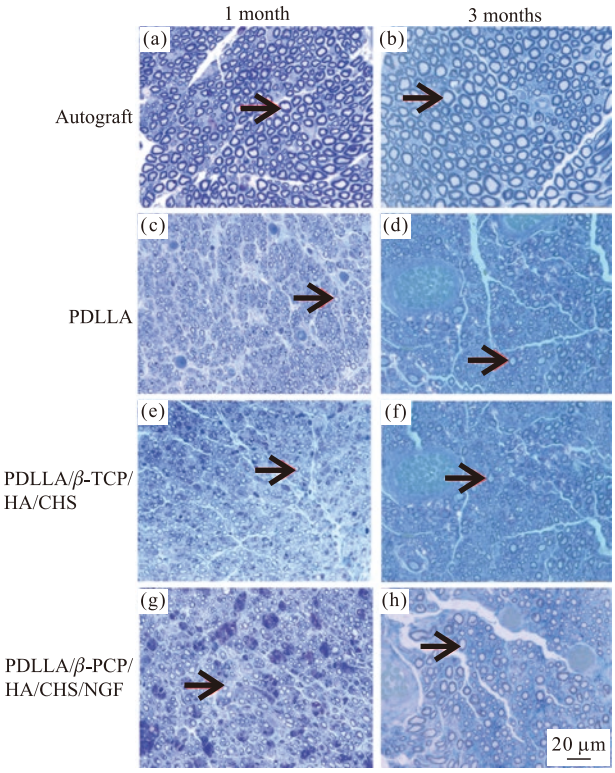

Fig.3 The regenerated nerve was stained with methylene blue at 1st month and 3rd month post-surgery. Arrows indicate the regenerated nerve fiber. Scale bar: 20 μm

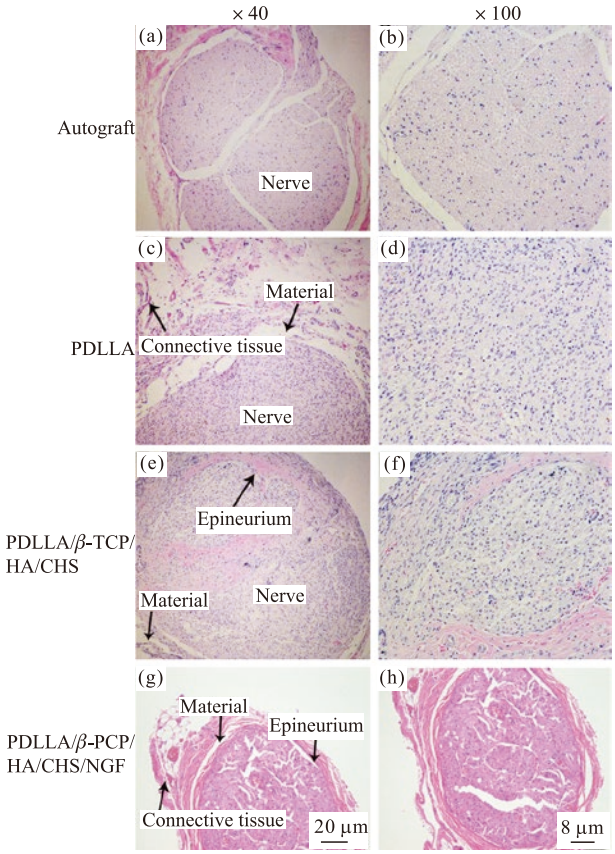

Fig.4 The regenerated nerves of four groups stained with HE at 1st month post-surgery. Scale bar: 20 μm (×40);8 μm (×100)

groups, which was in the following order: PDLLA > PDLLA/*β*-TCP/HA/CHS > PDLLA/*β*-TCP/HA/CHS/NGF, from most degraded to the least degraded conduit (Fig.4 and Fig.5). This illustrates that the degradation rate of the PDLLA/*β*-TCP/HA/CHS/NGF conduit is the slowest in comparison to the other conduits studied. In addition to degradation rate, we observed less vascular tissue invading into the PDLLA/*β*-TCP/HA/CHS/NGF conduits than the conduits of PDLLA and PDLLA/*β*-TCP/HA/CHS, indicating that the relatively compact structure of PDLLA/*β*-TCP/HA/CHS/NGF conduits prevented vascular growth into the conduit. Nerve tissue regeneration was observed to be more effective in the PDLLA/*β*-TCP/HA/CHS/NGF conduits than in the PDLLA and PDLLA/*β*-TCP/HA/CHS conduits, and was similar to the autograft controls.

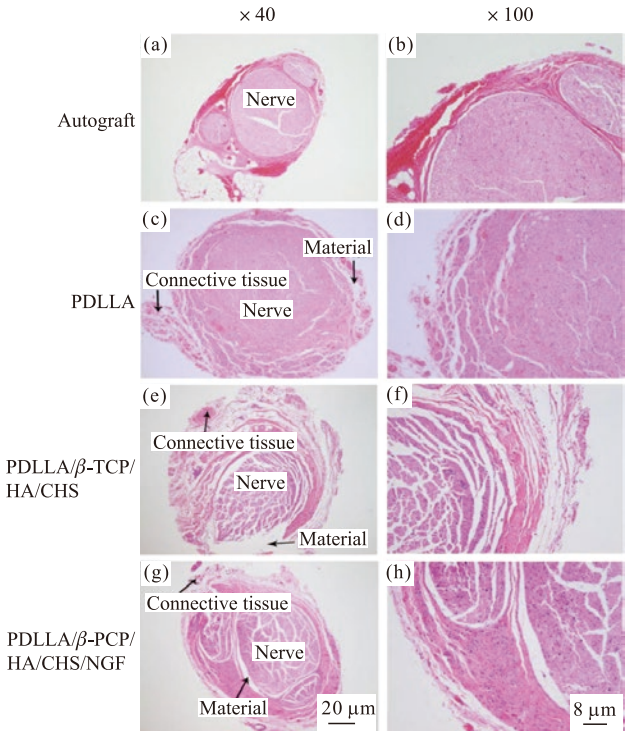

Fig.5 The regenerated nerves of four groups stained with HE at 3rd month post-surgery. Scale bar: 20 μm (×40);8 μm (×100)

3.3.3 NGF immunohistochemistry

After nerve injury, a large number of SCs near the end of the nerve fracture proliferate and secrete a variety of active substances, including NGF, which can promote the growth and development of nerve cells, protect neurons and promote damaged nerve repair. After nerve regeneration, NGF levels return to normal. Therefore, the degree of nerve recovery can be determined according to the positive expression of NGF.

The positive expression of NGF was stronger at 1 month post injury in PDLLA/*β*-TCP/HA/CHS and PDLLA/*β*-TCP/HA/CHS/NGF groups compared with 3 months and the PDLLA/*β*-TCP/HA/CHS/NGF group displayed higher expression of NGF than that of PDLLA/*β*-TCP/HA/CHS group (Fig.6). These results demonstrate that nerve repair was faster at 1st month than at 3rd month after implantation of the conduits. Moreover, compared with the other NGCs, the conduit containing NGF was most effective at promoting nerve repair.

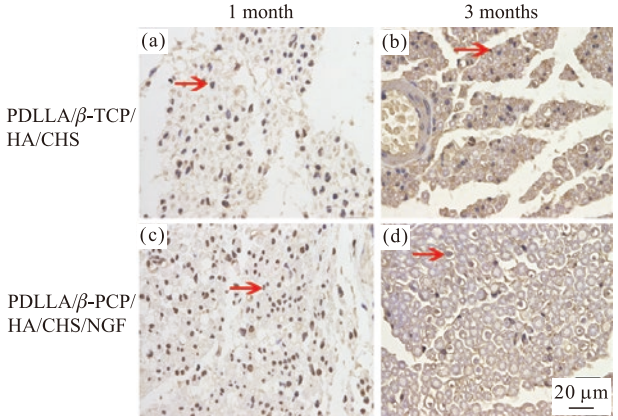

Fig.6 NGF immunocytochemistry of regenerated nerves taken from PDLLA/*β*-TCP/HA/CHS and PDLLA/*β*-TCP/HA/CHS/NGF groups at 1st month and 3rd months post-surgery. Arrows indicate the positive expression of NGF. Scale bar: 20 μm

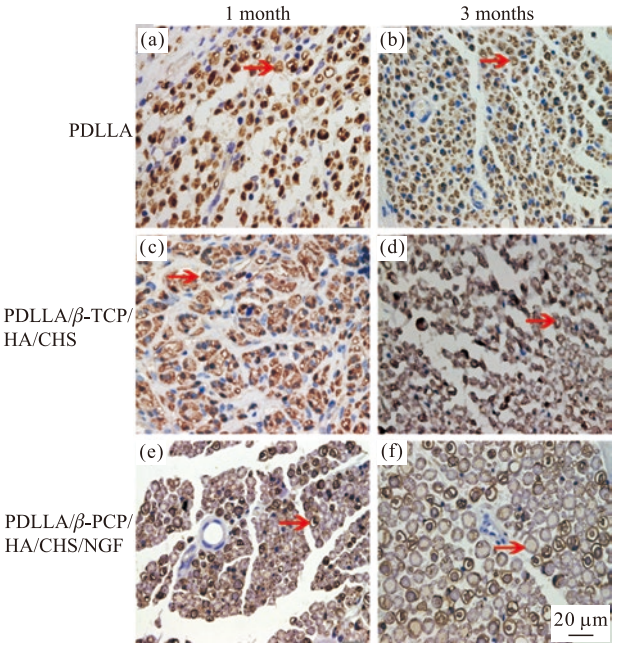

Fig.7 The S-100 immunohistochemical staining of regenerated nerves taken from PDLLA, PDLLA/*β*-TCP/HA/CHS and PDLLA/*β*-TCP/HA/CHS/NGF groups at 1st month and 3rd months post-surgery.Arrows indicate the positive reaction of S-100. Scale bar: 20 μm

3.3.4 S-100 immunohistochemistry

S-100, a relatively low molecular weight soluble

neuroglial protein, is only distributed in nerve fibers, which could provide nutrition for nerves. In the repair process of peripheral nerve, S-100 responds to the stress and reacts with SCs, participating in the process of the proliferation of nerve cells and nerve reconstruction. Therefore, the expression of S-100 can reflect the function of SCs in this process. The expression intensity of S-100 in myelin nerve fibers with different thickness and developmental maturity also differs, which allows for the developmental status of the regenerative nerve to be determined according to the level of S-100 expression.

Representative images of S-100 immunohistochemistry at 1 month and 3 months post injury are shown in Fig.7. Compared with the 1st month, S-100 positive expression in the SCs in the three conduits decreased inside the nerve bundle, and the neural structure was clear and more mature at 3rd month. Positive expression of S-100 was stronger in the PDLLA/*β*-TCP/HA/CHS/NGF and PDLLA/*β*-TCP/HA/CHS groups than that of the PDLLA group, and the regenerated nerve was stronger and more mature.

3.3.5 TEM analysis

Three months after surgery, the TEM results (Fig.8) show a large number of regenerated myelinated fibers in the autograft, PDLLA/*β*-TCP/HA/CHS and PDLLA/*β*-TCP/HA/CHS/NGF groups. Among them, the nerve fibers in the PDLLA/*β*-TCP/HA/CHS/NGF group regenerated more effeciently than those in the PDLLA/*β*-TCP/HA/CHS group. Three months after surgery, there was no significant difference (p>0.05) between the PDLLA/*β*-TCP/HA/NGF group and the autograft group.

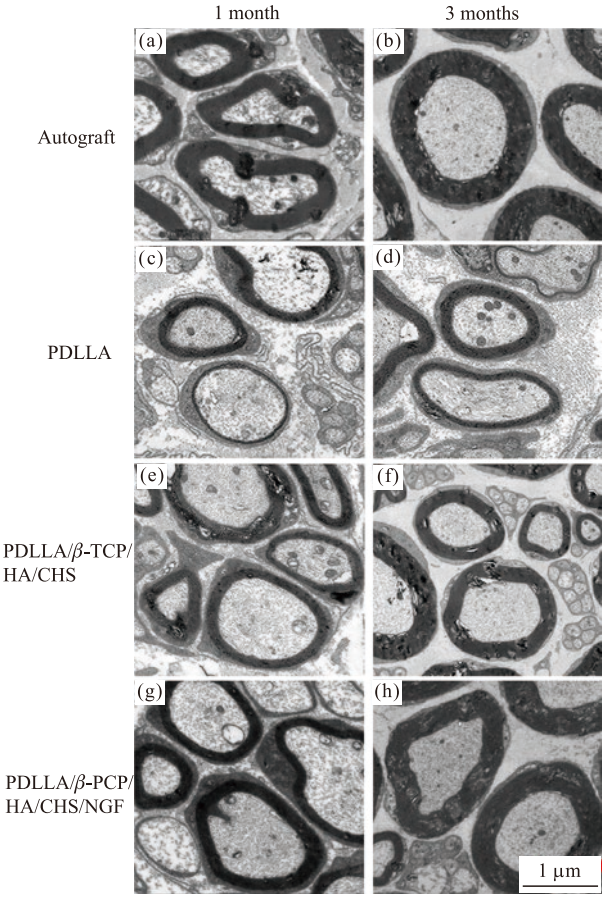

Fig.8 The regenerated nerves of four groups observed by TEM at 1st month and 3rd month post-surgery. Scale bar: 1 μm

4 Conclusions

In this study, the prepared PDLLA/*β*-TCP/HA/CHS/NGF nerve conduit, as well as the autologous nerve, PDLLA, PDLLA/*β*-TCP/HA/CHS NGCs were implanted into SD rats to compare their effects on the repair of a 10 mm nerve defect. Triceps wet weight, histological and immunological assessments showed that the regenerated neuromyelin sheath of the PDLLA/*β*-TCP/HA/CHS/NGF group was thicker and more uniform, which is similar to autograft controls and better than the PDLLA, PDLLA/*β*-TCP/HA/CHS groups. Furthermore, the PDLLA/*β*-TCP/HA/CHS/NGF NGCs are capable of slowly releasing NGF, which effectively promoted the regeneration of the peripheral nerve defect. In conclusion, the PDLLA/*β*-TCP/HA/CHS/NGF sustained-release nerve conduit is an excellent conduit material to induce nerve regeneration.

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