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Evaluation of a Novel Bioabsorbable PRGD/PDLLA/ β-TCP/NGF Composites in Repair of Peripheral Nerves

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Abstract: Peripheral nerve regeneration using a novel nerve conduit (PRGD/PDLLA/β-TCP/NGF) was evaluated, which was made of RGD peptide modified poly{(lactic acid)-co-[(glycolic acid)-alt-(L-lysine)]} (PRGD), poly(*d,l*-lactic acid) (PDLLA) and β-tricalcium phosphate (β-TCP). And the effectiveness was compared with that of PRGD/PDLLA/β-TCP, PDLLA and autograft in terms of nerve regeneration across a gap. Both of biodegradablity and cell-biocompatibility of the novel nerve conduit were evaluated in vitro. The results show that PRGD/PDLLA/β-TCP/NGF composite materials have better biodegradation properties and cell affinity than PDLLA, and could promote the RSC96 Schwann cells adhesion, proliferation and growth on the surface of materials. PRGD/PDLLA/β-TCP/NGF composite conduit was significantly superior to that of the PDLLA conduit in histological and axon morphologic index. PRGD/PDLLA/β-TCP/NGF conduit is more beneficial to nerve regeneration than PDLLA conduit. The biodegradable PDLLA/PRGD/β-TCP/NGF conduit has a good biocompatibility with rats tissue and it could effectively promote the nerve regeneration after bridging sciatic nerve defect of rats, the effect is as good as that of the autograft nerve, significantly superior to the PRGD/PDLLA/β-TCP conduit and PDLLA conduit. PDLLA/PRGD/β-TCP/NGF composite conduit is a potential ideal conduit.

Key words: peripheral nerve; nerve conduit; PDLLA/PRGD/β-TCP/NGF composite; regeneration

1 Introduction

Despite over 150 years of experience in modern surgical management of the peripheral nerve, repair of a nerve gap remains a problem in microsurgery. Widely accepted method by most surgeons is bridging the defect with an autologous donor nerve. This is associated with several disadvantages, including an extra incision for removal of a healthy sensory nerve ultimately resulting in a sensory deficit. Finally, donor graft material is limited, particularly for managing extensive lesions (*e g* brachial plexus), which requires several lengths of nerve graft. As a result, increasing efforts have been made in the last decade to better understand nerve regeneration and find alternatives to the autogenous nerve graft. Studies on nerve conduits for nerve regeneration have focused on the application of artificial nerve conduits in order to avoid sacrifice of a donor nerve. Several synthetic materials, either non-degradable[1] or biodegradable[2, 3], have been used as a nerve conduit. Non-degradable conduits is used mainly to remain in situ as foreign bodies after the nerve has regenerated. A second surgery might then be necessary to remove the conduits, causing possible damage to the nerve. Therefore, biodegradable conduits seem a more promising apparatus to reconstruct nerve gaps. However, biodegradable conduits that degrade as a function of time may lose their functional capability as a structural cuff. Neurotrophic growth factors play important roles in nerve regeneration and as a result, and there is a great clinical interest in addressing whether they can supplement damaged nerve and nerve repairs in order to enhance sensory or motor recovery, or alternatively to avoid excessive tissue inflammation and scarring[4]. Therefore, an ideal biodegradable conduit should maintain its structure integrity, permitting cell affinity and subsequent tissue ingrowth during the regenerative processes[5]. In the present study, a novel RGD peptide

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modification of poly {(lactic acid)-co-[(glycolic acid)-alt-(L-lysine)]}/ poly (d, l-lactic acid)/ β-tricalcium phosphate/nerve growth factor (PRGD/PDLLA/ β-TCP/NGF) composite nerve conduit was prepared. Its physical properties as well as biocompatibility and effectiveness as a guidance channel for peripheral nerve regeneration were evaluated.

2 Experimental

2.1 Preparation of the nerve conduits

The polymer RGD peptide modification of poly {(lactic acid)-co-[(glycolic acid)-alt-(L-lysine)]} (PRGD) was synthesized by the following steps. Firstly, (3S)-3-[4-(benzyloxycarbonylamino) butyl] morpholine-2, 5-dione (BMD) was synthesized by bromoacetyl bromide and N^{ε}-(benzyloxycarbonyl)-L-lysine. Secondly, poly {(lactic acid)-co-[(glycolic acid)-alt- (N^{ε}-benzyloxycarbonyl -L-lysine)]} (PLGLZ) was obtained by copolymerization of D, L-lactide and BMD. Then, poly {(lactic acid)-co-[(glycolic acid)-alt-(L-lysine)]} (PLGL) was synthesized by catalytic hydrogenation of PLGL. Finally, PLGL was modified with RGD peptide. PRGD/PDLLA/ β-TCP/NGF composite was prepared by using solvent volatilization method. The final length of PRGD/PDLLA/ β-TCP/NGF composites and PDLLA were fabricated to films and conduits respectively. The thickness of films was 0.15 mm. The final length of conduits was 14 mm, with an inner diameter of 2.0 mm and a tube wall thickness of 0.2 mm. The nerve conduits were sterilized with ultraviolet light for about 30 min for subsequent experiment and implantation.

2.2 Biodegradation of the never conduits

Both of PDLLA and PRGD/PDLLA/ β-TCP/NGF films were dried to constant weight and put into phosphate buffer solution (PBS, pH=7.40). The films were took out from PBS, rinsed by with distilled water after 2 w, 4 w, 6 w, 8 w, 10 w, 12 w, vacuum-dried to constant weight, and weighed respectively.

2.3 Cell-biocompatibility of the never conduits

RSC96 Schwann cells (Shanghai Institute of Cell Biology, Chinese Academy of Sciences) were cultured with Dulbecco's modified Eagle's medium (DMEM, GIBCO) with 10% fetal bovine serum (FBS, GIBCO), 1.0×10^5 U·L^{-1} penicillin (Sigma) and 100 mg/L streptomycin (Sigma). PRGD/PDLLA/ β-TCP/NGF and PDLLA films were cut into small circular pieces with a diameter of 16 mm and placed in a 24-well plate. The RSC96 cells were seeded on the films at a density of 1×10^6 cells/well, incubated in a humidified incubator at 37 ℃ and 5% CO_2. After 5 d, the films with cells attached to the surface were washed with PBS and then fixed with a 2.5% glutaraldehyde solution for 12 h at 4 ℃. Finally, the films with cells attached were examined by SEM (S-570, HTTACHI). Cell viability on the surface of films was characterized by MTT assay. The PRGD/PDLLA/ β-TCP/NGF, PRGD/PDLLA/ β-TCP, PRGD/PDLLA and PDLLA films were cut into small circular pieces with a diameter of 6.38 mm and placed in a 96-well plate. The RSC96 Cells were seeded in 96 well culture plates at a density of 1×10^5 cells/well, incubated in a humidified incubator at 37 ℃ and 5% CO_2. After culturing for 1 d, 3 d and 5 d, respectively, the supernatant was removed. 20 μL MTT solution (5 mg· mL^{-1}) was added to each culture well and incubated at 37 ℃ under 5% CO_2 conditions for 4 h. The absorbance at 490 nm was recorded by an automatic enzyme scanner (Thermo labsystem, Finland).

2.4 Animals and surgical procedure

Thirty-two Wistar rats of either sex, weighing 200-250 g were used and distributed into the following four groups: (A) PRGD/PDLLA/ β-TCP/NGF; (B) PRGD/PDLLA/ β-TCP; (C) PDLLA; (D) In situ autologous nerves. The rats of each group were removed upon sacrifice at various time points of 3 and 6 months. The rats were anesthetized by intraperitoreal injection of pentobarbital (mg·kg^{-1}). Following the skin incision, fascia and muscle groups were separated using blunt dissection, and the right sciatic nerve was severed into proximal and distal segments. The proximal stump was then secured with a single 9-0 atraumatic suture through the epineurium and the outer wall of the nerve conduits. The distal stump was secured similarly into the other end of the chamber. Both the proximal and distal stumps were secured to a depth of 1.0 mm into the chamber. The muscle layer was re-approximated with chromic gut sutures, and the skin was closed with silk sutures. All animals were housed in temperature (20 ℃) and humidity (45%) controlled rooms, and they had access to food and water ad libitum.

2.5 Electrophysiological analysis

To assess the recovery of nerve function, electrophysiological recordings were carried out under anesthesia before harvesting the nerve samples. Electromyogram/evoked potential systems (Keypoint, Danmark) were recorded to evaluate the global function of the descending and ascending systems. Stimulation was applied using a needle electrode placed through the skin in the region of the proximal nerve stem of conduit, and a recording electrode placed at the shank triceps. The peak latency and peak amplitude of the sciatic nerve action potentials were measured from the chart recordings, as

was the conduction velocity through the regenerated nerve.

2.6 Histological evaluation

Histological evaluations of nerve regeneration were made after sacrificing the rats. The observation periods ranged from 1 week to 6 months. The rats were deeply anesthetized with intramuscular ketamine hydrochloride (50 mg·kg^{-1}) and were then given an intravenously administered overdose of pentobarbital sodium before being perfused transcardially with a prewash of 0.1 M phosphate-buffered saline (PBS) followed by 1% glutaraldehyde in 0.1 M PBS. The nerves and artificial conduits on implantation sides were removed, including the reconstructed site. The tissue specimens were fixed in 1% glutaraldehyde in 0.1 M PBS for 2 h, postfixed in 2% osmium tetroxide for 12 h, dehydrated in a graded ethanol series and then embedded in Epon 812 resin. For light microscopy, 10 μm-thick serial sections were cut and stained with methylene blue (MB). Most of the nerve samples were sliced longitudinally to allow us to determine the regrowth from both nerve ends. The samples were stained with S-100 antibody. For transmission electron microscopy (TEM, Philips CM20, Netherland), the specimens removed from animals after 3 and 6 months of recovery were cut transversely into 100 nm-thick slices using an ultramicrotome and were stained with lead citrate and uranyl acetate by the Reynolds method. For scanning electron microscopy (SEM, JSM-5610LV, JEOL, Japan), the specimens were fixed by using dimethyl benzene.

Statistical significance was calculated using software SPSS10.0. Statistical significance was defined as $P \leqslant 0.05$. Data are presented as mean ± standard error.

3 Results and Discussion

3.1 Biodegradation analysis

The rate of weight loss of PRGD/PDLLA/β-TCP/NGF was 30%, 50% and 70% after soaking in PBS 4 w, 12 w and 24 w respectively. However, the weight loss of PDLLA was only 25% at 24 w. PRGD/PDLLA/β-TCP/NGF degraded faster than PDLLA during the soaking process. The degradation of low-molecular-weight PRGD and β-TCP resulted in PDLLA degraded quickly and promoted the weight loss.

3.2 Cell viability

RSC96 cells could attach and grow well on the films (Fig.1), both PDLLA and PRGD/PDLLA/β-TCP/NGF have good biocompatibility. The surface of PDLLA was smooth and displayed loosely attached cells. However, PRGD/PDLLA/β-TCP/NGF films had well-proportioned pores and promoted well both cell attachment and growth, that RSC96 cells could grow not only on the surface but also in the pore (Fig.1(b)). MMT results show that the absorbency of cell on the surface of PRGD/PDLLA/β-TCP/NGF and PRGD/PDLLA/β-TCP is higher than that of PDLLA and PRGD/PDLLA (Fig.2), and the cytoactive was in direct to the absorbency. The cell viability of the former is superior to that of the latter and is benefit to the adhesion and proliferation of RSC96 cells.

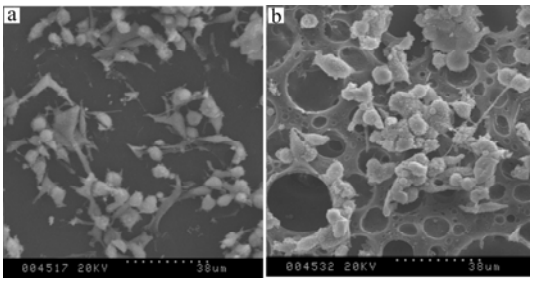

Fig.1 SEM images of RSC96 cells grow on the films after co-cultured ((a) PDLLA, (b) PRGD/PDLLA/β-TCP/NGF)

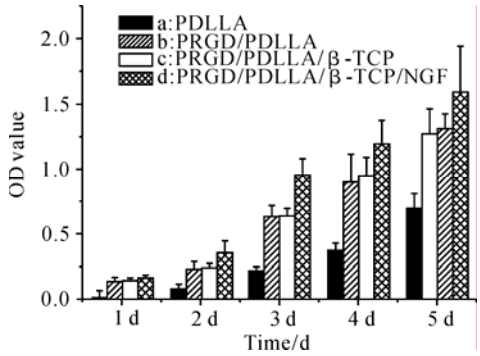

Fig.2 Results of MTT test

3.3 Gross view

None of the rats died due to surgically related or other causes before sacrifice. With respect to locomotor function, slight limping caused by bilateral foot drop was observed for up to 1 month after surgery, the operative site of all rats had a little myatrophy. By 2 months, no difficulty in walking was evident, the rats of A and D groups had obvious painful feeling in the operative site. There were no definite walking disturbances after 3 months, though the myatrophy symptom of A and D groups were more light than that of B and C groups after 3 months, and there no difference between A and D groups.

At 3 months postimplantation, nerves of A, B and C groups remained the initial shape and were thinned, without conglutination between conduits or surrounding tissue. Loose connective tissue containing plenty capillaries were formed on the conduit surface of A and B groups, the regenerate nerves connected the distal and proximal end, there was no visible scar appeared on the joint of nerves of A group and the middle piece nerve of C groups was very fine. For D groups, there was

low-grade conglutination between the autologous nerves and surrounding tissue, the nerves of the middle piece and distal end was the same size. At 6 months after implantation, the regenerate nerves of A group were similar to that of autogeneic nerves.

3.4 Electrophysiological evaluation

At 3 months postimplantation, evoked potential is detectable at the electrophysiological examination of all groups (Table 1). The sciatic nerve conduction velocity of A, B and D had significant difference ($P<0.05$) contrast to that of C group, and the difference difference between A and D was not significant ($P>0.05$), which confirmed that the nerve conduction velocity of PRGD/PDLLA/β-TCP had an evident improvement compared with that of pure PDLLA, and NGF was beneficial to improving the conductive function of nerve stem.

Table 1 Sciatic nerve conduction velocity postimplantation

Experiment group	3 m/(m • s^{-1})	6 m/(m • s^{-1})
A:PRGD/PDLLA/β	65.30±1.09 * △	69.43±0.7 * △
-TCP/NGF	62.24±0.59 * ▽	66.24±0.7 * ▽
B:PRGD/PDLLA/β-TCP	41.25±2.32	51.54±0.66
C:PDLLA	65.52±1.42	68.39±1.28
D:Autologous nerves		

3.5 Histological observations

At 3 months postimplantation, the nerve-fiber-density of A group was larger than that of B group. Both A and D groups had a mass of nerve fibers with regular round in shape, well-proportioned size and thick myelin sheath (1.10±0.11 μm and 1.08±0.12 μm). B group had plentiful nerve fibers with asymmetric size and thin myelin sheath (0.98±0.09 μm). C group had sparse nerve fibers with asymmetric size and thinner myelin sheath (0.94±0.07 μm) (Fig.3). For all groups, the diameter of nerves increased and the myelin sheath thickened after 6 months implantation (Fig.4). For A and B groups, the density of nerves increased and was lower than that of D group. The axon diameter and the thickness of myelin sheath of A group were as same as that of autologous nerves transplant (D group).

S-100 negative slice showed that there was no S-100 protein expression, which demonstrated no positive reaction. At the distal end of the regenerative nerve tissue of A, B, C and D groups, S-100-positive cells infiltrated into the tube from the proximal cut-end of the nerve funiculus, the distribution of the GFAP-positive cells was similar to that of S-100-positive cells, thereinto, A and D groups had the best positive reaction, B group took the second place, and S-100 cells were sporadic in C group (Fig.5).

At 6 months postimplantation, TEM images revealed that groups A and B had more regenerative nerves and Schwann cells than group C and were similar to group D (Fig.6). Schwann cells had an active function and abundant cytoplasm, and there were a plenty of endocytoplasmic reticulum, microbule, free ribosome and bioblast. In group A, the regenerate nerves had clear myelin layers structure and uniform thickness, which was approximately 47 layers and got close to the normal nerves (50 layers). For groups A and D, the regeneration nerves had intact myelin sheath, which had compact layers structure, and the axolemma closely bordered on the myelin membrane. The diameter of the myelinated axons the thickness of the myelin-wall on groups A and D was larger than that on groups B and C. TEM images revealed that the presence sparse regenerate myelinated fiber with thin myelin-wall in group C.

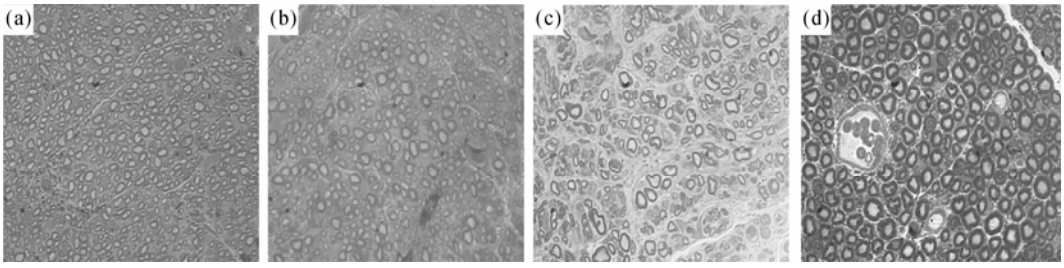

Fig.3 MB staining images for regenerative nerves after 3 months implantation ((a) A: PRGD/PDLLA/β-TCP/NGF; (b) B: PRGD/PDLLA/β-TCP; (c) C: PDLLA; (d) D: In situ autologous nerves)

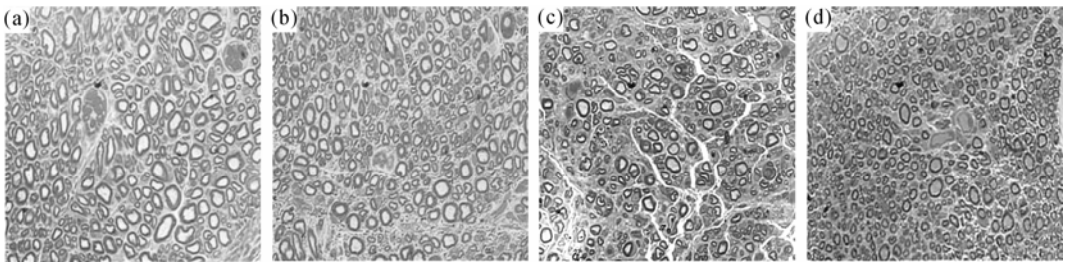

Fig.4 MB staining images for regenerative nerves after 6 months implantation ((a) A: PRGD/PDLLA/β-TCP/NGF; (b) B: PRGD/PDLLA/β-TCP; (c) C: PDLLA; (d) D: In situ autologous nerves)

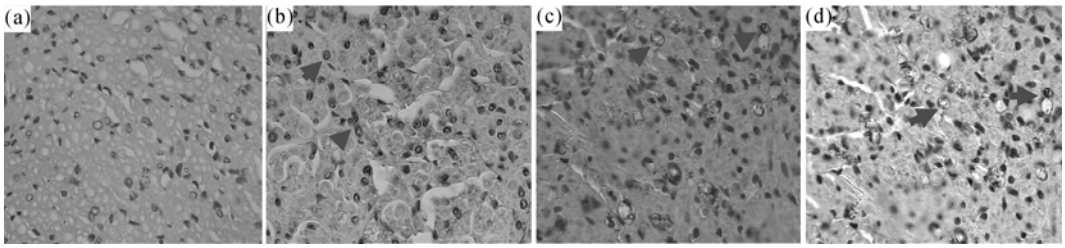

Fig.5 Immunocytochemical staining images of regenerative nerves at 6 months postsurgery ((a) S-100 negative slice, (b) A: PRGD/PDLLA/ β-TCP/NGF, (c) B: PRGD/PDLLA/ β-TCP, (d) D: In situ autologous nerves)

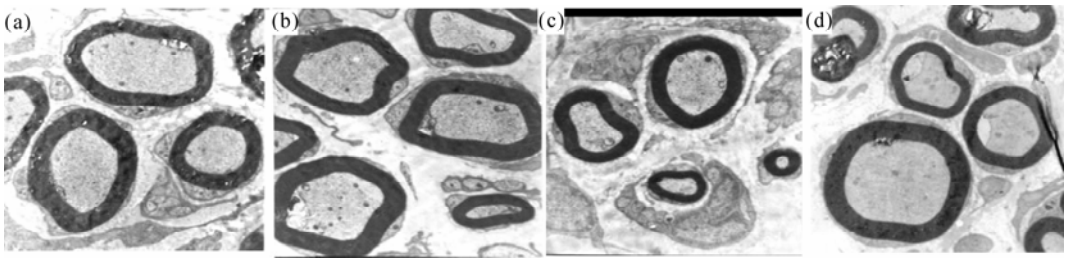

Fig.6 TEM images of the regeneration nerve after 6 months implantation ((a) A: PRGD/PDLLA/ β-TCP/NGF, (b) B: PRGD/PDLLA/ β-TCP, (c) C: PDLLA, (d) D: In situ autologous nerves)

SEM results reveal that PRGD/PDLLA/ β-TCP/NGF composite degrades more quickly than PDLLA(Fig.7). Many micropores appear on the surface of PRGD/PDLLA/ β-TCP/NGF after 3 months implantation. The number and size of micropores became larger on the surface of PRGD/PDLLA/ β-TCP/NGF after 6 months of implantation, resulted from the degradation of β-TCP and the releasing of NGF.

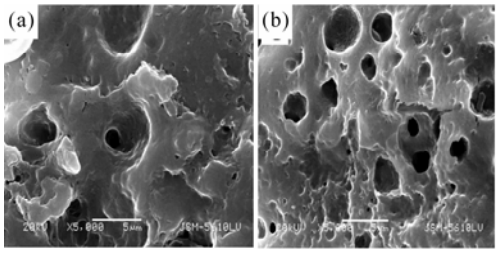

Fig.7 SEM images of PRGD/PDLLA/ β-TCP/NGF after implantation, (a) 3 months, (b) 6 months

Poly-DL-lactic acid (PDLLA) has been widely used in surgical repair, as carriers in drug delivery, and temporary matrixes or scaffolds in tissue engineering [6, 7] due to its superior biodegradability, biocompatibility, high mechanical properties, and excellent shaping and molding properties. However, its further application was limited in the fields of tissue engineering because its poor cell affinity. To improve the cell attachment on such a material, various approaches have been taken. Adsorption of adhesive proteins to PDLLA surface was usually attempted[8, 9], but the retention time of the adhesive proteins was too short for practical application. Poly (lactic acid-*co*-lysine) has been synthesized to incorporate free amines that were then used to graft RGD peptides to promote cell attachment[10, 11]. RGD is the activity sequence of extracellular matrix protein of the RGD and plays an important role in the mediated cell adhesion, migration and growth. Immobilization of RGD peptides on PDLLA surface is a good procedure. We synthesized (3S)-3-[4-(benzyloxycarbonylamino) butyl] morpholine-2, 5-dione (BMD) and copolymerized with D, L-lactide to give poly {LA-*co*-[Glc-*alt*-Lys (Z)]}. When the protective group was removed, poly [LA-*co*-(Glc-*alt*-Lys)] was obtained. Poly [LA-*co*-(Glc-*alt*-Lys)] was modified with RGD peptide in the presence of 1, Í-carbonyldiimidazole (CDI). In order to eliminate the side-effect of the acid degradation product of PDLLA, β-TCP was composited with PRGD/PDLLA due to its basic degradation product, good biodegradability and biocompatibility. It is possible to farther enhance the nerve regeneration by applying growth factors, and NGF was also compounded with the composite.

In contemporary neurosurgery, reconstruction with an autograft has been standard procedure for a nerve gap defect, and is now widely used daily in clinics as the gold standard[12]. Therefore, the present study was designed to determine whether the artificial nerve conduits can be used as an effective alternative to the conventional autograft, by comparing the performance of both, concurrently, in the same animal. In most of the previous reports, histological evaluation was mainly performed on transverse sections of the regenerated nerves. However, because the main function of a nerve is to transfer electrical signals rapidly to another part of the body, histological recognition of tissue recovery alone provides insufficient evidence of the degree of recovery achieved. We believe that it is necessary to assess not only the morphology, by histological examination, but also functional (electrophysiological) recovery. We therefore used the rat as an

experimental animal because it is possible to make appropriate electrophysiological evaluations in these animals, and intravenous administrations of muscle relaxant are easy to repeat.

The results of the present study provide interesting findings that β-TCP can neutralize the acidity which produced by degradation of PDLLA and PRGD, that availably adjust the pH value of degradation medium to keep at neuter. Adding PRGD and β-TCP can promote the degradation of the composite films, so that PRGD/PDLLA/β-TCP/NGF composite has better biodegradation and compatibility properties than PDLLA. PRGD/PDLLA/β-TCP/NGF also has a better cell affinity than PDLLA, which can promote RSC96 Schwann cells adhesion, proliferation and growth on the surface of materials. Regenerated nerve fibers of PRGD/PDLLA/β-TCP/NGF composite were significantly superior to that of PDLLA in histological, axon morphologic index and electrophysiology. PRGD/PDLLA/β-TCP/NGF is more beneficial to nerve regeneration than PDLLA, has good compatibility with rat's tissue and can effectively promote nerve regeneration after bridging sciatic nerve defect of rats. The effect is as good as that of autograft.

4 Conclusion

In comparison with autografting and PDLLA conduit, our newly developed nerve conduit enabled superior functional recovery. Although electrophysiological and physiological recovery reached nearly 100% of normal values after only 6 months, the results of this preliminary experiment suggests that the pattern of functional recovery was better on PRGD/PDLLA/β-TCP/NGF than on PDLLA and was as good as on the autograft. To confirm further the superiority of this conduit compared to autografting, more experimental studies are required to clarify whether it could be an effective alternative. Our results indicate that PRGD/PDLLA/β-TCP/NGF composite shows great promise and can serve as biodegradable scaffold for cell and tissue engineering.

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