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Slow-releasing rapamycin-coated bionic peripheral nerve scaffold promotes the regeneration of rat sciatic nerve after injury

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ABSTRACT

Aims: To investigate the effect of locally slow-released rapamycin (RAPA) from the bionic peripheral nerve scaffold on rat sciatic nerve regeneration in the early phase of nerve injury.
Main methods: Slow-releasing RAPA-polyhydroxy alcohol (PLGA) microspheres were prepared and tested for microsphere diameter and slow-release effect in vitro after loading onto nerve scaffold. A total of 48 male SD rats were randomly divided into control group and 3 experimental groups as follows: group 1: RAPA-PLGA scaffold; group 2: RAPA scaffold; and group 3: scaffold alone. In the control group, a 15 mm sciatic nerve was excised and religated reversely. In the experimental groups, the scaffolds were used to bridge a defect of 15 mm sciatic nerve. The outcome of nerve regeneration was evaluated using neurophysiological and neuromuscular morphological techniques.
Key findings: The RAPA-PLGA microspheres displayed a smooth exterior. The slow-release of RAPA in group 1 lasted for 14 days. The sciatic nerve function index (SFI) and electrophysiological and morphological features were examined 12 weeks after the surgery in all groups to reveal various degrees of ipsilateral sciatic nerve regeneration. The SFI values at 12 weeks showed no significant difference between the RAPA-PLGA scaffold and control groups; morphological observations revealed that the outcomes of nerve regeneration in the above 2 groups were similar and significantly better than those in the RAPA scaffold and scaffold alone groups.
Significance: RAPA-PLGA microsphere-loaded bionic peripheral nerve scaffold gradually released RAPA locally in the early phase of sciatic nerve regeneration, reduced the secondary nerve injury, and evidently promoted the regeneration of peripheral nerve.

Introduction

Repairing and replacement therapy of the injured long segment of the peripheral nerve is an effective therapeutic remedy with the use of bionic peripheral nerve scaffold, which represents a difficult task for clinicians. Great progress has been made in design and construction of scaffold based on tissue-engineering in recent years [1,2]. Existing reports and our previous work showed that highly-bionic tissue-engineered peripheral nerve scaffold provided temporary support to a long segment of impaired nerve and guided the regenerating axons to grow through the damaged segment [3,4]. And it has also been shown that certain regeneration effects were achieved in repairing 10 mm defect of rat sciatic nerve in some in vivo studies. However, there are still ample gaps present between the clinical requirement and the currently-achieved regeneration speed and functional recovery. The main obstacle is the inflammatory response developed in the injured peripheral nerves. Normally peripheral nerves are in an immune-privileged environment, while they are exposed to the inflammatory environment severe local inflammatory response and infiltration of inflammatory cells (dominated by leukocytes) after injury. In addition, different species of collagen protein and chitosan, as the major components of tissue engineering scaffolds, further worsen the inflammatory response in the bridging area. These inflammatory cells readily penetrate through the porous structure of the wall of the scaffolds and enter the interior microtubules to clear myelin debris and necrotic cells. However, these cells unfavorably impacted axons, hampering nerve regeneration [5–7]. The subsequent proliferation of the connective tissue and scar tissue formation may further aggravate traumatic neuroma, thus impeding the nerve regeneration.

Previous studies have shown that macrolide immunosuppressant can significantly promote the interaction between neuronal cells, and help the injured tissues build a conducive microenvironment for nerve repair [8–10]. However, the relatively large doses used, as well as the traditional route of drug administration are difficult to operate and hence not feasible for the routine clinical application [11,12]. Rapamycin (RAPA) is a new type of macrolide immunosuppressant possessing

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immunosuppressive effect on T lymphocytes and other immune cells by inhibiting the cell cycle process from G1 to S phase, via blocking the signal transduction of different cytokine receptors. Although RAPA, cyclosporin A, and FK506 have a similar structure and anti-rejection mechanism, RAPA is considered to have more advantages for its potent immunosuppressive action and that recommended dosage is less than one tenth of that of other immunosuppressants. Thus, RAPA has been employed as a preferred safe and effective immunosuppressive agent with minimal nephrotoxicity and no neurotoxicity [13]. More importantly, RAPA is chemically stable with high solubility in organic solvents, and its pharmacological effects as an immunosuppressant are not altered by encapsulating in a microsphere as slow-releasing agent. In addition, the low effective dose of RAPA also makes it an ideal candidate for our local slow-release technology.

These advantages of RAPA make it an ideal candidate for local slow-release in repairing the injured nerves. Thus, we prepared RAPA-PLGA microspheres and loaded them onto the scaffold in the current study, which was then implanted into the experimental animal at the early phase of the peripheral nerve injury. And then the benefit of local slow-release of RAPA in reducing the secondary nerve injury was investigated.

Materials and methods

Reagents, animals, and instruments

RAPA was purchased from Ruibio (Ingelheim, Germany); PLGA (RG503H, LA/GA = 50:50, Mw: 13,000–23,000) was from Boehringer Ingelheim (Ingelheim, Germany); type I collagen (C9879) and gelatin (G9382) were from Sigma-Aldrich (St. Louis, MO, USA); geniposide was the product of Challenge Bioproducts (Taiwan, China), Mek-5216k Hematology Analyzer was from Nihon Kohden (Tokyo, Japan); ACUSON Cypress digital echocardiography system was from Siemens (Harrisburg, PA, USA); Fluoro-Gold (FG) was from Fluorochrome (Denver, CO, USA); overhead blender RW20 was the product of IKA Works (Wilmington, NC, USA); alpha2-4 freeze dryer was from Martin Christ (Osterode, Germany); laser scanning confocal microscope was the product of Leica (Wetzlar, Germany); scanning electron microscope (SEM) was from Hitachi (Tokyo, Japan); Viking IV electrophysiological apparatus was the product of Nicolet (Madison, WI, USA); chromatography column was from Waters Corporation (Milford, MA, USA); and laser particle size analyzer was the product of Beckman (Pasadena, CA, USA) micro-speed adjusting instrument was self-made. A total of 48 male SD rats, weighing 207 ± 15 g, were provided by the Experimental Animal Center of Fourth Military Medical University, Xi'an, China.

Ethics statement

The authors state that they have obtained appropriate institutional review board approval and have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. All the experiments were carried out in strict accordance with the guidelines issued by the Ethical Committee of The Fourth Military Medical University. All the animals received humane care, all surgical procedures were performed under general anesthesia with infusion of 1% pentobarbital solution (0.4 mL/100 g body weight) and all efforts were made to minimize suffering. All operations were carried out under sterile condition, total experimental time was 6 to 8 months.

Preparation of slow-releasing RAPA-PLGA microspheres

The RAPA-PLGA microspheres were prepared by solvent evaporation method according to previous studies [14,15] and our pre-experiment. PLGA (2 g) was dissolved in 20 mL of dichloromethane, added with 200 mg of RAPA at a ratio of 1:10 (w/w), and mixed thoroughly by shaking. The oil phase of the above mixture was added dropwise into 0.5% polyvinyl alcohol (PVA) solution to obtain homogenized O/W emulsion under the action of a high pressure homogenizer (3000 rpm, 5 min); the emulsion was stirred continuously at room temperature (400 rpm, 4 h) to remove the volatile organic solvent, and was further centrifuged at 3000 rpm for 5 min to collect the microspheres, which were then washed 3 times and finally freeze-dried to obtain the RAPA-PLGA microsphere powder.

Determination of the morphology and the encapsulation efficiency of RAPA-PLGA microspheres

Appropriate amount of PLGA microspheres was fixed onto a clean dry silicon wafer and coated with gold under vacuum condition. The morphology and surface features of PLGA microspheres were observed under a current of 10 mA and an accelerating voltage of 5 kV. The size distribution of microspheres was measured using laser particle size analyzer. The encapsulation efficiency of RAPA in the microspheres was determined using high performance liquid chromatography (HPLC). Briefly, 10 mg microspheres were dissolved in 1 mL of acetonitrile, sonicated for 10 min to dissolve, and centrifuged at 3000 rpm for 15 min; the supernatant was collected and detected for drug content on Waters 2695 HPLC system under the following conditions: Waters symmetry C18 column (150 × 4.6 mm, 5 μm), isocratic elution, mobile phase (10 mM ammonium acetate–0.1% formic acid aqueous solution:0.1% formic acid methanol solution [10:90]), flow rate 1 mL/min, injection volume 20 μL, and detection wavelength 210 nm.

$$\text{Encapsulation efficiency}\ (\%) = (L_a/L_t) \times 100 \left(L_a : \text{actual drug content in microspheres}, L_t : \text{input content of drug}\right).$$

$$\text{Drug loading rate}\ \% = (P_t/M_t) \times 100 \left(P_t : \text{drug weight in microspheres}, M_t : \text{weight of microspheres}\right).$$

Preparation of RAPA-PLGA scaffold

Separately, chitosan (70 mg) and collagen I (280 mg) were weighed on an electronic scale, placed into 20 mL beaker, added with 10 mL of 3-time-distilled water and 20 μL of 4% acetic acid (pH 3.2), sealed, and placed in a 4 °C refrigerator to dissolve for 24 h. The above 2 solutions were then stir-mixed with homogenizer in ice-water bath stirred at 5000 rpm for 60 min. The thoroughly-mixed solution was then divided evenly into 3 parts. According to the predetermined microsphere drug loading rate, in group 1, 26 mg RAPA-PLGA microspheres were added into the mixed solution, to obtain a final RAPA content of 2 mg; in group 2, 2 mg of RAPA was directly added into the mixed solution; and in group 3, no ingredients were added. After slightly stirring, the mixture was quickly injected into a silicone tube (3 mm in diameter and 20 mm in length), both ends of which were occluded with a lead wire. The variously-treated silicone tubes were slowly placed into liquid nitrogen along the axial direction using a self-made micro-speed adjusting instrument at a speed of 2×10^{-5} m/s. After fully submerged, the mold was incubated in liquid nitrogen for 4 h, then removed, and placed onto a prechilled aluminum pan, which was then transferred to a vacuum dryer to freeze–dry the tubes under vacuum condition for 8 h to obtain the scaffold of nerve scaffold, which was then crosslinked in 1% genipin solution at 37 °C for 24 h [16,17]. The obtained scaffold with good tenacity was freeze-dried at −40 °C, 100 mTorr for 24 h again, until the scaffold was thoroughly dry and springy. Part of the material was sampled, fixed, gold-coated, and observed under SEM. The well-structured scaffold was segmented and sterilized under Cobalt-60 radiation.