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Rapid repair and regeneration of damaged rabbit sciatic nerves by tissue-engineered scaffold made from nano-silver and collagen type I

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ABSTRACT

A tissue-engineered scaffold with nano-silver and collagen type I was constructed and investigated for its ability to adsorb laminin and the usefulness in the repair and regeneration of damaged peripheral nerves in animals. The nano-silver scaffold displayed ideal microtubule structure under electronic microscope; even distribution of the nano-silver particles was also seen with energy spectrometry. After immersion in a laminin solution, the laminin-attached scaffolds were implanted into rabbits to repair a 10-mm injury of the sciatic nerve. At 30 days post-implantation, regeneration of the damaged nerve was evaluated by transmission electron microscopy, electrophysiological examination and fluoro-gold (FG) retrograde labelling. Compared with the control collagen-scaffold without nano-silver, the nano-silver-containing scaffold showed a higher rate of laminin adsorption, regenerated a nerve with a thicker myelin sheath and improved the nerve conduction velocity and nerve potential amplitude. FG retrograde labelled the newly grown axons in the spinal cord cortex anterior horn and the dorsal root ganglion. These results demonstrate the superior functionality of the nano-silver–collagen scaffold in the adsorption to laminin and subsequent regeneration of damaged peripheral nerves.

Introduction

The regeneration of defective or damaged peripheral nerves has been a big challenge for neuroscience and the use of biocompatible tissue-engineered scaffolds for this purpose has become a significant research topic. Bridging the nerve stumps through nerve conduits can induce the longitudinal outgrowth of the axons while avoiding the formation of neuroma. Since this approach provides a relatively isolated micro-environment for nerve regeneration, it is conducive for the repair of the peripheral ganglions.

Currently, tissue-engineered scaffolds are made of natural materials such as blood vessels and collagens, as well as synthetic polymers such as polylactic acid (PLA) and polyglycolic acid (PGA). Since PLA and PGA reduce pH values during their degradation and the lower pH then, in turn, can speed up the degradation process, this cycle thus produces a strong acidic environment deleterious to cellular functions. The construction of scaffolds with natural extracellular matrix (ECM) components has become the new direction of tissue engineering. Collagens and gelatine are the main components of ECM. Collagens can promote cell adhesion and proliferation. They are non-immunogenic and their degradation products are not cytotoxic. They have been shown to participate in the tissue-healing process and have been widely used in burn wound dressings. They have also been shown to induce and improve the regeneration of peripheral nerves.[4]

Cations at the surface have been shown to promote the interaction of the scaffold with the attached proteins thus increasing the amount of protein attachment; scaffolds made of cation-rich materials such as chitin achieved good laminin attachment.[18] Silver ions can constantly release divalent cations via ionisation equilibrium and the electrostatic repulsion can prevent aggregation of the particles to ensure their even distribution. Based on this finding, we chose the nano-silver as the supplement to engineer our scaffolds. Furthermore, the scaffolds prepared by freeze-drying methods exhibit high porosity and strong hygroscopicity, so we used these methods to prepare ours.[3]

To offer the experimental basis for the clinical applications of tissue-engineered nerves in the implantation and repairs of defective nerves, we constructed a nerve tissue-engineered scaffold using laminin as the seeding protein to promote nerve regeneration and the freeze-dried nano-silver–collagen as the scaffold material.[13,15] The resulting axial structural tissue-engineered nerves[12] were implanted to repair a 10-mm sciatic injury in adult rabbits; the outcome of the nerve regeneration was

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evaluated by morphological and electrophysiological examinations 30 days after implantation.

Materials and methods

Reagents and instruments

Collagen type I and gelatine were both from Sigma, USA; fluorogold (FG) was from Fluorochrome; US and Leica CM3050S CryoStat and TCS SP2 laser scanning confocal microscope were both from Leica, Germany. HITACHI S-3400 Scanning Electron Microscope, HORIBA 7021-H Energy Spectrometer and EMAX ENERGY software were used throughout this study. Nano-silver was purchased from Shanghai Tinaph Nano-Tech Co., Ltd.

Preparation of the collagen scaffold with or without nano-silver

Collagen type I (150 mg) and gelatine (75 mg) were dissolved into 0.05 mol l^{-1} acetic acid solution. After stirring at 18,000 rpm for 90 min at 4 °C, the solution was vacuumed under a negative pressure and stored overnight at 4 °C. The gel suspension was formed after thorough mixing and then applied to a 3 mm × 10 cm silica column. The materials were then divided into two groups. In the experimental group, 1/10 volume of nano-silver solution at a concentration of 20,100 ppm was added, and in the control group, there was no addition. The columns were then sealed at both ends and immersed into the coolant at the speed of 2×10^{-5} m s^{-1}, made possible by a home-made micro-speed controller. Later, both the gel suspension and the cooled product were placed in aluminium plates and then freeze-dried at −40 °C, 100 mTorr for 48 h. The dried collagen scaffolds were then cross-linked by Genipin, sterilised by cobalt^{60} sealed and stored.[5]

Observation of the interior of the scaffolds with a scanning electron microscope

After metalling and coating, the scaffolds were cut into both, cross-sections and longitudinal sections, for scanning electron microscope (SEM) examinations and the measurements of the diameters of the micro-pores. The distribution of the nano-silver particles was examined by energy spectrometry.

Adsorption of laminin by the scaffolds

Scaffolds of both groups were immersed in a laminin solution and were pressed to remove the air inside them and allow the protein suspension to enter the scaffolds. After 6 h, they were transferred to clean water and then gently pressed several times to remove most of the free protein molecules inside. They were then dried by freeze-drying, and cut into longitudinal sections for SEM observation of the laminin adsorption.

Surgery for bridging the defected sciatic nerves

Twenty male New Zealand rabbits were divided into two groups of 10. They were anaesthetised by intraperitoneal (i.p.) injection of 3% pentobarbital sodium at 30 mg kg^{-1}. After routine skin preparation and disinfection, the left sciatic nerves were bluntly separated along the spatium intermusculare and a 10-mm section was removed. The scaffold from each group was applied to the injury to bridge the gap and the surgical opening was fixed by non-traumatic suture. All procedures were carried out in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health publication no. 85-23, revised 1985).

Histological examinations

The regenerated nerve tissues were dissected 30 days after the surgery and cut into ultra-thin sections for toluidine blue staining and observations under a transmission electron microscope (TEM) by the standard protocols. They were then observed under a light microscope or by SEM for the density of axons and the thickness of the myelin.

Electrophysiological examination

Sciatic nerves were exposed 30 days after the above-mentioned surgery from both legs and subjected to electrophysiological examination with an electric physiological instrument *in vivo*. The nerve potential amplitude (AMP) and nerve conduction velocity were detected.

Retrograde dye FG labelling

Retrograde fluorescent dye FG was used to trace the injured peripheral axons. Rabbits were anaesthetised by i.p. injection of 3% pentobarbital sodium at 30 mg kg^{-1} 30 days after the surgery. After routine skin preparation and disinfection, sciatic nerves including the bridged sections were exposed. FG in 5% physical saline was injected for a total volume of 1 μl at four sites of the sciatic nerve under the 10-mm injury distal of the implant. A micro-sampler was used for the injection; after retaining it inside the injection sites for a few seconds, the sampler was retrieved slowly and the surgical opening was closed. Two weeks later, retrogradely labelled neurons in the dorsal root ganglia (DRG) and spinal cord ventral horn were assessed by measuring the number of FG-positive neurons under a UV illuminator (40× magnification for 20 fields).

Statistical analysis

All results are expressed as mean ± S.E.M. Statistical significance was evaluated using analysis of variance by using SPSS version 10.0 (SPSS, Inc., Chicago, IL, USA). A value of $p < 0.05$ was considered to denote statistical significance.

Results

Structures of the nano-silver–collagen scaffold

As described in the earlier section, collagen scaffolds containing nano-silver were constructed by freeze-drying. Under SEM, a newly constructed scaffold was in a cylindrical shape (Fig. 1A). Its cross-section showed microtubules of uniform diameters (Fig. 1B), and the longitudinal section displayed the parallel arrayed interstructure of microtubules (Fig. 1C). Since the diameters of these microtubules were about 20–80 μm, similar to that of the sciatic nerve, they may provide the passage for the nerve signal transduction that is essential for the function of nerve fibres. Energy spectrometry showed that the nano-silver particles distributed evenly inside the nano-silver–collagen scaffold (Fig. 2A) but none were found in the control scaffold (Fig. 2C). Energy spectrometry also confirmed the even distribution of silver particles inside the nano-silver–collagen scaffold but not the control scaffold (Fig. 2B).

Adsorption of scaffolds to laminin

Collagen scaffolds adsorbed laminin proteins after immersion in a laminin solution. Under SEM, laminin proteins were seen to distribute evenly and attach tightly to the inner surface of the