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Construction of nerve guide conduits from cellulose/soy protein composite membranes combined with Schwann cells and pyrroloquinoline quinone for the repair of peripheral nerve defect

Lihua Luo [a, b, 1], Li Gan [a, 1], Yongming Liu [a], Weiqun Tian [a], Zan Tong [a], Xiong Wang [c], Celine Huselstein [c], Yun Chen [a, *]

[a] Department of Biomedical Engineering, School of Basic Medical Sciences, Wuhan University, Wuhan 430071, China
[b] Center of Molecular Medicine, School of Medicine, Hubei University of Arts and Sciences, Xiangyang 441053, China
[c] Ingénierie Moléculaire et Physiopathologie Articulaire (IMoPA), UMR 7365 CNRS – Université de Lorraine, Biopôle, 54500 Vandœuvre-lès-Nancy, France

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ABSTRACT

Regeneration and functional reconstruction of peripheral nerve defects remained a significant clinical challenge. Nerve guide conduits, with seed cells or neurotrophic factors (NTFs), had been widely used to improve the repair and regeneration of injured peripheral nerve. Pyrroloquinoline quinone (PQQ) was an antioxidant that can stimulate nerve growth factors (NGFs) synthesis and accelerate the Schwann cells (SCs) proliferation and growth. In present study, three kinds of nerve guide conduits were constructed: one from cellulose/SPI hollow tube (CSC), another from CSC combined with SCs (CSSC), and the third one from CSSC combined with PQQ (CSSPC), respectively. And then they were applied to bridge and repair the sciatic nerve defect in rats, using autograft as control. Effects of different nerve guide conduits on the nerve regeneration were comparatively evaluated by general analysis, sciatic function index (SFI) and histological analysis (HE and TEM). Newly-formed regenerative nerve fibers were observed and running through the transparent nerve guide conduits 12 weeks after surgery. SFI results indicated that the reconstruction of motor function in CSSPC group was better than that in CSSC and CSC groups. HE images from the cross-sections and longitudinal-sections of the harvested regenerative nerve indicated that regenerative nerve fibers had been formed and accompanied with new blood vessels and matrix materials in the conduits. TEM images also showed that lots of fresh myelinated and non-myelinated nerve fibers had been formed. Parts of vacuolar, swollen and abnormal axons occurred in CSC and CSSC groups, while the vacuolization and swell of axons was the least serious in CSSPC group. These results indicated that CSSPC group had the most ability to repair and reconstruct the nerve structure and functions due to the comprehensive contributions from hollow CSC tube, SCs and PQQ. As a result, the CSSPC may have the potential for the applications as nerve guide conduits in the field of nerve tissue engineering.

1. Introduction

At present, peripheral nerve defects caused by various kinds of trauma have an obvious upward trend. Autologous nerve graft is the “golden standard” to repair the peripheral nerve defects [1,2]. However, autologous nerve graft is limited due to the lack of donator resources and an extra incision to remove a healthy sensory nerve from the donator [3]. With the rapid development of tissue engineering, many researchers have focused on the repair of peripheral nerve defects by using nerve guide conduits, which show great potentials for nerve regeneration and reconstruction of nerve functions [4,5]. Materials used as nerve guide conduits include non-degradable and degradable biomaterials. The nerve conduits prepared from non-degradable biomaterials remain *in situ* as a foreign body, causing a chronic tissue response, nerve compression, and infection [6,7]. As a result, the nerve guide conduits prepared from biodegradable materials have attracted much more attention [8]. An ideal nerve guide conduit must be biocompatible, biodegradable, soft and flexible, and semipermeable, which can provide a guidance cue *via* 3D tubular structure,

* Corresponding author. Fax: +86 27 68759142.
E-mail address: yunchen@whu.edu.cn (Y. Chen).
[1] Authors equally contributed to the work.

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prevent fibrous tissue from ingrowth and meet technical requirements for further production, sterilization, long-term storage and surgical handling [9]. It not only can give a support for axons regeneration, but also can guide the axons regeneration and construct a necessary microenvironment for nerve regeneration. For example, synthetic or natural biodegradable polymers such as laminin, collagen, poly-glycerol sebacate, polyglycolic acid (PGA), chitosan, silk and polylactic acid (PLA) [2,10–15] have been used as nerve guide conduits. However, some materials can bridge long gaps successfully and few conduits have the properties as expected to be an ideal nerve guide conduit [10]. It is necessary to explore and develop more new materials for the construction of nerve conduits with potential clinical applications to repair nerve defects.

Cellulose is the most abundant natural polymer, which is widely used in biomedical engineering fields due to its biodegradability, biocompatibility, and nontoxicity to cells and tissue [16]. Soy protein isolate (SPI) is an attractive plant protein with potential applications in biomedical fields because of its good bioactivity, biodegradability, biocompatibility and processability [17]. A series of cellulose/soy protein composite membranes with suitable mechanical properties and biocompatibility have been prepared from cellulose and SPI in our previous work [18]. Due to the good processability of cellulose/SPI composite membranes, it is easy to fabricate hollow tubes from the cellulose/SPI composites. Therefore, based on the processability, mechanical properties, cytocompatibility and biodegradability of cellulose/SPI composites, we hypothesized that the hollow tubes prepared from cellulose/SPI composite membranes may meet the demands in structure and performance as nerve conduits.

Schwann cells (SCs) are crucial components in the microenvironment of peripheral nerve regeneration [19]. SCs play an important role during nerve regeneration through the production of growth factors and the excretion of extracellular matrix [20]. Considering the importance of SCs in nerve regeneration process, SCs were always used as seed cells and co-cultured with polymer-based tubes for the construction of nerve conduits [21]. At the same time, neurotrophic factors (NTFs) show positive function for nerve regeneration [14,22]. As a result, nerve guide conduits were usually constructed using biocompatible polymer tubes as conduit matrix, SCs as seed cells and NTFs as nerve growth nutrition. Pyrroloquinoline quinone (PQQ) is one kind of NTFs, which has been proved able to be used to enhance SCs proliferation and migration [23,24]. However, its potentials for nerve repair when combined within polymer conduits were rarely investigated [25,26]. Thus, in this work, we tried to construct a new kind of nerve guide conduit using cellulose/SPI composite membranes as conduits, SCs as seed cells and PQQ as NTF. The potentials of this new nerve conduit for the repair of peripheral nerve defect were evaluated *in vivo*. It was expected that the cellulose/SPI conduit, SCs and PQQ will respectively play important and different roles for the promotion of nerve regeneration during the nerve repair process.

2. Materials and methods

2.1. Preparation of hollow tubes based on cellulose/SPI composite membranes

Cellulose/SPI solution was prepared according to our previous work [18]. Briefly, cellulose and NaOH/urea aqueous solution were separately pre-cooled in the refrigerator freezer. When the temperature of NaOH/urea aqueous solution dropped to −12 °C, cellulose were quickly immersed into the solvent and then vigorously stirred for about 5 min to get a transparent cellulose solution. The concentration of cellulose was 3.5 wt%. SPI was dissolved in the above NaOH/urea aqueous solution to get a slurry with SPI content of 3.5 wt%. The cellulose solution and SPI slurry were mixed and stirred to obtain cellulose/SPI mixture solution containing 30 wt% of SPI, based on weight percent of SPI in total mass of cellulose and SPI. The cellulose/SPI solution was stirred at room temperature for 30 min and degassed at 10 °C by centrifugation for 10 min at 7500 × g. The degassed solution was injected into a special tubular mold and coagulated into 5 wt% acetic acid aqueous solution for 5 min to obtain cellulose/SPI hollow tubes with inner diameter of 1.5 mm and outer diameter of 1.8 mm. This kind of hollow tube could be sterilized by autoclaving and then used as nerve guide conduit (coded as CSC) for the repair of nerve defect in the rat.

2.2. Construction of nerve guide conduits from CSC and co-cultured SCs

To construct nerve guide conduits from cellulose/soy protein tubes and SCs, SCs of neonatal Sprague–Dawley rats were used as seed cells and cultured into the above sterilized CSC according to procedures described previously [27]. After 90% confluence, the SCs were digested with 0.25% trypsin and the density was adjusted to 1×10^7 cells/mL. The pre-wetting sterilized conduits were put into 24-well plate and 100 μL cell suspensions were seeded onto the inner wall of CSC. After 3 h, another 900 μL DMEM culture medium were added into the well, and incubated at 37 °C, 5% CO_2 humidified atmosphere for 24 h. This kind of nerve guide conduits constructed from CSC and co-cultured SCs were coded as CSSC.

2.3. Construction of nerve guide conduits from CSC, SCs and PQQ

The sterilized CSC was placed into the 24-well plates. SCs were detached and re-suspended in DMEM medium (1×10^7 cells/mL). The cell suspensions were injected into CSC (100 μL for one conduit) and kept in CO_2 incubator for 3 h. Then PQQ (15 nmol/L, 100 μL) was injected into CSC and incubated for another 3 h. Finally, SCs maintenance medium was added to cover the conduits and incubated overnight before surgery. This kind of nerve conduits constructed from CSC, SCs and PQQ were coded as CSSPC.

2.4. Surgical procedure

96 Sprague–Dawley (SD) male rats (200–250 g) purchased from the Wuhan University Laboratory Animal Center (WHULAC, China) and acclimatized in the animal care facility for two weeks prior to surgery. The experiments were divided into 4 groups, including CSC, CSSC, CSSPC and control groups. The animal experimental procedure was shown in Fig. 1A–D. The rats were anesthetized by intraperitoneal administration of 10% urethane sodium at a dose of 1 mL per 100 g body weight. After the anesthesia, the hair on the lateral thigh of the rats was shaven and the skin was treated with 70% alcohol solution. The incision extended from the lateral femoral oblique and the muscle tissue was split. Then the sciatic nerve was exposed in visual field. The nerve was cut with a sterile blade and the distance of defects about 6.0–8.0 mm. The three kinds of nerve guide conduits with 10 mm length were used to bridge the nerve gap by 8-0 nylon sutures, respectively. Muscle and skin were closed with interrupted absorbable sutures. According to the variety of nerve guide conduits, the experimental groups were coded as CSC, CSSC and CSSPC groups, respectively. The control group was the autograft, which was rotated 180°, using to bridge the nerve defect. Post-operative animals were conventional breeding.

2.5. General analysis

After surgery, the experimental skin ulcers of foots and toes, the hindlimb muscle atrophy, the joint stiffness and flexion situation

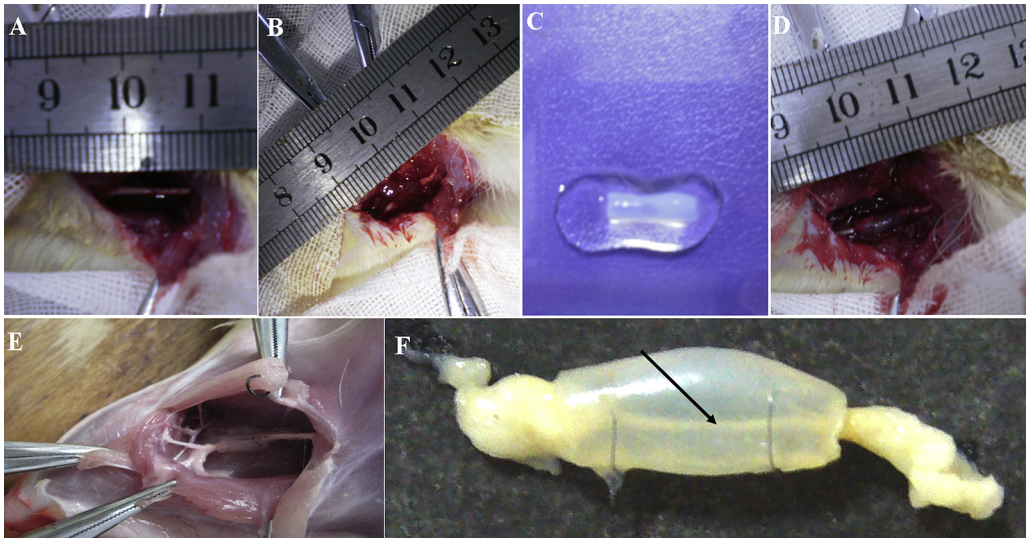

Fig. 1. Sciatic nerve of the rat (A), construction of animal model with peripheral nerve defect (B), nerve guide conduit from hollow nerve conduit (C), and bridge and repair of sciatic nerve defect in rat using nerve guide conduit (D), macroscopic view of nerve conduit with regenerative nerve fiber in CSSPC group in rat (E), and the harvested regenerative nerve fiber 12 weeks after surgery (F). Arrow shows regenerative nerve connected the far-end and near-end of the original injured nerve.

were observed by naked eyes. 12 weeks after surgery, the incision tissue was split, in order to observe the surrounding tissue, scar formation, and the relationships of nerve conduits and regenerated nerve.

2.6. Sciatic function index (SFI)

12 weeks after animal surgery, the motor function of animals was evaluated by walking tract analysis [28]. The animals were placed in a darkened cage of walking pathway ending and white papers were placed on the bottom of the tract of appropriate dimensions. During experiment, all animals were allowed several conditioning trials because of often stopping to explore the corridor in the beginning. And then, the animals walked steadily to the darkened cage [29]. The hind feet of animals were dipped with Indian ink, when the animals walking through the tract would leave their hind footprints on the paper. The sciatic functional index (SFI) was calculated as following equation [30]:

$$SFI = -38.3 \times (EPL - NPL)/NPL + 109.5 \times (ETS - NTS)/NTS + 13.3 \times (EIT - NIT)/NIT - 8.8$$

Where E and N were the experimental and normal sides, respectively; PL was the print length, meaning distance from the heel to the third toe; TS was the toe spread, meaning distance from the first to the fifth toe; IT was the intermediary toe spread, meaning distance from the second to the fourth toe. If SFI value is 0, it means the repair effect is normal; If SFI value is −100, it indicates total impairment [28].

2.7. Histological analysis

After conventional feeding for 12 weeks, the animals were euthanized, the regenerated nerve specimens were washed with PBS and fixed with 4% paraformaldehyde for 6 h, dehydrated by increasing concentrations of ethanol, embedded in paraffin, cut into 4–6 μm thickness and stained with hematoxylin-eosin (HE). The sections were observed by light microscopy (LM, CKX41, Olympus, Japan).

In order to observe the detailed axon and myelin sheath regeneration of the nerve in the conduits, the specimens were fixed with 2.5% glutaraldehyde before 2 h, and then fixed with 1% osmium tetroxide another 2 h, dehydrated by ethanol progressively, embedded in Epon812, ultrathin sliced to 50–60 nm, stained with lead–uranium and observed by a transmission electron microscope (TEM, H-600, Hitachi, Japan).

2.8. Statistical analysis

The data got from CSC, CSSC and CSSPC nerve guide conduits in TEM evaluations were average value ± SD. Student's *t*-test was used to evaluate the differences among the conduits. $p < 0.05$ was considered as statistically significant.

3. Results

3.1. General observation

During the experimental period, all animals survived and remained in normal state. No inflammatory signs or adverse tissue reactions were observed. Fig. 1E and F illustrated the regenerative nerve fibers passing through the nerve conduits. In this work, the nerve conduits fabricated from cellulose/SPI composite membranes showed high transparency. As a result, the newly regenerative nerve fibers in the hollow conduits could be directly and clearly seen by naked eyes. 12 weeks after surgery, the rats were anesthetized, incised from the original incision and the sciatic nerve were exposed. It was found that the nerve conduits slightly adhered to the surrounding tissues and were easily peeled. As shown in Fig. 1F, in the middle of the nerve conduits, the new axons generated and completely connected the two ends of the sciatic nerve.

3.2. Sciatic function index (SFI)

Further SFI check was performed and the results were shown in Fig. 2. From the results of walking tract analysis, the control group showed a higher SFI score than the three experimental groups. In the control group, SFI value was −64.73 ± 3.96. In the experimental groups, the order of SFI value from high to low was: CSSPC group (−71.60 ± 3.85), CSSC group (−80.59 ± 2.04), and CSC group (−86.13 ± 4.07). There was no significant difference between the control group and CSSPC group, but there was obviously difference

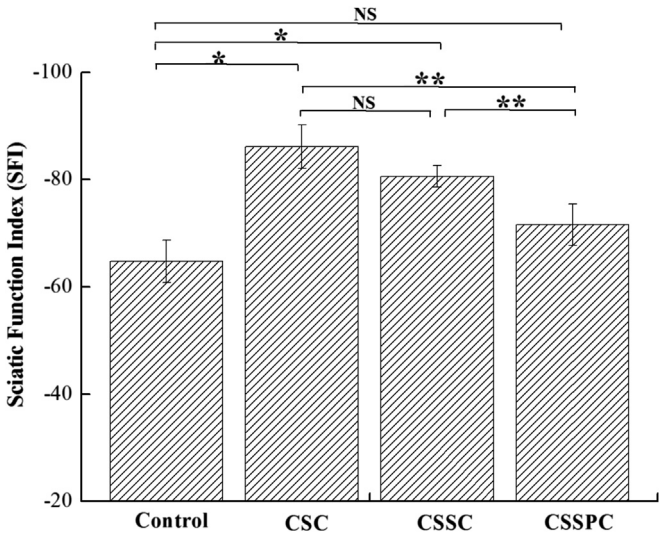

Fig. 2. The sciatic function index (SFI) of the control group and experimental groups. $^{*}p < 0.05$ and $^{**}p < 0.05$ means significant difference, NS means no significant difference.

between the control group and CSSC as well as CSC groups ($p < 0.05$), indicating more motor recovery from the repair with nerve conduits in CSSPC group than that in CSSC and CSC groups.

3.3. Histological analysis

Fig. 3A showed the HE images of cross-sections cut from the middle part of the regenerative nerve fibers in the different groups after 12 weeks surgery. In the control group, the nerve fibers arrayed regular and surrounded by lots of blood vessels. New nerve fibers were formed and passed through the nerve conduits in all experimental groups. In CSC group, the regenerative nerve fibers looked scattered and there were lots of blood vessels and matrix materials infiltration. In CSSC and CSSPC groups, the new nerve fibers grew more regularly. Among the three experimental groups, the number of regeneration nerve fibers in CSSPC group was the most, and a largest number of new capillaries had regenerated, indicating that CSSPC group exhibited better nerve regeneration than CSSC and CSC groups.

The HE images of regenerative nerve fibers along the longitudinal direction of nerve were shown in Fig. 3B. In CSC group, scattered regenerative axons extended from the nerve of proximal to the distal direction along the conduit and lots of matrix materials

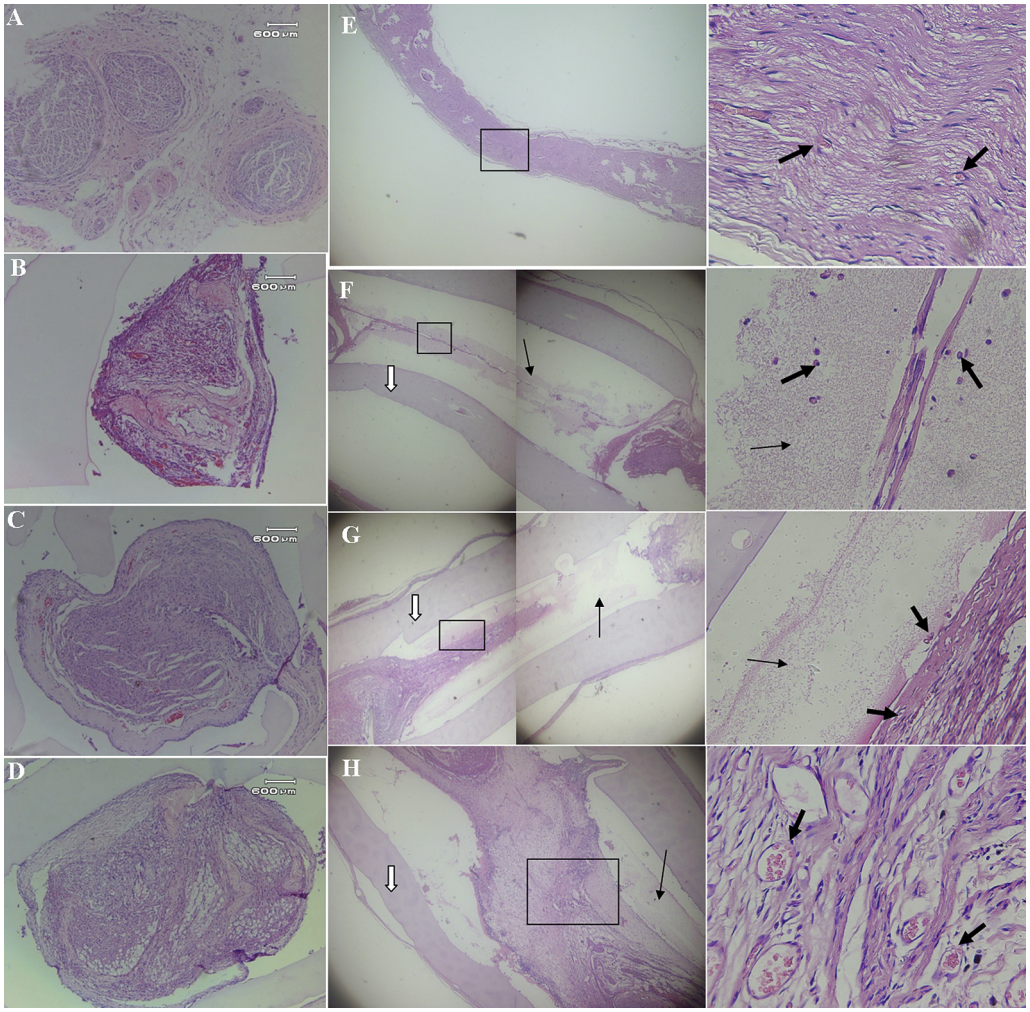

Fig. 3. HE-stained images of cross-sections and along the longitudinal directions cut from sciatic nerves in control group (A, E) and from the regenerative nerves after 12 weeks of surgery in CSC (B, F), CSSC (C, G) and CSSPC (D, H) groups. cross-sections: A, B, C and D; longitudinal directions: E, F, G and H. (Wide arrows: blood vessels; narrow arrows: matrix materials; white arrows: cellulose/SPI blend nerve conduit.)

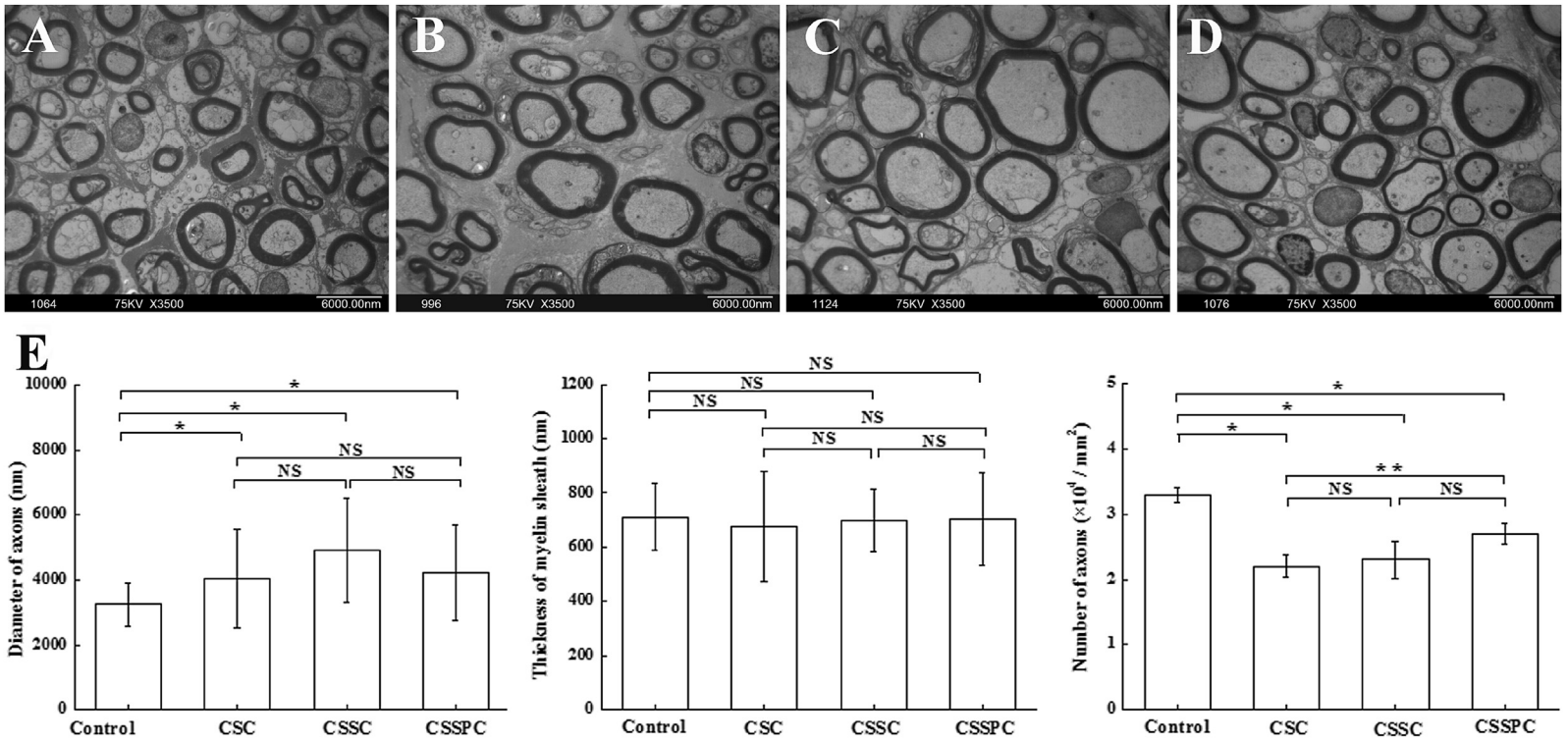

Fig. 4. TEM images of the sciatic nerve fibers in control group (A) and of the middle from regenerative nerves after 12 weeks of surgery in CSC (B), CSSC (C) and CSSPC (D) groups. Diameter of axon, thickness of myelin sheath and number of axon of the regenerative nerves calculated from TEM images (E). $^{*}p < 0.05$ and $^{**}p < 0.05$ means significant difference, NS means no significant difference.

filled in the conduit. In CSSC group, the regenerative axons were in a bundle, arranging regularly and there were many new vessels and matrix materials formatted in the distal of the conduit. In CSSPC group, the new nerve fibers connected fully from the near-end to the far-end, the density of regeneration axons and the number of the new blood vessels was highest, which were consistent with the results from the images of cross-sections.

TEM images of nerve fibers cut from the middle of the regenerative nerve after 12 weeks surgery were shown in Fig. 4A–D and the diameter of axon, thickness of myelin sheath and number of axon of the regenerative nerves calculated from TEM images were illustrated in Fig. 4E. Many new-formed myelinated and non-myelinated nerve fibers could be observed in the TEM images of regenerative nerves. In the control group, the morphology of axons was normal, and vacuolization and swell occurred rarely. In CSC group, the number of the axons was the least and lots of vacuolus were formed, including a few swollen and abnormal axons. Comparing with CSC group, the diameter of regenerative axon in CSSC group was the most. A few vacuolization of axons and some swollen and abnormal axons were still observed in CSSC group. Among the three experimental groups, the number of axons in CSSPC group was the most, while the swell or vacuolization of the myelin sheath was the least. It indicated that the best nerve regeneration happened in CSSPC group. According to Fig. 4E, the diameter of axons in the three experimental groups was much larger than that in the control group. There was no significant difference of thickness of myelin sheath between the control group and the experimental groups. The number of axons in the three experimental groups was less than that in the control group. Altogether, CSSPC group has the second largest diameter of axons, the largest thickness of myelin sheath and most number of axons among the three experimental groups.

4. Discussion

Autologous nerve graft is the “golden standard” for the repair of nerve defects in clinic. Due to associated donor site morbidity, neuroma formation and infections with autografts, several alternatives to bridge the nerve gap have been investigated [31]. Cellulose and SPI are natural polymers with good biocompatibility and biodegradability, having been widely used in biomedical fields [16]. In our previous work [18], it was proved that the cellulose/SPI composite membranes had suitable mechanical properties and cytocompatibility as biomaterials. Especially, cellulose/SPI membranes containing 30% of SPI not only showed porous structure, but also exhibited suitable mechanical properties at wet state. Therefore, based on the comprehensive consideration of cytocompatibility, biodegradability, mechanical properties and processability, this kind of cellulose/SPI membranes containing 30% of SPI (coded as CSC) was chosen as the basic tube.

SCs are the major cells in the nervous system and can support the repair and regeneration of injured peripheral nerves. Recently SCs have been transplanted alone or in combination with synthetic nerve guide conduits in order to improve the regeneration of peripheral nerves [27]. PQQ is a low molecular weight co-factor of microbial quinoprotein enzyme, acting as an antioxidant to prevent cell from injury [25]. It has been demonstrated that a certain concentration of PQQ (1–100 nmol/L) could improve the expression of mRNA on the SCs and enhance the proliferation and migration of SCs *in vitro* [24]. Therefore, in this work, cellulose/SPI membranes (CSC) was used as hollow tube, SCs as seed cells and PQQ as nerve growth factor to construct a new kind of nerve guide conduit (CSSPC).

At 12 weeks, we could directly see the regenerative nerve passing through the two-ends in CSSPC group because of its high transparency (Fig. 1F). In most of the literature, the regenerative nerve fibers couldn't be directly seen because the conduits were not transparent. In those cases, the conduits must be cut open for the observation of regenerative nerve [32]. So, the basic nerve conduit fabricated from cellulose/SPI composites can be used to “*in situ*” confirm the success of formation of regenerative nerve fiber in the rat. Based on the results from SFI, histological analysis, diameter of axon, thickness of myelin sheath and number of axon sheath, it was found that the CSSPC group which combined with CSC, SCs and PQQ had better regeneration of peripheral nerve than those in the CSSC group and CSC group, but less than that of the control group. In this study, autograft nerve was used as conduit to repair the injured nerve in the control group, which is much closer to the “golden standard” of clinic. Therefore, the repair of peripheral nerve in control group is better than that of the all experimental groups. In CSSPC group, the new nerve fibers showed regular and completely connection from the proximal end to the distal end. Only little vacuolar, swollen and abnormal axons occurred in the CSSPC group. The reconstruction of muscle function in the CSSPC group was better than that in CSSC and CSC groups. The results indicated that SCs combined with PQQ played more important role for the regeneration of injured peripheral nerves than SCs alone did. The combinatory strategy of using PQQ and SCs together with the CSC nerve conduit may represent a novel avenue for nerve tissue engineering and regeneration.

Conflict of interest

None.

Acknowledgments

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