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End-to-side neurorrhaphy using an electrospun PCL/collagen nerve conduit for complex peripheral motor nerve regeneration

Bu-Kyu Lee [a,b], Young Min Ju [a], Jae-Gu Cho [a,c], John D. Jackson [a], Sang Jin Lee [a], Anthony Atala [a], James J. Yoo [a,*]

[a] Wake Forest Institute for Regenerative Medicine, Wake Forest School of Medicine, Medical Center Boulevard, Winston-Salem, NC 27157, USA
[b] Department of Oral and Maxillofacial Surgery, Asan Medical Center, College of Medicine, University of Ulsan, Asan Beongwon-gil 89, Songpa-ku, Seoul, Republic of Korea
[c] Department of Otolaryngology-Head and Neck Surgery, Korea University Guro Hospital, 148 Gurodong-ro, Guro-gu, Seoul, Republic of Korea

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ABSTRACT

In cases of complex neuromuscular defects, finding the proximal stump of a transected nerve in order to restore innervation to damaged muscle is often impossible. In this study we investigated whether a neighboring uninjured nerve could serve as a source of innervation of denervated damaged muscle through a biomaterial-based nerve conduit while preserving the uninjured nerve function. Tubular nerve conduits were fabricated by electrospinning a polymer blend consisting of poly(ε-caprolactone) (PCL) and type I collagen. Using a rat model of common peroneal injury, the proximal end of the nerve conduit was connected to the side of the adjacent uninjured tibial branch (TB) of the sciatic nerve after partial axotomy, and the distal end of the conduit was connected to the distal stump of the common peroneal nerve (CPN). The axonal continuity recovered through the nerve conduit at 8 weeks after surgery. Recovery of denervated muscle function was achieved, and simultaneously, the donor muscle, which was innervated by the axotomized TB also recovered at 20 weeks after surgery. Therefore, this end-to-side neurorrhaphy (ETS) technique using the electrospun PCL/collagen conduit appears to be clinically feasible and would be a useful alternative in instances where autologous nerve grafts or an adequate proximal nerve stump is unavailable.

1. Introduction

Muscle denervation can cause permanent muscular atrophy and eventual functional disability. Coaptation of transected nerves is critical in avoiding these problems [1]. In cases of complex neuromuscular defects created by gunshot wounds or malignant tumors, finding the proximal stump of a transected nerve for use in coaptation is often impossible [2]. In these circumstances, end-to-side neurorrhaphy (ETS) using an adjacent uninjured nerve (with or without an autologous nerve graft) can be used as an alternative method to reinnervate the denervated muscle. ETS was originally introduced as early as 1903 for facial nerve reconstruction using the spinal accessory nerve as a donor source [2,3]. This technique was reintroduced in 1994 and has been used in clinical and experimental practices for nerve repair cases [2,3]. In ETS, the distal nerve stump, termed as the "recipient nerve", is coapted to the sidewall of an uninjured nerve, termed as the "donor nerve" [3,4]. Theoretically, axonal regeneration in the ETS model, which occurs through collateral sprouting, originates in the donor nerve and progresses towards the recipient stump. This may allow muscle reinnervation while preserving the donor nerve function, and is particularly useful when the proximal stump of an injured nerve cannot be found [3,5]. Consequently, an autologous nerve graft is usually used as a standard procedure in ETS cases to repair the gap between the donor and the recipient nerves. However, excessive tension on the nerve frequently results in impaired microvascular flow in the nerve tissue and excessive scarring at the repair site [6–8]. This is especially true for grafts other than autologous donor nerve.

Despite a long history and high demand for this technique, clinical application of ETS is still limited and even controversial. This is mainly due to the less likelihood of sidewall donor axonal sprouting to the recipient nerve and possible donor muscle damage following donor nerve axotomy. From a clinical point of view, these problems often escalate when an autologous nerve graft is used due

Abbreviations: ETS, end-to-side neurorrhaphy; ETE, end-to-end neurorraphy; NGC, nerve guidance channel; NR, non repair group; ETSC, repair group with ETS and a conduit; AchR, acetylcholine receptor; CMAP, compound muscle action potential; EMG, electromyogram; GA, gastrocnemius muscle; AT, anterior tibialis muscle; EDL, extensor digitorium longus muscle; NMJ, neuromuscular junction.

* Corresponding author. Tel.: +1 336 713 7294; fax: +1 336 713 7290.
E-mail address: jyoo@wakehealth.edu (J.J. Yoo).

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to harvest site morbidity such as pain, scarring, neuroma formation and permanent loss of sensation in the area innervated by the donor nerve. Furthermore, a sensory nerve graft taken from the sural or greater auricular nerves, which are the most popular donor nerve sources for autologous nerve grafts, is known to be often ineffective for motor nerve regeneration [9,10]. This is due to the structural differences between motor and sensory nerves [9,10]. Thus, to improve the understanding of axonal regeneration through a nerve conduit in the ETS model and to avoid major pitfalls related to autologous nerve graft in ETS, we hypothesized that a simple tubular nerve conduit would guide the axonal nerve growth in the ETS model and reduce the known complications in ETS procedures.

We previously developed a composite tubular conduit by hybridizing high molecular weight poly(ε-caprolactone) (PCL) with type I collagen using electrospinning technique [11,12]. The electrospun PCL/collagen conduits are non-cytotoxic, possess adequate biomechanical properties (4.0 MPa tensile strength and 2.7 MPa elasticity) and provide a favorable environment that supports the growth of cells. We hypothesized that this electrospun tubular conduit system could be used as a nerve guidance channel for complex motor nerve regeneration. In this study we have developed a technique in which a neighboring uninjured nerve serves as a source of axonal sprouting through a PCL/collagen conduits to induce reinnervation and restoration of denervated muscle function. To confirm the feasibility of using this method, we tested these conduits *in vitro* to determine their biocompatibility with nerve cells as well as in a rat nerve injury model. We examined the levels of nerve function restoration using various assessment modalities, including walking track analysis, electrophysiologic tests, moist muscle weights, and histological evaluations of the muscle and nerve tissues.

2. Materials and methods

2.1. Fabrication and characterizations of electrospun PCL/collagen nerve conduits

Nanofibrous tubular nerve conduits were fabricated by electrospinning with a polymer blend of PCL and collagen type I as described previously [11] (Fig. 1). Briefly, the nerve conduits were electrospun using a 1:1 (weight ratio) polymer blend of PCL (Inherent viscosity = 1.77 dL/g, Lactel Absorbable Polymers, Pelham, AL, USA) and collagen type I derived from calf skin (Elastin Products Co., Owensville, MO, USA) in 1,1,1,3,3,3-hexafluoro-2-propanol (HFP, Sigma Chemical Co., St. Louis, MO, USA). The electrospinning set-up included a syringe pump, a high-voltage supply, and a rotating mandrel. A positive voltage (20 kV) was applied to the system using a power supply (Spellman high Voltage, Hauppauge, NY, USA). The PCL/collagen blend solution was delivered through an 18-gauge blunt tip syringe needle at a constant flow rate of 3 ml/h using a syringe pump. The collecting mandrel consisted of a stainless steel rod (0.8 mm diameter). The distance between the syringe tip and the mandrel was 10 cm, and the rotation rate of the mandrel was approximately 1000 rpm. The electrospun PCL/collagen nerve conduits were cross-linked in the vapor of a 2.5% glutaraldehyde solution at room temperature for 6 h. The electrospun nerve conduits were then gas sterilized with ethylene oxide (Amsco®Eagle® 3017 EO Sterilizer, STERIS Co., Mentor, OH, USA) at 30 °C for 36 h prior to use. All chemical reagents were obtained from Sigma Chemical Co. unless stated otherwise.

The electrospun PCL/collagen conduits were examined using scanning electron microscopy (SEM; Model S-2260N, Hitachi Co. Ltd., Japan). Briefly, the electrospun conduits were sputter-coated with gold (Hummer™ 6.2, Anatech Ltd, Denver, NC, USA) to a thickness of 10–15 nm. Images were acquired using an environmental SEM operating at an accelerating voltage of 20 kV with a 10 cm working distance. The SEM images were analyzed using UTHSCSA ImageTool 3.0 (Freeware provided by the University of Texas Health Sciences Center at San Antonio, TX, USA) to determine average fiber diameters. Twenty random fibers per image ($n = 12$) were used to calculate the mean and standard deviation of fiber diameters. Pore areas were measured by a subjective approximation of surface pores in the SEM images (average and standard deviation calculated from twenty random measurements per image, $n = 12$).

2.2. In vitro biological evaluations of electrospun nerve conduits

2.2.1. Cytotoxicity assessment

In order to determine whether the electrospun conduits could support the growth of nerve cells, NG108-15 neuroblastoma cross glioma hybrid cells (ATCC, HB-12317, Manassas, VA, USA) were seeded onto the electrospun PCL/collagen conduits, and these constructs were maintained in cell culture plates for testing via the direct contact method [13]. Briefly, the conduits ($5 \times 5 \times 0.3$ mm^3) were incubated at 37 °C for 30 min with culture medium and then they were placed at the center of each well of a 24-well plate that contained subconfluent monolayers of NG108-15 cells. Wells containing only cells (no conduit) served as negative controls. Pieces of latex, which has been shown to be toxic to many cell types *in vitro*, were cut into the same size as the conduit material and placed into some wells to serve as positive controls. Cell–material contact was maintained at 37 °C in 5% CO_2, and the culture medium was changed every 3 days. The AlamarBlue® assay was performed on days 1, 3 and 7 to determine cell viability in each well. The AlamarBlue® reagent (Invitrogen Co., Carlsbad, CA, USA) was added to the cell culture medium at a ratio of 1:10 (v/v). The plates were then incubated at 37 °C for 1 h. A 200 μl aliquot of the medium/AlamarBlue® solution from each well was then placed into a 96-well plate, which was read on a SpectraMax M5 fluorimeter (Molecular Devices, CA, USA) at $\lambda = 560$ nm excitation, $\lambda = 590$ nm emission. The amount of fluorescence in each sample is proportional to the number of living cells in the well. These results were reported as a percentage of the fluorescence obtained with supernatant from negative control wells ($n = 5$). All reagents for cell culture were purchased from Invitrogen (Gibco® Cell Culture, Carlsbad, CA, USA).

2.2.2. Cell adhesion and proliferation

For cellular adhesion and proliferation studies, PCL/collagen was electrospun onto plastic coverslips (10×10 mm^2) to form two-dimensional nanofiber structures. Briefly, plastic cover slips were sonicated in ethanol and allowed to vacuum dry. The electrospun PCL/collagen- covered plastic coverslips were placed into 6-well plates, gas sterilized by ethylene oxide at 30 °C for 36 h, and incubated at 37 °C for 30 min in culture medium prior to cell seeding. To evaluate the initial stage of *in vitro* cellular adhesion to the nerve conduit material, the coverslips covered with electrospun PCL/collagen fibers were seeded with NG108-15 cells in culture dishes. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM without sodium pyruvate) supplemented with 10% fetal bovine serum (FBS), 0.1 mM hypoxanthine, 400 nM aminopterin, 0.016 mM thymidine, 1.5 g\L sodium bicarbonate and 1% penicillin/streptomycin at 37 °C in 5% CO_2, and the culture medium was changed every 3 days. The biological activity of the nerve conduit material was also tested by observing the ability of cells to proliferate on the material by the AlamarBlue assay®. Briefly, NG108-15 cells (2×10^4 cells/cm^2) were seeded onto the electrospun PCL/collagen nanofibers in the wells of 24-well culture plates for up to 7 days. The adhesion and proliferation of the cells on the nerve conduit material were assessed by the intensity of fluorescence obtained, which was directly proportional to the metabolic activity of the cell population. The fluorescence intensity was recorded for quantification ($n = 5$) of cells within the nerve conduits.

To assess cell morphology on the conduit material, NG108-15 cells that had been seeded on electrospun PCL/collagen nanofibers were examined by SEM. At each time

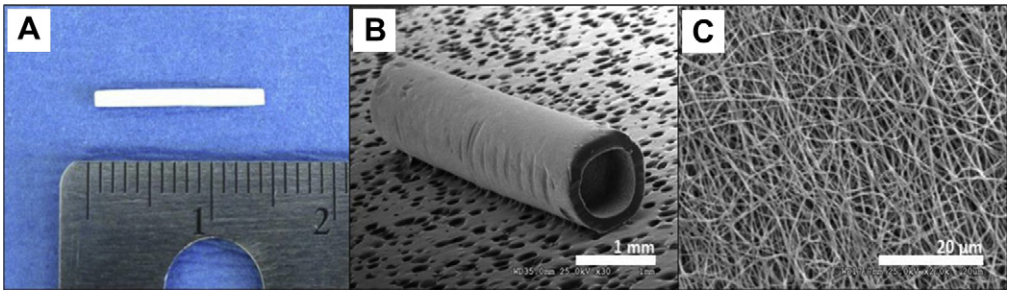

Fig. 1. (A) The gross appearance and SEM images of electrospun PCL/collagen conduits: (B) entire (×30) and (C) surface (×2.0 K).