Long term peripheral nerve regeneration using a novel PCL nerve conduit

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HIGHLIGHTS

\begin{itemize}
  \item We have developed a novel poly e-caprolactone peripheral nerve conduit.
  \item Our experimental in vivo model compares PCL nerve conduit repair with autograft.
  \item Long term outcomes display comparable re-innervation of end organ muscle and skin.
\end{itemize}

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ABSTRACT

The gold standard in surgical management of a peripheral nerve gap is currently autologous nerve grafting. This confers patient morbidity and increases surgical time therefore innovative experimental strategies towards engineering a synthetic nerve conduit are welcome. We have developed a novel synthetic conduit made of poly e-caprolactone (PCL) that has demonstrated promising peripheral nerve regeneration in short-term studies. This material has been engineered to permit translation into clinical practice and here we demonstrate that histological outcomes in a long-term in vivo experiment are comparable with that of autologous nerve grafting. A 1 cm nerve gap in a rat sciatic nerve injury model was repaired with a PCL nerve conduit or an autologous nerve graft. At 18 weeks post surgical repair, there was a similar volume of regenerating axons within the nerve autograft and PCL conduit repair groups, and similar numbers of myelinated axons in the distal stump of both groups. Furthermore, there was evidence of comparable re-innervation of end organ muscle and skin with the only significant difference the lower wet weight of the muscle from the PCL conduit nerve repair group. This study stimulates further work on the potential use of this synthetic biodegradable PCL nerve conduit in a clinical setting.

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1. Introduction

The management of peripheral nerve gap following injury is a surgical challenge. The gold standard in clinical practice for surgical repair of a peripheral nerve gap remains an autologous nerve graft [2,10]. However, the use of autologous nerve graft confers morbidity such as loss of sensation and scarring at the donor site, can be time-consuming and a technical challenge for the surgeon, is a finite resource, and nerve size mismatch has to be overcome by using multiple lengths of nerve graft within the defect. The functional recovery of this reconstruction remains deficient and these injuries can have a profound and permanent impact on the patient and their ability to perform activities of daily living as well as preventing return to work [8,14]. Poor outcomes may be a direct consequence of slow, insufficient and misdirected axonal outgrowth at the site of injury [1,4]. Whilst innovative experimental strategies have been employed to engineer a synthetic nerve conduit to bridge the defect, nothing yet has proven good enough to be used routinely in clinical practice [3,6,20]. Conduits approved for current clinical use include collagen and synthetic polyester-based materials; however, their use has been limited due to the lack of good clinical data, relative expense and inability to bridge large nerve gaps [9].

Recently, the synthetic biomaterial poly e-caprolactone (PCL) has demonstrated great promise as a novel nerve conduit which is ultrathin with a pitted structure formed by solvent casting on a glass slide and sealed with a controlled heating method [13,15]. This manufacturing process is low cost, reproducible, allows easy sterilisation with unique properties allowing flexibility and sufficient strength for clinical handling. Furthermore, it possesses...
many features that would suggest an ideal nerve conduit; in particular it is biodegradable, non-toxic, non-immunogenic, and may incorporate supportive cells into its structure. In short-term studies, excellent nerve regeneration has been demonstrated with the use of PCL conduit to bridge a 1 cm nerve gap such that a large volume of axons consistently bridged the 1 cm gap at only 2 weeks post-injury [16]. In order to understand the potential for clinical translation of this nerve conduit, it is crucial to demonstrate long-term outcomes such as re-innervation of end organ skin and muscle and compare this to the current clinical gold-standard. This study sought to test the long-term regeneration of an in vivo peripheral nerve injury when repaired with a PCL nerve conduit as compared to an autologous graft across a 1 cm nerve gap in a rat sciatic nerve injury model.

2. Materials and methods

2.1. Experimental design

We compared experimental peripheral nerve repair of a 1 cm gap using empty PCL conduit versus autologous nerve graft. Outcome measures compared were axonal counts and myelination in the conduit or autograft and in the distal stump; end organ muscle fibre size and weights; and end organ skin re-innervation.

2.2. Preparation of PCL conduits

Poly e-caprolactone (PCL), conduits were prepared as previously described [15]. Briefly, 3% (w/v) of PCL (Sigma Aldrich, US) was dissolved in dichloromethane (Fisher Scientific, UK) and spread evenly on degreased borosilicate glass cover slip. Following complete solvent evaporation, the films were treated in 10 N NaOH for 1 h with gentle shaking and washed in distilled H₂O. Films were cut into rectangular sheets and rolled around a 16G intravenous cannula (Abbocath®, Abbott Ireland, Republic of Ireland), which standardised the internal diameter of the conduits at 1.6 mm, more than 1.5 times the diameter of rat sciatic nerve, thus allowing space for post-injury swelling. Conduits were sealed by controlled heating while still mounted on the cannula and then sterilised using UV radiation.

2.3. Surgical procedures and groups

The animal care and experimental procedures were performed in accordance with the terms of the United Kingdom Animals (Scientific Procedures) Act 1986 and the number of animals used was kept to a minimum. Surgical procedures were performed under isoflurane general anaesthesia on young adult male Sprague–Dawley rats (180–220 g). All animals underwent sciatic nerve transection and a 10 mm nerve gap was created at the level Sprague–Dawley rats (180–220 g). All animals underwent sciatic nerve transection and a 10 mm nerve gap was created at the level of the mid-femur. In the autologous nerve graft group (n = 5) the proximal and distal nerve stumps of the transected nerve were secured 2 mm within the PCL conduit using 4 intermittent 9-0 Ethilon epineurial sutures (Ethicon, US). The conduit had been cut to 14 mm length in order to maintain a 10 mm gap between the nerve stumps. The wound was closed in layers and post-operative analgesia was given as 4 μg buprenorphine intramuscularly. The animals were caged in a temperature and humidity controlled room with a 12-hour light/dark cycle, and food and water provided immediately. After 18 weeks survival, animals were deeply anaesthetised with isoflurane and perfused transcardially with 0.9% heparinised saline (0.9% NaCl, 50 units/ml heparin) followed by 4% paraformaldehyde in 0.1 M phosphate buffer solution (PBS). The sciatic nerves including PCL conduit were harvested and post-fixed in 4% paraformaldehyde before being frozen in OCT embedding medium (VWR, UK) and stored at −40 °C. For axon counts, 2–3 mm long sciatic nerve specimens were excised at 3 mm distance from the proximal and distal nerve graft/conduit interfaces. These nerve segments were fixed in 3% glutaraldehyde, post-fixed in 1% osmium tetroxide (OsO₄) in 0.1 M cacodylate buffer (pH 7.4), dehydrated in acetone and embedded in Vestopal. Both ipsilateral and contralateral medial gastrocnemius muscles were weighed and then frozen in OCT using isopentane cooled with liquid nitrogen and foot pad skin was post-fixed in Zamboni’s solution before being frozen in OCT embedding medium (VWR, UK) and stored at −40 °C.

2.4. Axon counts

Semi-thin transverse sections of proximal and distal nerve segments were cut on a 2128 Ultratome (LKB, Sweden) and counterstained with Toluidine Blue. Myelinated axons in the these segments were counted at 1000× final magnification using the fractionator probe in Stereo Investigator™ 6 software (MicroBrightField, Inc., USA).

2.5. Axonal regeneration

Random longitudinal nerve sections were cut with a thickness of 14 μm and collected onto glass slides coated with Vectabond (Vector, Peterborough, UK). The sections were permeabilised in 0.2% Triton-X in PBS and washed twice in PBS for 5 min. They were then blocked with normal goat serum (Sigma, UK; 1:100 dilution) and the primary antibody (protein gene product 9.5, PGP 9.5, Dako, UK; 1:1000) was applied overnight. The following day, the sections were washed three times in PBS and a fluorescein goat anti-rabbit conjugated secondary antibody (Vector Laboratories, UK; 1:100) was applied. The sections were finally washed carefully in PBS and mounted on glass slides with Vectashield. After staining, each section was examined by fluorescence microscopy using a 20× objective. Measurements were carried out at 5 mm from the proximal stump and images were captured using an Olympus BX60 inverted fluorescence microscope and a monochrome camera (Evolutio QEI, MediaCybernetics, Bethesda, USA). The immunostaining area was then quantified using Image Pro Plus Imaging Software (Media Cybernetics). Images were converted into grayscale and thresholded. The immunostaining area was finally evaluated automatically by the software and expressed as μm². The final staining area was expressed as the ratio between the staining area and the total frame area.

2.6. Muscle analysis

Sixteen micron transverse sections of gastrocnemius muscles from the contra-lateral and operated sides were cut on a cryostat and samples were then fixed in 4% (w/v) paraformaldehyde for 15 min at room temperature, washed briefly in phosphate buffered saline (PBS) and permeabilised in 0.1% (v/v) Triton X-100 in PBS for 30 min. Samples were blocked with normal serum and then incubated with monoclonal primary antibodies raised against fast and slow myosin heavy chain protein (NCL-MHCf and NCL-MHc, Novocastra, Peterborough, UK; both 1:20) for 2 h at room temperature. Each slide was also co-incubated with rabbit anti-laminin antibody (Sigma, Poole, UK; 1:200). After rinsing in phosphate-buffered solution, secondary goat anti-rabbit and goat anti-mouse antibodies Alexa Fluor 488 and Alexa Fluor 568 (Invitrogen, The Netherlands; 1:100) were applied for 1 h at room temperature in the dark. The slides were cover-slipped with Prolong anti-fade mounting
Fig. 1. Myelination morphology is very similar between the autograft and PCL conduit nerve repair groups (A, scale = 20 μm). Furthermore, the axonal regeneration seen within the autograft and PCL nerve conduit by PGP immunohistochemical staining was similar (B, scale = 50 μm). Myelinated axon counts demonstrated similar numbers in the distal stumps of both groups 7729 ± 1308 and 6414 ± 1544 axons in autograft and conduit respectively (C). The percentage of regenerating axons within the autograft (48 ± 2%) and PCL nerve conduit (52 ± 3%) were not significantly different (D).
Fig. 2. Immunohistochemistry of fast and slow muscle fibres from the medial gastrocnemius muscle demonstrating similar sizes of muscle fibre (A, scale = 50 μm). The area (B) and diameter (C) of fast and slow muscle fibre types was expressed as a percentage of the contralateral side and did not demonstrate a significant difference between the autograft and the PCL nerve conduit repair groups. Fast muscle fibre area was 84.0 ± 3.3% in the autograft group and 72.6 ± 7.0% in the PCL conduit group; slow muscle fibre area was 71.2 ± 7.0% in the autograft group and 76.5 ± 7.8% in the PCL conduit group. Fast muscle fibre diameter was 84.8 ± 1.6% in the autograft group and 77.8 ± 5.7% in the PCL conduit group; slow muscle fibre diameter was 74.3 ± 3.6% in the autograft group and 78.0 ± 4.7% in the PCL conduit group.

2.7. Skin re-innervation

Systematic random samples of 15 μm cryosections from ipsilateral foot pad skin were collected onto glass slides coated with Vectabond. Sections were permeabilised in 0.2% Triton-X (BDH Laboratory Supplies, Bristol, UK), and stained by indirect immunohistochemistry using primary antisera against polyclonal rabbit antisera for protein gene product 9.5 (PGP 9.5; 1:1000) and polyclonal rabbit calcitonin gene-related peptide (CGRP; Sigma, UK; 1:1000). PGP antibodies identify all nerve fibre types whilst CGRP antibodies are specific for C and Aδ sensory fibres. Staining was visualised using a fluorescein isothiocyanate-conjugated secondary
antibody (conjugated goat anti-rabbit serum; 1:100). After staining, each section was examined by fluorescence microscopy using a 20× objective. For each antibody, a random sample of six visual fields, including epidermis and dermis was captured for analysis using an Olympus BX60 inverted fluorescence microscope and images acquired using a monochrome camera. The immunostaining area in each captured image was quantified by image analysis using Image-Pro Plus Imaging software as previously described [7]. First the colour immunofluorescent image was converted into greyscale; then the epidermis and dermis was outlined by tracing its margin. An intensity threshold was applied to differentiate bright immunostaining from any low-level background autofluorescence, and the immunostaining area was calculated automatically.

2.8. Statistics

GraphPad Prism 4© software (GraphPad Software, San Diego, CA, USA) was used to calculate statistics. An unpaired student t-test was used to compare the two experimental groups. All data was expressed as mean ± SEM. A value of $P<0.05$ was considered to be statistically significant.

3. Results

No adverse events were encountered in the use of the PCL conduit in the 18-week survival period and there was no evidence of conduit degradation at this time point.

3.1. Axon counts

Specimens of the distal nerve were harvested 3 mm from the graft/conduit nerve interface and contained numerous myelinated nerve fibres (Fig. 1). There were not any marked differences in appearance and distribution of myelinated fibres between the autograft and PCL conduit experimental groups (Fig. 1A) and quantitative analysis demonstrated that similar numbers of axons were found in the distal stumps of both groups (Fig. 1C). Proximal stump axon counts were significantly higher for both groups.
In order to measure and compare the extent of axonal regeneration within the nerve repaired with conduit or autograft, the area highlighted by PGP immunohistochemical staining (Fig. 1B) was expressed as a proportion of the area within the conduit or autograft (Fig. 1D). This demonstrated that there was no significant difference between the volumes of regenerating axons within the nerve repaired with conduit and the nerve repaired with autograft.

3.2. Muscle fibre size and weights

The area and diameter of both fast and slow muscle fibre types was measured after immunohistochemical staining of the medial gastrocnemius muscle (Fig. 2). There were no statistically significant differences between the autograft and the PCL groups for muscle fibre area or diameter. The wet muscle weight of the medial gastrocnemius muscle supplied by the nerve repaired with PCL graft was significantly heavier than the wet weight of medial gastrocnemius muscle supplied by the nerve repaired with autograft, which was expressed as a proportion of the area within the conduit [60.24 ± 2.6% and 48.58 ± 3.1% of contralateral sides respectively, P < 0.05].

3.3. Skin re-innervation

Skin re-innervation was measured by CGRP and PGP immunohistochemical staining of the ipsilateral footpad skin (Fig. 3). There was nerve regeneration into the dermis of all sections examined. There were no statistically significant differences between the autograft and the PCL groups for the immunostaining area of CGRP or PGP.

4. Discussion

Autologous nerve grafting remains the clinical gold standard for repair of a nerve gap; however, much promising work has been reported in the development of synthetic nerve conduits to fulfil this role. This study provides evidence of comparable long-term histological outcomes in in vivo nerve gap repair with autograft or a novel PCL nerve conduit; thereby stimulating further work on the potential use of this synthetic biodegradable PCL nerve conduit in the clinical setting. In our previous in vivo study of this nerve conduit, we demonstrated that a large volume of axons consistently bridged a 1 cm rat sciatic nerve gap at only 2 weeks post-injury [16]. At 18 weeks there was a similar volume of regenerating axons within the nerve autograft and PCL conduit repair groups, and similar numbers of myelinated axons in the distal stump of both groups. Furthermore, there was evidence of comparable re-innervation of end organ muscle and skin with the only significant difference the lower wet weight of the muscle from the PCL conduit nerve repair group. Given that there is a similar muscle fibre size and number of myelinated axons between the two groups, the mechanism of a different muscle weight is unclear; however, the long survival period in this study may conceal differences in aspects of regeneration that could have been detected at earlier time points, and may be more critical in a human model [19]. Regeneration is slower and over much longer distances in humans than in rats, which confers increased susceptibility to target organ atrophy. However, the results of the PCL conduit are favourable when compared with recent studies examining collagen and fibrin nerve conduits versus autologous nerve grafting [11,18]. After 18 weeks survival, there were no adverse incidents and the PCL conduit remained intact with no evidence of degradation. This indicates that the conduit could provide support for axonal regeneration over longer distances and longer periods of time. This is useful in a human model whereby nerve conduits may need to provide support for many months of nerve guidance.

It is clear that further experimental studies and innovation will be required before a bioengineered conduit demonstrates superiority to an autologous nerve graft. This may be realised with the advent of stem cell integration within conduits and the manipulation of the conduit lumen to bear physical or chemical cues in order to aid axonal regeneration [5,13,17]. Features such as this will attempt to recreate the cellular and molecular ‘regenerative milieu’ of an autologous nerve graft within a biomaterial nerve conduit.

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