Delivery of chondroitinase ABC and glial cell line-derived neurotrophic factor from silk fibroin conduits enhances peripheral nerve regeneration

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Abstract

Nerve conduits are a proven strategy for guiding axon regrowth following injury. This study compares degradable silk–trehalose films containing chondroitinase ABC (ChABC) and/or glial cell line-derived neurotrophic factor (GDNF) loaded within a silk fibroin-based nerve conduit in a rat sciatic nerve defect model. Four groups of silk conduits were prepared, with the following silk–trehalose films inserted into the conduit: (a) empty; (b) 1 μg GDNF; (c) 2 μg ChABC; and (d) 1 μg GDNF/2 μg ChABC. Drug release studies demonstrated 20% recovery of GDNF and ChABC at 6 weeks and 24 h, respectively. Six conduits of each type were implanted into 15 mm sciatic nerve defects in Lewis rats; conduits were explanted for histological analysis at 6 weeks. Tissues stained with Schwann cell S-100 antibody demonstrated an increased density of cells in both GDNF- and ChABC-treated groups compared to empty control conduits (p < 0.05). Conduits loaded with GDNF and ChABC also demonstrated higher levels of neuron-specific protein 9.5 (p < 0.05). In this study we demonstrated a method to enhance Schwann cell migration and proliferation and also foster axonal regeneration when repairing peripheral nerve gap defects. Silk fibroin-based nerve conduits possess favourable mechanical and degradative properties and are further enhanced when loaded with ChABC and GDNF. Copyright © 2014 John Wiley & Sons, Ltd.

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Keywords drug delivery; peripheral nerve; chondroitinase ABC; GDNF; silk fibroin; nerve conduit

1. Introduction

Peripheral nerve injury occurs in 2–3% of all major traumas and also results from complications of surgery (Ichihara et al., 2008; Millesi, 1998; Siemionow et al., 2010). Although complete recovery can occur after mild injuries, outcomes remain unsatisfactory following complete transection. Once cut, nerves are able to slowly regrow, locate and reconnect to the structures they previously controlled (Siemionow et al., 2010). When an axon is disconnected from its cell body, its distal segment gradually deteriorates through a process called Wallerian degeneration (Lundborg, 2000; Navarro et al., 2007). Damaged neurons exhibit alterations in cellular structure and increased metabolic activity, which can lead to impeded regeneration (Dawson et al., 2003; Toth, 2009). If regrowth and re-innervation do not occur within 12–18 months, there is little chance of functional recovery (Dow et al., 2004; Ngeow, 2010). Maximizing recovery appears to lie within optimizing the quantity, quality and speed of nerve regeneration following repair.

Nerve autografting is the most commonly used surgical technique for repair of peripheral nerve gaps and remains the clinical gold standard (Ichihara et al., 2008). Success
arises from the presence of Schwann cells and basal lamina endoneurial tubes, which provide neurotrophic factors and surface adhesion molecules to recruit and guide regenerating axons, respectively (Myckatyn and MacKinnon, 2004). Studies have reported full restoration of sensation following autograft repair, but recovery of motor function is often < 40% (Nichols et al., 2004). Disadvantages of the autograft technique include limited donor nerve, loss of function at the donor site and donor site morbidity (e.g. scarring and neuroma formation).

Nerve guides offer an alternative strategy for spanning gap defects and promoting nerve recovery, a fact confirmed both experimentally and clinically (Ichihara et al., 2008; Taras and Jacoby, 2008). Well-engineered biodegradable guides provide a scaffold for regeneration while retaining the necessary mechanical stiffness to remain open while under pressure from the surrounding tissues, all without causing damage to the nerve stumps and adjacent structures. An ideal guide is also semi-permeable, allowing for controlled exchange between intra- and extrachannel environments; the ability to retain key neurotrophic factors within the lumen of the guide has been identified as a key factor in augmenting nerve growth response rates (Lundborg, 2000; Meek and Coert, 2002; Taras and Jacoby, 2008).

Silk-based guides are a relatively new alternative, showing promise in experimental models, but are still in development for clinical use (Ghaznavi et al., 2010). Silk fibroin, an FDA-approved material already utilized in numerous medical technologies, is characterized by a slow degradation rate, strong mechanical strength and high biocompatibility (Madduri et al., 2010; Uebersax et al., 2007; Yang et al., 2009). Silk guides possess many promising physical and biological properties necessary for promoting nerve repair, e.g. high biocompatibility, slow degradation rates, mechanical stiffness and selective permeability, and exhibit superior performance in bridging gaps across proximal-to-distal nerve stumps when compared to a collagen nerve guide (Ghaznavi et al., 2010).

Many studies have shown that it is possible to improve the performance of nerve guides by enabling them to deliver growth factors and other cellular materials (Pfister et al., 2007; Rovak et al., 2004). Biodegradable materials have a significant advantage in that as they degrade, they can release trophic factors from the material. Glial cell line-derived neurotrophic factor (GDNF), one of the dopaminergic factors identified from neurons of the midbrain (Iwase et al., 2005), promotes the proliferation, migration and differentiation of Schwann cells, as well as increasing axonal sprouting and enhancing growth from injured nerve stumps (Airaksinen and Saarma, 2002; Henderson et al., 1994; Paratcha and Ledda, 2008; Yamada et al., 2004). Researchers have developed silk fibroin conduits loaded with GDNF and other growth factors to boost functional recovery of injured peripheral nerves (Madduri et al., 2010; Yang et al., 2007a, 2007b). A recent study has shown that differential gradients of GDNF are able to drive axonal growth down silk conduits in experimental models of peripheral nerve repair (Lin et al., 2011).

If delivering neurotrophic factors results in enhanced regeneration of peripheral nerves, neutralizing naturally occurring inhibitory factors should also prove a successful strategy to enhance growth and boost functional recovery. Chondroitinase ABC (ChABC), a glycosidase with strong specificity for glycosaminoglycans, has been shown to enhance nerve regeneration (Crespo et al., 2007; Kehoe et al., 2012; Kwon et al., 2011). The mechanism is through cleavage of chondroitin sulphate proteoglycans, major inhibitory regulators of axonal regeneration after nerve regeneration. Interestingly, single injections of ChABC at the site of peripheral nerve repair have shown benefit in a hind limb vascularized composite allotransplantation model, leading to increased axonal number and fibre density across the anastomosis site (Tuffaha et al., 2011).

The development of successful ChABC-based clinical therapies has been limited by the stability of the enzyme, the best results demonstrating retention of enzymatic activity at 4 weeks in vitro (Lee et al., 2010). Trehalose, a natural α-linked disaccharide formed by an α,α-1,1-glucoside bond between two α-glucose units, is implicated in anhydrobiosis (i.e. the ability of plants and animals to withstand prolonged periods of desiccation) and has been shown to significantly enhance the stability and temperature tolerance of proteins and DNA (Barreca et al., 2013; Colaco et al., 1992). In this study we report a new method of fashioning thin silk fibroin films incorporating trehalose and use this method to stabilize and deliver multiple trophic factors to the regenerating nerve. The drug-eluting silk films are easily inserted inside nerve conduits, enabling a new method of treating peripheral nerve injuries.

2. Materials and methods

2.1. Fabrication of silk fibroin nerve guide and GDNF/ChABC silk film

A 6–8% w/v silk fibroin aqueous solution was obtained from Bombyx mori silkworm cocoons using previously described procedures (Kim et al., 2004; Li et al., 2006). Briefly, the silkworm cocoons (supplied by Tajima Shoji Co., Yokohama, Japan) were extracted in 0.02 M sodium carbonate solution, rinsed in distilled water, dissolved in 9.3 M lithium bromide and dialysed against distilled water, using a Slide-a-Lyzer dialysis cassette (MWCO 3500, Pierce, Rockford, IL, USA) for 48 h. The resulting 6–8% w/v fibroin solution was concentrated by placing the dialysis cassette on the benchtop and allowing water to evaporate through the dialysis membrane to produce a 20–30% w/v silk fibroin aqueous solution. All silk fibroin solutions were stored at 4°C until used to make silk nerve conduits or drug-eluting films. All reagents, unless otherwise specified, were of analytical grade and obtained from Sigma-Aldrich (St Louis, MO, USA).

Porous silk fibroin conduits were prepared according to previously described protocols, with slight modification (Ghaznavi et al., 2010). Briefly, tubes were formed by
dipping stainless steel mandrels (2 mm diameter; McMaster-Carr, Elmhurst, IL, USA) into concentrated aqueous silk fibroin solutions (20–30% w/v) blended with 7 wt% poly(ethylene oxide) (PEO) at a ratio of 98:2 (wt%) silk fibroin:PEO. The stainless steel mandrels were dipped into the silk–PEO blend and, when evenly coated, dipped in methanol to crystallize the amorphous silk protein into a water-stable conformation, characterized by anti-parallel β-sheets (Jin and Kaplan, 2003). Tubes were alternately dipped in the silk–PEO blend and methanol until the steel wire was evenly coated with a thickness of ~0.6 mm (3–5 times). The coated wires were left to dry overnight before being hydrated with distilled water to remove the tubes from the wires, and placed in distilled water to extract the PEO, leaving porous silk conduits. The guides were cut to a length of ~17 mm and stored submerged in water until film implantation.

The drug-eluting silk fibroin/trehalose films were manufactured under sterile conditions. Silk solution was diluted to 2% w/v and sterile-filtered. The dilute solution was then concentrated to ~25% w/v via centrifugation in sterilized Amicon ultra centrifugal filters (EMD Millipore, Billerica, MA, USA). The sterile ~25% w/v silk fibroin solution was then mixed with an equal volume of sterile 1 M trehalose in distilled water; 1 μg GDNF and/or 2.0 U ChABC (Sigma-Aldrich) was optionally added to the silk–trehalose solution. Films were fabricated by casting 50 μl silk–trehalose solution on 17 mm × 8 mm polydimethylsiloxane blocks and allowing to air-dry in a laminar flow hood.

Prior to assembly of the nerve guides, the silk fibroin conduits were autoclaved. The nerve guides were assembled in a laminar flow hood using sterile gloves. The silk–trehalose films were rolled around a 1.5 mm diameter steel mandrel (McMaster-Carr, Elmhurst, IL, USA) and then inserted into the silk conduit (see Figure 1). The 8 mm length of the rolled film covered nearly half the length of the inner lumen of the conduit. A simple gradient distribution is obtained by inserting the drug-eluting film into only half of the conduit, with this half serving as the distal aspect of the guide when implanted. Four sets of six silk nerve guides were prepared with different drug-eluting silk–trehalose films, as follows: group 1, empty vehicle; group 2, 1.0 μg GDNF; group 3, 2.0 U ChABC; and group 4, 1.0 μg GDNF and 2.0 U ChABC. The sterile nerve guides were stored at ~80°C until ready for implantation.

2.2. Nerve guide drug delivery

GDNF release was quantified using an enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN, USA), according to the manufacturer’s instructions; a total of six nerve conduits were prepared with 1 μg GDNF loaded into silk–trehalose films inserted within each guide. The guides were incubated at 37°C for 6 weeks in 125 μl phosphate-buffered saline (PBS) solution supplemented with 0.01% w/v sodium azide. The incubated solution with released GDNF was collected on days 0, 1 and 3 and at weeks 2, 3, 4, 5 and 6 and frozen at ~22°C. An eight-point calibration curve of GDNF (2000–15.6 pg/ml) was linearized by plotting the log of the GDNF concentration vs the optical density at 450 nm, and used to determine the GDNF released at each time point. Data were collected using a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA, USA). Absorbance values were obtained at 450 nm, with background correction completed by subtracting the absorbance at 540 nm; three replicates were tested in parallel.

An unsaturated disaccharide produced by the ChABC-mediated digestion of the chondroitin sulphate (CS) absorbs light at 232 nm, allowing ChABC activity to be quantified based on the slope of the resulting absorbance curve (Yamagata et al., 1968). A standard curve of ChABC activity was prepared by mixing 100 μl of a known ChABC solution with 10 μl 5 mg/ml CS in PBS solution in a UV-transparent cuvette (Brandtech, Essex, CT, USA). The cuvette was immediately inserted into a SpectraMax M2 UV-vis Spectrophotometer (Molecular Devices, LLC, Sunnyvale, CA, USA) and maintained at 37°C, while the absorbance of the solution at 232 nm (A232) was read every 1 s for 5 min. A linear fit was applied to the absorbance curves between 1 and 5 min; the data were truncated at the point where the rise in A232 ceased (i.e. indicating exhaustion of the substrate). The slope of the A232 line for each known ChABC solution was plotted against the known concentration, and a linear fit was obtained (data not shown). To test release from the nerve conduits, silk–trehalose films with 2.0 U ChABC were fabricated as described above, loaded into silk nerve conduits and placed into 125 μl PBS maintained at 37°C. At each time point, 100 μl PBS release solution was collected and replaced.

Figure 1. Fabrication procedure for nerve conduits: (1) porous silk nerve guides were made by dip-coating a 2 mm mandrel in concentrated silk–PEO solution; the guides were cured using methanol immersion after each coating, and autoclaved to produce sterile guides; (2) silk–trehalose solutions were prepared with or without activ-ites (i.e. GDNF and/or ChABC), sterile-filtered, cast onto a silicone sheet, dried to a flexible film and rolled over a 1.5 mm mandrel; (3) the film insert was guided into the silk–PEO nerve guide, the insert mandrel was removed and the assembled device was water-annealed for 2 h. [Colour figure can be viewed at wileyonlinelibrary.com]
The collected solution was added to a UV-transparent cuvette along with 10 μl 5 mg/ml CS. The slope of \( A_{232} \) was used to calculate the ChABC release by way of the previously constructed standard curve; three replicates were tested in parallel.

### 2.3 In vivo studies

Lewis rats \( n = 24 \) were housed individually and received a standard rat chow (Rodent Laboratory Chow 5001, Purina Co., USA) and water ad libitum in the Division Laboratory Animal Resources facilities of the University of Pittsburgh. All in vivo experiments were approved by the University of Pittsburgh Animal Care facility. The rats were maintained in a room at constant temperature with a 12 h light–12 h dark cycle. Following a 1 week acclimatization period, the animals were randomly divided into four groups, as follows: group 1, silk fibroin nerve guide with empty silk–trehalose film \( n = 6 \); group 2, silk fibroin nerve guide with silk–trehalose film containing 1.0 μg GDNF \( n = 6 \); group 3, silk fibroin nerve guide with silk–trehalose film containing 2.0 U ChABC \( n = 6 \); and group 4, silk fibroin nerve guide with silk–trehalose film containing 1.0 μg GDNF and 2.0 U ChABC \( n = 6 \).

### 2.4 Surgical procedure

Each animal was weighed and anaesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg) intraperitoneally. The right hind limbs were shaved and cleansed with 70% ethanol. While under anaesthesia, a 3 cm skin incision 5 mm below and parallel to the femur was made through the panniculus carnosus, exposing the fascia of the underlying hind limb musculature. The midpoint of the biceps femoris muscle belly was visualized and split parallel to the muscle fibres, with a combination of blunt and sharp dissection. The entire length of the right sciatic nerve from its exit from the pelvis to its bifurcation at the knee was exposed in a bloodless field (see Figure 2). Under a surgical microscope a 5 mm segment from the middle of the sciatic nerve was excised, the stumps were allowed to retract and a 17 mm nerve guide was used to bridge the resulting gap. Two 9-0 nylon transverse mattress sutures were used at each nerve stump to secure the guide in position. Each nerve stump was inserted 1 mm into the lumen of the conduit, resulting in a 15 mm gap. The conduit was orientated such that the silk–trehalose film was located adjacent to the distal nerve stump. The conduit lumen was filled with normal saline and the muscle was closed with four interrupted absorbable 4-0 Vicryl sutures; the skin was then closed with a single running absorbable 4-0 Vicryl suture. The rats were returned to their housing location after recovery from the anaesthetic on a warming pad. The rats received injections of buprenorphine intraperitoneally (0.1 mg/kg) every 12 h for postoperative analgesia for a total of 72 h.

The rats were followed for 6 weeks, monitoring the wound healing closely in the first week. After 6 weeks, the animals were euthanized, using carbon dioxide inhalation and cervical dislocation. Careful dissection through the previous incision was done to expose the sciatic nerve with the graft. All harvested grafts and conduits were intact with no anastomotic disruptions or fractures along the length the guide. The graft was harvested en bloc, leaving at least 5 mm of the nerve stump at each end. Explanted samples were tied to a toothpick to prevent contraction and to preserve the orientation during the processing of the tissues, utilizing a black suture to mark the proximal end and a white suture the distal end. Tissues were immediately placed in sterile PBS upon harvest and fixed on the day of harvest.

### 2.5 Gastrocnemius muscle weight

Gastrocnemius muscles were harvested from both legs of each animal following removal of the nerve guides. In both the injured and uninjured limbs, the gastrocnemius muscles were carefully dissected and isolated from other surrounding muscles and the overlying skin. The muscles were immediately placed in sterile PBS upon harvest and later on the day of harvest were removed from PBS, blotted on dry gauze to remove any residual moisture and weighed.

![Figure 2. Representative surgical photographs of silk fibroin guide implantation: (A) exposure of the sciatic nerve; (B) implanted guide secured with 9–0 nylon sutures. (Colour figure can be viewed at wileyonlinelibrary.com)](image_url)
2.6. Histology and immunohistochemistry

The harvested nerve grafts were first fixed for 6 h in 4% paraformaldehyde, then overnight in 0.1% osmium tetroxide (to aid in sectioning of the tissues), and stored in PBS with 0.2 g glycine/100 ml at 4°C until they were processed (Scipio et al., 2008). All nerve samples were cross-sectioned at the level of the proximal nerve stump, proximal third of the conduit, the middle third of the conduit, the distal third of the conduit and the distal nerve stump (see Figure 3); the sections were paraffin-embedded in preparation for immunohistochemical analysis.

Sections (5 μm) were cut from all paraffin nerve blocks and affixed on glass slides. The sections were deparaffinized by three immersions in xylene, then hydrated by descending concentrations of ethanol (100%, 90% and 70%) to PBS. Osmium tetroxide was etched away from these sections with immersion in 3% hydrogen peroxide solution for 1 h. The slides were then blocked in goat serum and incubated overnight with either monoclonal rabbit S100 antibody (Sigma, 1:800) or mouse PGP 9.5 antibody (Abcam, 1:800). After washing, a secondary fluorescent goat anti-rabbit IgG Cy3-labelled antibody (Sigma; 1:400) or goat anti-mouse IgG Cy3-labelled antibody (Jackson Immuno; 1:400) was used to allow for quantitative detection of the fluorescent label at the S-100 protein or neuroendocrine marker PGP 9.5, respectively.

The five samples from each section of all treatment groups were evaluated at ×20 and ×40 magnification, using a fluorescent microscope (Olympus Provis AX-70; Olympus America) equipped with a camera (Olympus U-MAD 2, Olympus, Japan). The images were captured using the program MagnaFire 2.1B (Olympus) and analysed using ImageJ software; image thresholds were used to identify the S-100 or PGP 9.5 staining of the entire native nerve calculated using the following formula:

\[
\text{Target protein (\%)} = \frac{\text{target protein pixel area/native nerve pixel area}}{100}
\]

2.7. Statistical analysis

All results are shown as mean ± standard deviation (SD) and five specimens from each sample were utilized as described in the above sections. All data from this experiment were analysed by a two-tailed, unequal-variance t-test performed in Excel v 14.3.9 (Microsoft): \( p < 0.05 \) was considered significant; *statistical significance at this level in the accompanying figures.

3. Results

3.1. Drug release

The release of GDNF from the silk–trehalose films inserted within the silk fibroin conduits was characterized by slow steady release over a 6 week period (see Figure 4A). At 1 week, 10% of the total GDNF drug load was recovered. Over the subsequent 5 weeks, the recovered GDNF reached approximately 20% of the initial drug load. A plateau in GDNF release was not observed at the conclusion of the 6 week study, indicating ongoing active release of drug. The activity of ChABC released from the silk–trehalose films within the silk fibroin conduits was characterized by a large burst that occurred within the first hour of incubation in 37°C PBS (see Figure 4B). At the 1 h time point, almost 15% of the loaded enzyme activity was recovered. Over the next 5 h, the recovered enzyme activity reached approximately 18.5% and at 24 h 20% of the loaded enzyme activity had been recovered. Negligible active ChABC was recovered after 24 h.

3.2. Gastrocnemius muscle weight

As an indicator of functional recovery, gastrocnemius muscle weight was measured; the weight from the injured right side was compared to the uninjured left side. Figure 5 shows the gastrocnemius weights at 6 weeks for different groups of silk nerve guides that were implanted in rat sciatic nerve injuries. Significantly different gastrocnemius muscle weights were observed between the injured and uninjured legs in all groups (\( p < 0.0001 \)). A significant difference was also observed between the gastrocnemius muscle weights in the injured legs of the control and ChABC treatment groups (0.328 ± 0.044 and 0.383 ± 0.037 g, respectively; \( p < 0.02 \)). No significant differences were detected between the remaining treatment groups (\( p > 0.05 \)).

3.3. Immunohistochemical staining

There were no fractures along the lengths of the conduits or dislodgement of the sciatic nerve stumps from their insertion sites in all of the treatment groups. Samples were paraffin-embedded, sectioned and stained with either anti-S-100 or anti-PGP9.5 fluorescent antibodies in order to identify and quantify these protein targets of interest in the regenerating nerve and to assess the quality of the regeneration in the conduit (see Figure 3).

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Nerve sections stained with S-100 antibody demonstrated significant differences between the four groups at various points along the lengths of the specimens (see Figure 6, Table 1). Higher degrees of staining are indicative of greater infiltration and proliferation of Schwann cells within the regenerating nerve, and calculation of percentage target protein across multiple samples allows for statistical comparison amongst the treatment groups. The pattern of S-100 staining seen was largely endoneurial and, as expected, was most easily seen in the proximal and distal nerve stumps shown in Figure 6; this pattern is consistent with previous published studies (Ghaznavi et al., 2010; Lin et al., 2011). The proximal nerve stumps clearly demonstrated greater S-100 staining in both the ChABC- and GDNF/ChABC-treated groups compared to control and GDNF. S-100 staining in the proximal conduit was highest in the combined GDNF/ChABC group relative to all others. In the middle conduits, all the treatment groups stained higher than the control, while GDNF/ChABC stained higher than either ChABC or GDNF alone. In the distal conduits, all treatments stained higher than the control, and ChABC demonstrated significantly higher S-100 staining than GDNF. In the distal nerve stumps the ChABC group demonstrated higher S-100 staining than all the other treatment groups. Not all significant differences listed in Table 1 may be readily depicted in Figure 6, due to variation amongst the intensities of the samples.

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Figure 4. Drug release form nerve conduits incubated at 37°C in PBS: (A) GDNF release from nerve conduits containing silk–trehalose films loaded with 1 μg GDNF; (B) ChABC activity recovered from nerve conduits containing silk–trehalose films loaded with 2 U ChABC. Each data point is the average of six nerve conduits; error bars represent SDs.

Figure 5. Gastrocnemius muscle weights for injured and uninjured legs; error bars represent SDs.

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Figure 6. Representative images (×40 magnification) of silk conduits fluorescently stained with antibody for S-100 protein. [Colour figure can be viewed at wileyonlinelibrary.com]
Nerve sections stained with PGP9.5 antibody demonstrated significant differences between the four treatment groups in all areas that were assessed along the lengths of the specimens, with the exception of the distal nerve stump (see Figure 7, Table 2). Higher degrees of PGP9.5 staining are indicative of greater axon numbers and/or diameters, as the protein is expressed in the cytoplasm of the projecting axons. The pattern of PGP9.5 staining observed is consistent with an axonal pattern and is similar to previous published studies (Ghaznavi et al., 2010; Lin et al., 2011). The proximal nerve stumps demonstrated greatest PGP9.5 staining in the GDNF/ChABC-treated group. The proximal conduits showed equivalent PGP9.5 staining in the ChABC/GDNF and ChABC groups, which was greater than the control and GDNF. In the middle conduit, GDNF and ChABC treatments were equivalent and stained higher than the control group. In the distal conduit all treatments stained higher than the control, while GDNF demonstrated significantly higher PGP9.5 staining than either ChABC or GDNF/ChABC. There were no significant differences in PGP9.5 staining in the distal nerve stump. Not all significant differences listed in Table 2 may be readily depicted in Figure 7, due to variation amongst the intensities of the samples.

4. Discussion

In this paper, we report the fabrication of a silk fibroin nerve conduit incorporating a novel silk–trehalose film for delivery of trophic factors to a regenerating nerve, and stained higher than the control group. In the distal conduit all treatments stained higher than the control, while GDNF demonstrated significantly higher PGP9.5 staining than either ChABC or GDNF/ChABC. There were no significant differences in PGP9.5 staining in the distal nerve stump. Not all significant differences listed in Table 2 may be readily depicted in Figure 7, due to variation amongst the intensities of the samples.

Table 1. S-100 protein percentage of nerve tissue sections

<table>
<thead>
<tr>
<th>Region</th>
<th>Control</th>
<th>GDNF</th>
<th>ChABC</th>
<th>GDNF/ChABC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Middle conduit (MC)</td>
<td>9.482 ± 1.022*</td>
<td>15.092 ± 4.719*</td>
<td>14.712 ± 2.608*</td>
<td>16.463 ± 1.436*</td>
</tr>
<tr>
<td>Distal nerve (DN)</td>
<td>14.736 ± 2.145*</td>
<td>10.942 ± 4.401*</td>
<td>16.901 ± 4.780*</td>
<td>13.313 ± 2.462*</td>
</tr>
</tbody>
</table>

GDNF, glial cell line-derived neurotrophic factor; ChABC, chondroitinase ABC. Values in the same row without the same superscript are significantly different ($p < 0.05$).

Figure 7. Representative images (×40 magnification) of silk conduits fluorescently stained with antibody for neuroendocrine marker PGP9.5. [Colour figure can be viewed at wileyonlinelibrary.com]

Table 2. PGP9.5 Protein percentage of nerve tissue sections

<table>
<thead>
<tr>
<th>Region</th>
<th>Control</th>
<th>GDNF</th>
<th>ChABC</th>
<th>GDNF/ChABC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proximal conduit (PC)</td>
<td>13.950 ± 2.412*</td>
<td>15.552 ± 4.606*</td>
<td>19.860 ± 5.531*</td>
<td>20.453 ± 2.750*</td>
</tr>
<tr>
<td>Middle conduit (MC)</td>
<td>12.913 ± 2.552*</td>
<td>17.965 ± 4.447*</td>
<td>21.373 ± 6.657*</td>
<td>17.643 ± 5.042*</td>
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<tr>
<td>Distal conduit (DC)</td>
<td>14.693 ± 2.108*</td>
<td>20.697 ± 4.493*</td>
<td>21.597 ± 2.558*</td>
<td>17.876 ± 1.859*</td>
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<tr>
<td>Distal nerve (DN)</td>
<td>15.428 ± 2.199*</td>
<td>15.094 ± 2.227*</td>
<td>14.596 ± 4.091*</td>
<td>14.241 ± 2.326*</td>
</tr>
</tbody>
</table>

GDNF, glial cell line-derived neurotrophic factor; ChABC, chondroitinase ABC. Values in the same row without the same superscript are significantly different ($p < 0.05$).
and assess its performance in a critical-sized rat sciatic nerve gap injury model. GDNF and ChABC are delivered from the nerve conduits in quantities and over time frames previously shown useful for nerve regeneration and repair. We have chosen to assess nerve regeneration at 6 weeks in this study, because reliable histological outcomes, specifically changes in S-100 and PGP9.5 staining, are readily apparent at this time point. With our conduit we demonstrate not only a method to enhance Schwann cell migration and proliferation, but also foster axonal regeneration across peripheral nerve gap defects.

GDNF delivery to a damaged peripheral nerve has proved successful in boosting nerve repair responses (Kokai et al., 2010; Lin et al., 2011; Patel et al., 2007). Stable steady release of GDNF was achieved from the silk guides; these results are comparable to previously published results obtained by our group using double-walled microsphere drug-delivery reservoirs (Lin et al., 2011). In this study we have again confirmed the beneficial effect of a GDNF-loaded nerve conduit. However, our intention was to further augment this response with the addition of ChABC to the nerve guide.

We have been able to stabilize and release ChABC from our nerve guides and extend the therapeutic window of the enzyme. The large burst of active ChABC that, based on the in vitro data, was released in the first 24 h after implantation may have provided a similar effect to the single doses of enzyme previously shown to positively affect outcomes after nerve transection (Tuffaha et al., 2011). The fact that minimal active ChABC was recovered after 24 h could be due to a combination of thermal inactivation of the enzyme, or active enzyme remaining bound within the silk–trehalose film. There is some evidence that ChABC activity is maintained longer in vivo than under in vitro physiological conditions, with one study showing that the enzyme remained active for over 10 days after injection (Lin et al., 2008). This suggests that our in vitro release study may underestimate the amount of enzyme released from the nerve conduits in vivo. Furthermore, cellular interactions with the silk film would likely lead to increased release as the biomaterial was broken down. The fact that multiple parameters quantifying nerve regeneration were increased in the ChABC groups indicates that this may be the case.

Muscles are known to undergo atrophic changes in response to nerve injury, and muscle weight can be used as a simple measure of functional recovery. In our previous studies, nerve guides with GDNF loaded in double-walled microspheres have not significantly altered gastrocnemius muscle weight loss at a 6-week time point (Lin et al., 2011, 2012). On the other hand, Kokai et al. (2011) observed that the gastrocnemius muscle weight from animals treated with isografts as a positive control and a control poly(caprolactone) guide without growth factor, albeit at 16 weeks post-surgery. Our results suggest that rats treated with ChABC have a small but statistically significant reduction in gastrocnemius muscle weight loss at 6 weeks after surgery (see Figure 5). This result was not observed in the group treated with combined GDNF/ChABC.

Migration and proliferation of Schwann cells into an area of damaged or even missing nerve creates a temporary bridge, across which axon regeneration can proceed; neurotrophic factors, such as GDNF, have been shown to enhance this process (Henderson et al., 1994). Degradation of inhibitory products, specifically chondroitin sulphate proteoglycans, can reduce glial scar and similarly enhance cellular migration and proliferation into areas of damaged and missing nerve (Crespo et al., 2007; Ikegami et al., 2005). We observed that ChABC treatment alone increases S-100 protein expression with similar or greater efficacy to GDNF treatment in each case, suggesting that ChABC also supports Schwann cell migration as well as enhances axonal regeneration. We would expect to observe a synergistic effect in the S-100 staining results from our experiment, with the GDNF/ChABC group displaying greater staining intensity throughout the length of the nerve guides. In all sections except for the distal conduit and distal stump we observed significantly higher S-100 staining from the GDNF/ChABC treatment (see Table 1). Effects in the distal conduit may not be as pronounced, due to loss of neurotrophic agents out of the distal end of the conduit around the nerve stump and into the surrounding tissues; this may explain why the trend was not continued down the entire length of the guide. Furthermore, in the distal conduit the higher degrees of staining were observed with ChABC compared to GDNF. This may suggest that, rather than utilizing gradient distributions of ChABC, which have previously been shown to be superior with GDNF (Lin et al., 2011), uniform distributions of ChABC along the length of the guide may be preferable.

Axon regeneration across a gap defect in a nerve initiates from the proximal uninjured tissues. As the proportion of PGP 9.5 protein increases in nerve tissues, the axon density and overall number concurrently increase. Again, we would expect to observe a synergistic effect in the PGP9.5 staining results from our experiment, with the GDNF/ChABC group displaying greater staining intensity along the length of the nerve guides. At our 6 week time point, ChABC treatments do show higher PGP9.5 staining relative to GDNF in the proximal and middle portions of the conduit (see Table 2). Unfortunately, combined GDNF/ChABC does not show any significant improvement over ChABC alone. However, close examination does reveal a steady increase in PGP9.5 staining in both GDNF and ChABC groups, reaching maximum values in the distal and middle portions of the conduits, respectively, and overall higher intensities are associated with ChABC. It may be noted, however, that glial scar formation also serves important functions for injury repair, including reducing the overall immune response and limiting cellular degradation (Silver and Miller, 2004). The large dosing of ChABC at the distal end of the conduit may have contributed towards to lower PGP9.5 expression observed in the distal conduit and distal stump. This again may suggest that ChABC should be utilized in uniform distributions along the length of the nerve conduit.
When combining therapeutic agents, it is necessary to consider not only the amounts to be administered but also the spatial and temporal interactions of the agents. Our results do indicate that both ChABC and GDNF do increase Schwann cell migration and axonal regeneration. However, the results we obtained were not consistent with our initial hypothesis. There are two issues to consider with the use of agents such as ChABC: (a) degradation and inactivation of the enzymatic activity; and (b) spatial distribution of the agent. Schwann cell migration and proliferation would occur prior to axon regeneration, and we do see increased PG9.5 staining along the length of the guide. Axonal regeneration would subsequently occur and we do see increased PG9.5 staining, but only in the proximal portion of the conduit. It is possible that the ChABC activity had fallen too low to be effective when axonal regeneration was occurring. It is also possible that limitations could have been overcome by utilizing a uniform distribution.

5. Conclusion

Silk-based nerve conduits are biocompatible and possess favourable mechanical properties along with slow degradation rates making them attractive for repair of peripheral nerve gap defects. Furthermore, silk films represent an attractive candidate material from which to stabilize and deliver neurotrophic agents inside a nerve guide to the regenerating nerve. Our results demonstrate silk-fibroin-based nerve conduits containing silk films loaded with GDNF and ChABC to be a promising strategy for the treatment of peripheral nerve gap injuries. Future studies will involve optimizing the distribution of ChABC and further increasing its duration of activity to enhance axonal regeneration when delivered with GDNF.

Conflict of interest

The authors have declared that there is no conflict of interest.

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