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Silk Fibroin Conduits: A Cellular and Functional Assessment of Peripheral Nerve Repair

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Abstract

Novel silk fibroin conduits were designed with appropriate porosity for peripheral nerve repair. The aim of this work was to utilize these conduits to examine cell inflammatory responses and functional recovery in a sciatic nerve defect model.

45 randomized Lewis rats were utilized to create an 8-mm defect bridged by a silk guide, commercial collagen guide, or an autograft. After 1, 4 and 8 weeks, macrophage recruitment, percentage of newly formed collagen, number of myelinated axons, and gastrocnemius muscle mass were evaluated. Following 8 weeks, ED1+ cells in autograft and silk conduits decreased to < 1% and 17% of week 1 values, respectively. Collagen formation revealed no difference all measured time points, suggesting a similar foreign body response. Myelinated axon counts within the silk guide revealed a greater number of proximal spouts and distal connections than collagen guides. Gastrocnemius weights demonstrated a 27% decrease between silk and autografts after 8 weeks.

This study demonstrates that, in addition to tailorable degradation rates, our silk conduits possess a favorable immunogenicity and re-myelination capacity for nerve repair.

Introduction

In clinical practice, peripheral nerve injuries comprise approximately 5% of all open wounds of the limbs.¹ Such injuries require therapies that maximize neuronal regeneration and are necessary to optimize a patient's functional recovery. Currently, the clinical gold standard for repair of large peripheral nerve defects is autologous nerve grafts. Previous work has shown that this technique appears to be the best option for bridging a nerve gap in clinical situations.² The autograft serves as a physical guide composed of morphologically native biomaterial, which allows for the progression of "sprouting" axons from the proximal end to distal nerve stump. However, using an autograft to repair a nerve defect, the size of the defect can be a limiting factor to optimal repair. During repair, altered fascicular architecture in the graft may cause the axons to scatter during regeneration and thereby prevent regeneration. Other limitations include the number of donor sites available for graft harvest

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and there may be significant morbidity such the loss of donor site nerve function, the formation of a painful neuroma, or even hyperesthesia at the area of sensory loss. These disadvantages associated with autologous nerve graft repair have lead to the study of synthetic nerve guides as a method of repair.

Biodegradable nerve guides have proven to be a successful alternative to native nerve grafts for the repair of relatively short nerve defects in clinical studies. The advantages of this type of therapy includes the ability of the guide to direct the outgrowing nerve fibers towards the distal nerve stump while preventing the formation of a neuroma by inhibiting the ingrowth of fibrous tissue. It is paramount to identify a guide that possesses a slow degradation time, high tensile strength, and no cellular toxicity for successful peripheral nerve repair.

In this study, we describe the use of a silk fibroin conduit as a biodegradable nerve guide. Silk fibroin, derived from *Bombyx mori* silkworm cocoons, has been well characterized in many biomedical applications.^{3–5} Previous work has shown that the silk protein is biocompatible, degrades slowly in the body, has high tensile strength, minimum swelling and has minimal cytotoxic properties.^{6, 7} Here, the immunogenic profile and neuroregenerative capacity of silk fibroin as a nerve guide was evaluated *in vivo* in a rat nerve defect model. The parameters for evaluation included an assessment of macrophage infiltration and percentage of collagen formed. The assessment of neuronal regeneration was based on nerve histomorphometric parameters, measurements from nerve sections such as fiber and axon areas, myelin sheath thickness, fiber density, and g-ratio. Furthermore, an indirect measure of functional recovery following a nerve gap lesion was assessed by the gastrocnemius weight ratio.

As a comparison to the silk nerve guide, an Food and Drug Administration-approved collagen type I guide was used. When used in repair of short nerve gaps less than 10 mm, tubulization with collagen has demonstrated comparable neuroregenerative performance to that of nerve autografts in rodent and nonhuman primate experimental models. As an additional control, an autograft group was included to serve as the positive control for the study.

We hypothesized that the silk guide would have a low immunogenic reaction based on macrophage infiltration and collagen formation after the implantation of the guide for repair of the gap defect. We also hypothesized that the neuroregenerative capacity and functional recovery after eight weeks would be comparable or superior to that of a collagen type I guide.

Material and Methods

Preparation and Sterilization of Silk Guide

Porous silk tubes were prepared according to previously described protocols, with slight modifications.⁸ Tubes were formed by dipping stainless steel wire (1.6 mm diameter, Type 304V, Small Parts, Miami Lakes, FL) into concentrated silk fibroin (20–30% w/v) blended with 7 wt% poly(ethylene oxide) (PEO) at a ratio of 98/2 (wt%) silk fibroin/PEO. The silk fibroin/PEO blends were gently mixed with a micropipette tip before sonicating for 10 minutes. The stainless steel wires were dipped into the silk/PEO and, when evenly coated, dipped in methanol to transform the amorphous silk solution into the β -form silk fibroin conformation, characterized by anti-parallel β -sheets.⁹ Tubes were alternately dipped in the silk/PEO blend and methanol until the steel wire was evenly coated with a tube wall thickness of ~0.6 mm (3–5 times). Tubes were prepared by repeating the alternate dipping process 2–3 times, followed by 1–2 dips in an unloaded 98/2 (wt%) silk/PEO blend. The coated wires were then left to dry overnight before being cut at each end, placed in a

surfactant solution to remove the tubes from the wires, and placed in distilled water to extract the PEO from the silk/PEO tubes, leaving porous silk tubes. Before use, the guides were immersed in 100% ETOH for 15 minutes followed by PBS washes until its insertion.

Animals

Forty-five male Lewis rats (250–350 g) were randomly assigned to the four following groups: Group 1: Autograft (nerve autograft) ($n=15$); Group 2: Silk nerve Guides ($n=15$); Group 3: Collagen nerve guide ($n=15$); Group 4: Control (unoperated contralateral leg samples randomly analyzed from the 45 animals) ($n=6$). The animals were housed in the Division laboratory Animal Resources facilities of the University of Pittsburgh and given food and water ad libitum. All experiments were performed in accordance with the National Institutes of Health *Guide for Care and Use of Laboratory Animals* and were approved by the University of Pittsburgh Animal Care facility.

Surgical Procedure

Each animal was weighed and anesthetized with pentobarbital (50mg/kg) intraperitoneally. The animals were shaved and the surgical area cleaned with 70% ethanol. A longitudinal gluteal skin incision parallel to the femur was performed. Muscles were divided to expose the sciatic nerve from the sciatic notch to the point of bifurcation. Once the nerves were isolated and bleeding controlled with electrocautery, an 8 mm nerve segment of the sciatic nerve was resected. In group 1, autograft ($n=15$), after the removal of the nerve segment, the 8mm nerve was used as a nerve graft and was sutured to both stumps with 10-0 Prolene sutures (Ethicon, Somerville, NJ), with conventional epineurial techniques. In group 2 and 3 ($n=15$ each), segmental nerve resection was performed and a 1 cm nerve guide was used to bridge the defect. The muscle and skin was sutured with 4-0 Vicryl (Ethicon, Somerville, NJ). In group 4 ($n=6$) the contralateral unaffected nerve was harvested to serve as a control sample (Figure 1). Animals in each group were euthanized at weeks 1 ($n=3$), 4 ($n=6$), and 8 ($n=6$). At harvest, all grafts and conduits were intact with no anastomotic disruptions or fractures along the length the guide. All tissue were fixed in 3% paraformaldehyde and cross-sectioned at the level of the proximal stump, proximal third area of the graft or regenerated area, the mid-graft or conduit, distal third area of the graft or regenerated area, and the distal stump (Figure 2).

Histology and Gastrocnemius muscle harvest

Masson's Trichrome

Five micron sections were prepared from nerve samples from group 1 (autograft), group 2 (silk), and group 4 (controls) at all time points. Group 3 was excluded from this analysis as the guide was composed of collagen thereby confounding the identification of newly formed collagen. Based on a protocol of Scipio et al., a Masson's trichrome kit was used to stain specifically for collagen (American Master Tech Scientific, Inc, Lodi, CA).¹⁰ The samples were evaluated at 100x magnification using a 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) analyzed with the software MetaMorph Off-Line Version 6.2 (Universal Imaging Co.) image thresholds were used to identify the collagen staining, with total collagen percentage of the entire sample calculated using the following formula (Figure 3):

$$\text{Collagen Percent} = (\text{Collagen Pixel Area} / \text{Total sample Pixel area}) \times 100$$

Immunohistochemistry

Five micron sections were incubated with monoclonal ED1 antibody (Serotec), after which a secondary fluorescein isothiocyanate (FITC)-labeled anti-mouse antibody allocates the green fluorescent label at the activate macrophages. The samples were evaluated at 600x magnification and imaged using the microscope as described above. Six images were taken per section and a total count (TC) of positive stained ED1 cell was made. The adjusted count (AC) was then calculated from the TC using the below formula (Figure 4):

$$AC = (TC/6) \times (\text{Area of the Nerve} / \text{Total Field Area})$$

Gastrocnemius Weight Ratio

After nerve harvest from all animals, a longitudinal incision was performed in the lower leg, allowing extraction of the muscle. The gastrocnemius weight ratio (GWR) was determined using the following formula:

$$GWR = (\text{experimental muscle weight} / \text{Contralateral muscle weight}) \times 100$$

Histomorphometry

Five micron sections were stained with 1% toluidine blue dye, and mounted on slides for imaging with a Hitachi CCD KP-M1AN digitizing camera mounted on a Zeiss microscope with a manually controlled stage. A 100x oil immersion objective lens was used to produce a digital image at a final magnification of 1000x. Based on the semi-automated technique detailed by Hunter et al., images were captured using a Leco IA32 Image Analysis System (Leco, St. Joseph, MI).¹¹ Thresholds were applied to six fields from each section to identify myelin features following manual removal of non-myelin features. A total fiber count (TFC) was derived from the total area of the sciatic nerve and the number of fibers counted per mm² of field area to the entire nerve; G-ratio (GR) was calculated from the axon width and fiber width (FW); the percentage of neural tissue (PN) was defined as the percent of cross-sectional area of nerve specimen containing axons, myelin, and Schwann cells; and the Fiber Density (FD) was calculated from the total area of the nerve segment analyzed (Figure 5). All formulas used are detailed below:

$$TFC = (\text{Fiber count} / 6) \times (\text{Area of the Nerve} / \text{Total Field Area})$$

$$GR = \text{Axon width} / \text{Fiber width}$$

$$FW = \text{Axon width} + \text{Myelin Width}$$

$$PN = ((\text{fiber count} \times \text{fiber area}) / \text{Total Field Area}) \times 100$$

$$FD = (\text{fiber count} / \text{Total Field Area}) \times 1000000$$

Statistics

Differences in the percent collagen, histomorphometric parameters, gastrocnemius weight ratio, and adjusted ED1 counts between groups were evaluated with one-way analysis of variance. When the analysis of variance demonstrated statistical significance ($p < 0.05$), specific group comparisons were performed for the variable by means of Tukey-Kramer post hoc analysis. Values of $p < 0.05$ were considered statistically significant. All statistical tests were performed with StatView 5.0 (SAS Institute, Cary, NC) and data are reported as mean $\pm$ SEM.

Results

Histomorphometry

Eight weeks after implantation, examination of the distal nerve in the silk group demonstrated mean fiber counts of 1947.90 ± 430.43 as compared to the collagen group with a mean fiber count of 380.81 ± 45.20 ($p < 0.05$) (Figure 6). The autograft group showed a mean fiber count of 3004.81 ± 282.44 , a significant difference from the collagen group but showed no statistical difference to the silk group. The g-ratio after eight weeks showed no statistical difference between the three groups at the distal nerve. However, at the mid-conduit, the silk group had a mean value of 0.275 ± 0.5 compared to the autograft 0.447 ± 0.01 and collagen 0.463 ± 0.01 ($p < 0.05$). Results at the distal anastomosis also demonstrated statistically significant differences with a calculated value for the silk of 0.307 ± 0.05 , collagen with 0.405 ± 0.01 , and autograft at 0.432 ± 0.01 ($p < 0.05$). Density at eight weeks reveals a significantly higher fiber density at the mid-conduit for the autograft (11316.21 ± 1686.56), than the silk (4960.17 ± 1733.85) or collagen (5941.94 ± 1224.64) groups. At the distal anastomosis, the density of the silk (11331.47 ± 5371.16) was significantly higher ($p < 0.05$) than that of the collagen (3693.91 ± 1008.145) and autograft (8107.89 ± 1388.94) groups, but there appeared to be no differences in fiber density at the distal nerve. The percent nerve data demonstrated a higher percentage of neural tissue at the distal anastomosis in silk group ($15.42\% \pm 7.27\%$) vs. the collagen group ($5.38\% \pm 1.3\%$). Both the silk and collagen showed no statistical differences at the mid-conduit and distal nerve.

ED1 Macrophage infiltration

After the first week the ED1+ macrophages were significantly higher ($p < 0.05$) in the autograft group (proximal nerve, 437.94 ± 52.64 ; mid-conduit, 964.48 ± 68.69 ; distal anastomosis, 623.80 ± 100.79 ; distal nerve 319.20 ± 48.45) at all sections vs. the silk (proximal nerve 169.56 ± 225.69 , mid-conduit, 290.83 ± 49.47 , distal anastomosis, 494.38 ± 66.42 ; distal nerve 102.30 ± 18.14) and collagen groups (proximal nerve, 1.80 ± 1.01 ; mid-conduit, 20.30 ± 14.63 ; distal anastomosis, 19.77 ± 9.51 ; distal nerve, 4.05 ± 1.14) except in the proximal anastomosis where the silk (1106.91 ± 161.54) had a higher count than the autograft (964.48 ± 68.69) and collagen groups (70.35 ± 14.63) (Figure 7). A significant decrease ($p < 0.05$) in the number of ED1+ macrophages occurred in all sections of the autograft between weeks 1 and 8 to approximately .25%–6.2% of the Week 1 levels. A significant decrease in the number of ED1+ macrophages after 8 weeks was also observed in proximal, proximal anastomosis, and distal nerve sections of 0.17%, 2.3% and 1.9% of the original week 1 levels. A decrease in number of ED1+ macrophages was also shown in the mid-conduit and distal sections but was not statistically significant ($p > 0.05$). There was no statistically significant difference between the week 1 and week 8 numbers of macrophages in the collagen guides. At eight weeks, the ED1+ macrophages persisted at significantly higher levels ($p < 0.05$) in the silk group at the mid-conduit (184.12 ± 21.03) and distal anastomosis (154.39 ± 24.44) than both the autograft (mid-conduit, 39.43 ± 5.77 ; distal anastomosis, 8.17 ± 1.20) and collagen groups (mid-conduit, 2.04 ± 1.64 ; distal anastomosis, 3.19 ± 0.96).

Collagen Composition

After one week, the percent of collagen was statistically similar for the autograft groups at the proximal and distal nerve sections (Figure 8). However, there was significantly more collagen ($p < 0.05$) in the proximal anastomosis ($22.91\% \pm 3.80\%$), mid-conduit ($23.88\% \pm 6.32\%$), and distal anastomosis ($23.80\% \pm 4.10$) of the autograft vs. the silk group ($7.18\% \pm 3.22\%$; mid-conduit, $2.28\% \pm 0.94$; distal anastomosis, $3.00\% \pm 1.09\%$). However, after eight weeks, both groups showed no significant difference at all sections. A comparison between

first and eighth week time points of the autograft samples revealed a significant decrease ($p<0.05$) in the collagen percentage in only the proximal and distal anastomosis of 10.0% and 16.0%, respectively. In the silk groups, however, a significant increase ($p<0.05$) was seen in the proximal and distal anastomosis after eight weeks of 11.7% and 10.7% respectively.

Gastrocnemius Weight Ratio

For the gastrocnemius weight ratio, there was only a significant difference ($p<0.05$) between the autograft ($42.24\% \pm 7.79\%$) and the silk groups ($26.32\% \pm 3.92\%$) after eight weeks (Figure 9). No statistical differences were found between the experimental groups at all time points.

Discussion

Consistent with previously published work,^{12, 13} silk fibroin conduits support peripheral nerve regeneration and have neuroregenerative capacity similar to FDA-approved, commercially available nerve guides. In this study, we further analyzed the inflammatory response of the guides. The silk fibroin nerve guides in this study have easily controlled, tailorable degradation rates, which are beneficial for nerve repair over varying gap lengths. The immunogenic profile and biocompatibility of silk guides are similar to those of host tissue and comparable to other protein-based nerve guides.⁷ Based on ED1 data, macrophages were observed at week one, and decreased to $< 5\%$ of the first week values. Wang et al., showed a similar pattern in cellular infiltration of guides which were constructed from silk fibroin.¹⁴ We hypothesize that this decrease in number of ED1⁺ cells can be attributed to the silk guide's unique degradation rate as observed after harvest. Qualitative examination of the silk guides revealed little degradation of the macro-architecture. According to Vert et al., foreign body reactions that involve slowly degrading materials will elicit a milder inflammatory reaction which involves macrophages.¹⁵ Comparatively, the collagen guides revealed a much milder foreign body reaction at all time points. As shown by Srivastava et al., a semi-quantitative analysis described a mild tissue response to implanted collagen after two weeks.¹⁶ The disparity in macrophage response may also be due to the different degradation rates of the two guides.

The local amount of collagen type I produced by fibroblasts was minimal after 1 week and increased significantly after 8 weeks within the silk group. The peak percentage of collagen reveals the ongoing colonization of the guide at the coaptation sites by proliferating fibroblasts. Ilaria et al showed that after 45 weeks, the silk undergoes a deeper remodeling of newly produced collagen type I fibers, via the activity of metalloproteinases, gelatinases, and cathepsins; at which time the amount of collagen significantly decreased.¹⁷ However, due to our 8 week time point, it would be premature to identify silk as fibrogenic *in vivo*. This limitation in our assessment indicates that further investigation of silk fibroin at a longer time point is necessary in order to evaluate its fibrogenic properties.

The evaluation of the gastrocnemius weight ratio resulted in no significant difference between the collagen and silk groups, with both measuring less than the autograft group. The lack of appropriate gross motor reinnervation can be attributed to the early time point of evaluation.^{18, 19}

The morphometric data suggests that silk possesses comparable neuroregenerative potential similar in regenerative capacity to a collagen-based guide. Studies have shown that inhibition of the repair process by local milieu may occur at the distal stump.²⁰ In the collagen group, at the 8 week time point, spontaneous sprouting occurred from the proximal end, which resulted in an increase in axon count. The observed increase in axon count

reduced from the proximal to the distal stumps in the guide. It has been shown that during the repair process, the proximal end sprouts enter the guide and come into contact with Schwann cell bound to the surfaces at the distal end. However, not all the proximally sprouting axons are successful in establishing a distal end connection. Those that fail to connect at the distal portion will degenerate and lead to a reduction in axon count over time. As noted in previous studies, this phenomenon has been shown during the repair process of the gap defects by nerve guides.²¹ In our study, the silk group demonstrated spontaneous sprouting in greater number at the proximal sections and distal sections but was significantly lower in the guide itself. The discrepancy between the silk and collagen may be due to the micro-architectural differences between the conduits. The lower counts in the mid- and distal sections may be due to this specific silk guide formulation's inherent fragility and tensile strength in vivo. This limitation in our guide may have led to a mechanical obstruction of the axons extending towards the distal stump. This obstruction could lead to a decrease in axonal connections, and further lead to a decrease in axon counts. But most importantly, at the distal-most section, the amount of myelinated fibers was significantly greater than the collagen guide. As the fiber counts increased in the distal sections, the fiber density also concurrently increased. The more connections that were formed from mid to distal sections, the greater number and density of myelinated fibers we observed. This difference at the distal section indicates that more axons have formed connections with proximal sprouts and began an earlier regenerative process in the silk group than the collagen group. The observed difference in number of myelinated axons within the two guides may also be an indication of the difference in migration and attachment of Schwann cells.

In summary, though no difference was seen in the percentage of nerve tissue, fiber density and myelin maturity at the distal sections in comparison to both nerve guide groups, the silk guides proved to be comparable to the collagen groups in promoting axonal maturing and regeneration. The results of this study suggest that while the silk guides do not enhance the factors necessary to promote myelination from the proximal to the distal nerve stump, they do possess both the physical and biological properties to promote nerve repair from the proximal to distal nerve stumps when compared to an autograft and collagen nerve guide. Future experimentation is required to elucidate the advantage of silk nerve guides and if the addition of myelin promoting factors to the nerve guide may enhance myelination.

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Figure 1.

A) An 8 mm nerve segment used as autograft [group 1] B) A 1 cm Silk nerve guide sutured in place. C) A 1 cm Collagen nerve guide sutured in place.

Figure 2.

Nerve sections of a proximal stump nerve stump labeled Proximal Nerve (PN), proximal third of the graft labeled Proximal Anastomosis (PA), the mid-conduit (MC), the distal third of the graft labeled Distal Anastomosis (DA), and the distal nerve stump labeled Distal nerve (DN).

Figure 3.

A) Masson's Trichrome of nerve section at 100x B) Digital threshold of collagen pixel area (green) C) Total nerve sample Pixel area. Bar = 0.1 mm.

Figure 4.

A) Immunohistochemical staining of nerve section for ED1+ cells (green), nuclear staining DAPI (red), 100x. Bar = 0.1mm B) Arrowheads indicate ED1+ (green) with nuclear staining DAPI (red), 600x. Bar = 0.6 mm

Figure 5.

A) Original grey bit digital image of nerve section, 1000x. B) Threshold image of axon bit plane (red) with myelin (green). C) Box image of myelin (my=red) and axon (ax=green). Bar = 0.05 mm

Figure 6.

A) Week 8, fiber count comparing myelinated axons counted at all nerve sections for all groups. B) Week 8, Percentage of nerve in all harvested grafts from all nerve sections for all groups. C) Week 8, G-ratio comparing ratio of axon width to fiber width for all nerve sections for all groups. D) Week 8, Fiber density comparing the fibers per mm^2 at all nerve sections for all groups. All data reported as mean $\pm$ SEM, * = $p < 0.05$.

Figure 7.

A) Week 1 ED1⁺ cell counts at all nerve sections for all groups. B) Week 8 ED1⁺ cell counts at all nerve sections for all groups. All data reported as mean $\pm$ SEM. * = $p < 0.05$.

Figure 8.

A) Week 1 percentage of collagen in nerve grafts at all nerve sections for autografts and silk groups. B) Week 8 percentage of collagen in nerve grafts at all nerve sections for autografts and silk groups. All data reported as mean $\pm$ SEM, * = $p < 0.05$.

Figure 9.

A) Gastrocnemius weight ratio for all groups at all time points. All data reported as mean $\pm$ SEM, * = $p < 0.05$.