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Acellular Nerve Allografts in Peripheral Nerve Regeneration: A Comparative Study

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Abstract

Background—Processed nerve allografts offer a promising alternative to nerve autografts in the surgical management of peripheral nerve injuries where short deficits exist.

Methods—Three established models of acellular nerve allograft (cold-preserved, detergent-processed, and AxoGen® -processed nerve allografts) were compared to nerve isografts and silicone nerve guidance conduits in a 14 mm rat sciatic nerve defect.

Results—All acellular nerve grafts were superior to silicone nerve conduits in support of nerve regeneration. Detergent-processed allografts were similar to isografts at 6 weeks post-operatively, while AxoGen®-processed and cold-preserved allografts supported significantly fewer regenerating nerve fibers. Measurement of muscle force confirmed that detergent-processed allografts promoted isograft-equivalent levels of motor recovery 16 weeks post-operatively. All acellular allografts promoted greater amounts of motor recovery compared to silicone conduits.

Conclusions—These findings provide evidence that differential processing for removal of cellular constituents in preparing acellular nerve allografts affects recovery *in vivo*.

Keywords

peripheral nerve regeneration; nerve tissue engineering; tissue processing; acellular nerve allograft; decellularization

Introduction

Peripheral nerve injuries represent one of the most common causes of sensorimotor deficits and lost productivity in adults.¹ In cases of simple peripheral nerve defects, sufficient functional recovery can be attained through tension-free, end-to-end coaptation of residual nerve stumps. In contrast, functional reconstruction of large complex nerve defects typically requires the use of interpositional autologous nerve grafts (autografts). While autografting

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represents the gold standard treatment for complex peripheral nerve injuries, nerve autografts present a number of clinical limitations. Apart from the paucity of expendable nerve tissue, harvesting autologous nerves results in significant donor site morbidity, increased risk of infection, and longer intraoperative times. As a result, great interest has been placed in the development of effective alternatives to nerve autografts in the management of peripheral nerve injuries.

Allogenic nerve tissue (allografts) is one of the most promising substitutes for nerve autografts. Cadaveric nerve allografts are available in great abundance and offer the potential for size/length and motor/sensory specificity. They contain both viable donor Schwann cells (SC) and endoneurial microstructure that provide the same level of regenerative support as nerve autografts with appropriate immunosuppression.²⁻⁷ Unfortunately, clinical implementation of fresh nerve allografts is limited by the concomitant need for systemic immunosuppression, which predisposes graft recipients to opportunistic infections, neoplasia, and toxicity-induced side effects.^{8,9} Processing nerve allografts to remove cellular components offers an attractive means of circumventing these limitations by reducing graft immunogenicity. However, the lack of viable SCs limits the acellular allografts to short gap distances of 3cm. Little consensus exists as to which processing technique best preserves the natural regenerative capacity of peripheral nerve tissue and maximizes functional recovery *in vivo*.

Multiple methods exist for preparing acellular nerve grafts from allogenic donor nerve tissue, including lyophilization, cold-preservation, freeze-thawing, detergent processing, and irradiation.¹⁰⁻¹³ Despite inherent differences, all processing techniques simultaneously aim to 1) reduce graft immunogenicity by eliminating cellular constituents, and 2) enhance regenerative capacity through preservation of native extracellular matrix (ECM).¹⁴ Prolonged cold-preservation of donor nerve tissue remains one of the most widely studied and effective methods of accomplishing these aims. Arising from preservation techniques developed for use in organ transplantation, cold-preservation of nerve allografts in University of Wisconsin (UW) solution at 4°C has previously been demonstrated to effectively eliminate the antigenicity of peripheral nerve allografts.¹⁵⁻¹⁷ Specifically, seven weeks of cold-preservation in UW solution effectively decellularizes nerve allografts and leads to complete suppression of interferon- γ production in circulating lymphocytes following transplantation.¹⁷⁻¹⁹ Cold-preservation additionally results in effective conservation of native SC basal laminae and nerve ECM, enabling robust axonal regeneration through transplanted acellular allografts.¹⁷ Clinical application of cold-preservation techniques has remained limited due to extended processing times (~seven weeks) and poor mechanical properties of these friable acellular grafts. As a result, cold-preserved nerve allografts have been primarily limited to investigational use as a research tool.

Detergent-processing is an alternative method of preparing acellular nerve allografts. Detergent-processing techniques were initially developed in an effort to more effectively remove cellular remnants from donor nerves while avoiding the destructive effects of freeze-thawing on nerve ultra-structure.^{12,13} While early protocols relied heavily on the use of sodium deoxycholate, Triton X-100, and deionized water to decellularize nerve grafts, more recent studies have optimized the detergent-processing using less aggressive chemical treatments.¹⁴ Specifically, nerve allografts repeatedly exposed to solutions of deionized water, sulfobetaine-10 (SB-10), and Triton X-200 / sulfobetaine-16 (SB-16) over a period of four days demonstrated superior preservation of native ECM and equivalent levels of decellularization compared to previous chemical processing techniques.¹⁴ Donor nerve allografts processed utilizing this optimized detergent-processing technique have subsequently been shown to support significantly greater densities of regenerating axons

than both thermally decellularized and chemically decellularized nerve allografts when implanted in rat sciatic nerve.^{14,20} While presenting added benefits over standard cold-preservation techniques, *in vivo* studies have yet to elucidate whether the modest increase in regenerative capacity facilitated by detergent-processing translates to improvements in functional nerve regeneration and recovery following transplantation.

In contrast, proprietary processing techniques utilized by commercial laboratories represent the only method of allograft preparation successfully applied in a clinical setting. Avance® nerve grafts (AxoGen® Inc., Alachua, FL) represent the only commercially-available allograft. Despite limited disclosure of applied decellularization techniques, AxoGen®-processed nerves are known to undergo a combination of treatments including chemical decellularization (detergent-processing), and gamma irradiation. Additionally, AxoGen®-processed nerve allografts undergo enzymatic digestion of chondroitin sulfate proteoglycan (CSPG), a known inhibitor of axonal growth, through acute treatment with chondroitinase ABC. Prior studies have demonstrated that treating donor nerve tissue with chondroitinase ABC effectively reduced the quantity of CSPG, and increased axonal regeneration through resulting acellular grafts *in vivo*.^{21,22} Human nerve tissue processed using this combinatorial technique has subsequently been utilized to repair an increasing number of median, lingual, and common digital nerve defects. Despite growing clinical popularity, current research suggests that even AxoGen®-processed nerve allografts are still inferior to fresh autografts in their ability to support nerve regeneration.²³ As a result, acellular allografts most likely possess much shorter critical lengths (the length beyond which the nerve grafts cannot support nerve regeneration) than fresh nerve isografts. Further investigation of the critical length of such acellular allografts is greatly needed to direct development of more effective techniques for allograft preparation and shape current clinical guidelines regulating the use of available grafts. The need for additional studies on the critical length limitations of acellular allografts is highlighted by the fact that acellular allografts up to 70mm in length (more than twice the critical length of available nerve conduits) are currently being utilized for clinical nerve reconstruction in the absence of supporting data.

In this study, three established models of acellular nerve graft were comparatively evaluated to assess the degree to which differences in processing technique modulate nerve graft efficacy and to identify a low cost alternative to commercially available AxoGen®-processed nerve allografts for investigational use. Cold-preserved, detergent-processed, and AxoGen®-processed nerve allografts were compared to size and length-matched gold standard nerve autografts and silicone nerve guidance conduits in their ability to functionally repair a standardized nerve defect in laboratory animals. Graft efficacy, defined as the capacity to facilitate functional nerve regeneration and motor recovery, was assessed via histomorphometric analysis of regenerative nerve and evoked muscle force measurement. The immunogenicity of cold-preserved²⁴, AxoGen®-processed,²³ and detergent-processed²⁰ nerve allografts has been studied previously. All three processing techniques have been shown to reduce the host immune response of major histocompatibility complex mismatched allografts to the level of an isograft. This study does not evaluate the effect of the immune response of each graft on nerve regeneration.

Materials and Methods

Animals

Adult male Lewis rats weighing 225-250 g (Harlan Sprague-Dawley, Indianapolis, IN) were utilized as nerve graft recipients and as donors of nerve isografts. Adult male Sprague-Dawley rats weighing 225-250 g (Harlan Sprague-Dawley, Indianapolis, IN) were utilized as donors for detergent-processed and cold-preserved nerve allografts. Adult male Brown Norway rats were used as donors for AxoGen-processed grafts. Brown Norway (RT1^{l+n}

MHC) and Sprague-Dawley (RT1^b MHC) rats were specifically selected as allograft donors to ensure a complete major histocompatibility complex mismatch between donor and recipient Lewis (RT1^I MHC) rats. All animal procedures were performed in strict accordance with institutional and National Institutes of Health guidelines.

Experimental Design

Sixty adult male Lewis rats were randomized into five groups (I-V) of twelve animals each (n = 8 for histology, n = 4 for functional analysis). Group I served as the positive control in which sciatic nerve defects created in recipient animals were repaired with a 14 mm reversed nerve isograft obtained from an isogenic source. Groups II, III, and IV served as experimental groups, wherein sciatic nerve defects were repaired with 14 mm detergent-processed nerve allografts, Avance® AxoGen®-processed nerve allografts, and cold-preserved nerve allografts, respectively. Group V served as the negative control, as sciatic nerve defects created in recipient animals were repaired with empty 16 mm silicone nerve guidance conduits. The conduit repair incorporated one millimeter on each end to yield a 14 mm nerve gap repair. Six weeks post-operatively 8 animals from each group (I-V) were euthanized, and the sciatic nerve was harvested en bloc for histomorphometric evaluation. Sixteen weeks post-operatively four animals from each group (I-V) were re-anesthetized prior to undergoing functional assessment of nerve regeneration. An additional group of four healthy unoperative animals served as controls for the functional assessment. Mean values acquired upon assessment of experimental groups (I-V) were then normalized to the mean values acquired upon assessment of the unoperative control group, respective to each individual metric. Any use of the term unoperative control in this paper refers to a healthy control and functional state equivalent to the state of the animal prior to nerve transection and surgical repair.

Surgical Procedures

Rats were anesthetized by subcutaneous injection of ketamine (75 mg/kg, Ketaset®, Fort Dodge Animal Health, Fort Dodge, IA) and medetomidine (0.5 mg/kg, Dormitor®, Orion Corporation, Espoo, Finland). Sciatic nerves were harvested bilaterally from donor animals for cold-preservation, detergent-processing, and immediate use as fresh nerve isografts. Donor animals were subsequently euthanized via an intracardiac injection of sodium pentobarbital (>200 mg/kg, Somnasol™, Butler Animal Health Supply, Dublin, OH). Recipient animals underwent exposure of the right sciatic nerve through a gluteal muscle-splitting incision prior to transection of the nerve 5 mm proximal to the trifurcation. Recipient nerves were then repaired with either a 14 mm reversed isograft (Group I), 14 mm processed nerve allograft (Groups II, III, IV), or 16 mm silicone nerve guidance conduit (Group V). Nerve grafts were microsurgically sutured to the proximal and distal nerve stumps using one 10-0 nylon suture (SharpPoint™, Surgical Specialties Corp., Reading, PA), and secured with fibrin sealant (TISSEEL™, Baxter International Inc., Deerfield, IL). Empty silicone conduits were similarly interposed and secured to the proximal and distal nerve stumps using two 10-0 nylon sutures, such that 1 mm of the host nerve was present in either end of the conduit. As a result, recipient nerves in all groups were challenged with a consistent 14 mm nerve gap. Following implantation, the incision was irrigated, and the muscle fascia and skin were closed in two layers using 6-0 polyglactin (Vicryl™, Ethicon, Somerville, NJ) and 4-0 nylon suture (Ethilon™, Ethicon, Somerville, NJ), respectively. Anesthesia was reversed with a subcutaneous injection of atipamezole HCl (1 mg/kg, Antisedan®, Orion Corporation), and animals were closely monitored prior to returning to the central housing facility.

Six weeks post-operatively, animals designated for histomorphometric analysis were re-anesthetized in order to harvest the sciatic nerve en bloc. Specifically, recipient nerves were

transected 4-5 mm proximal and 4-5 mm distal to interposed nerve grafts or interposed nerve conduits. Explanted nerves were marked with one proximal suture and stored in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) at 4° C prior to histomorphometric analysis. Following explantation, all animals were euthanized via intracardiac injection of sodium pentobarbital (>200 mg/kg, Somnasol™).

Sixteen weeks post-operatively, animals designated for functional assessment were re-anesthetized and prepared for *in situ* measurement of evoked muscle force. Recipient sciatic nerves were re-exposed as previously described. The distal portion of the right extensor digitorum longus (EDL) muscle was exposed through a skin incision extending from the dorsum of the foot to the knee. The distal tendons of the EDL muscle were subsequently transected and sutured to a metal S-hook using 5-0 nylon suture (Ethilon™, Ethicon). Both incisions were bathed in saline-soaked gauze to prevent desiccation prior to *in situ* functional assessment. Following testing, all animals were euthanized via intracardiac injection of sodium pentobarbital (>200 mg/kg, Somnasol™).

Preparation of Detergent-Processed Nerve Allografts

Sciatic nerve allografts harvested from donor Sprague Dawley rats were chemically processed and decellularized using a series of detergents as described by Hudson et al. 2004.²⁰ Briefly, nerves were placed in 15 mL conical tubes containing 7 mL of deionized distilled water and agitated at room temperature for seven hours. The solution was replaced with a 10 mM phosphate-buffered 50 mM sodium solution (PBS) containing 125 mM sulfobetaine-10 (SB-10) (Sigma, St. Louis, MO), and nerves were agitated for 15 hours. Nerves were then washed with PBS for 15 minutes, transferred into a PBS solution containing 0.6 mM sulfobetaine-16 (SB-16) (Sigma, St. Louis, MO) and 0.14% Triton X-200 (Sigma, St. Louis, MO), and agitated for 24 hours. Nerves were rinsed 3 times in PBS for a period of 5 min, placed in the PBS solution containing SB-10, and agitated for 7 hours. Nerves were then washed again in PBS, transferred to the solution containing SB-16 and Triton X-200, and agitated for 15 hours. Finally, nerves were washed with PBS for 15 minutes and stored at 4° C prior to implantation.

Preparation of Cold-Preserved Nerve Allografts

Sciatic nerve allografts harvested from donor Sprague Dawley rats were independently processed and decellularized using a method of cold-preservation, described previously.¹⁹ Briefly, explanted nerves were placed directly into sterile, six-well tissue culture plates containing UW solution (NPBI International BV, Emmer Compascuum, The Netherlands), penicillin G (200,000 U/L), regular insulin (40 U/L), and dexamethasone (16 mg/L). Nerves were then stored in solution under sterile conditions at 4° C for a period of seven weeks prior to implantation, during which time the preservation solution was changed weekly.

Avance® AxoGen®-Processed Nerve Allografts

The Avance® processed nerve allografts used in this study were a generous gift from AxoGen® Inc. (Alachua, FL). AxoGen®-processed allografts consisted of sciatic nerves acquired from Brown Norway rats processed by AxoGen® Inc. using a proprietary technique, described previously.²³ Briefly, harvested allografts were processed and decellularized using a series of detergents¹⁴, as discussed above, prior to enzymatic digestion of chondroitin sulfate proteoglycans and sterilization via gamma-irradiation. AxoGen®-processed allografts were then delivered and stored at -80° C prior to implantation.

Histomorphometry and Electron Microscopy

Harvested sciatic nerves were processed and analyzed as described previously.²⁵ Briefly, nerves were post-fixed with 1% osmium tetroxide, serially dehydrated in ascending concentrations of ethanol, and embedded in Araldite 502 (Polyscience Inc., Warrington, PA). Blocked nerves were cut into 1 μ m thin cross-sections using an ultramicrotome and stained with 1% toluidine blue for light microscopy imaging and qualitative analysis. A blinded observer measured total number of nerve fibers, nerve fiber width (μ m), percent neural tissue ($100 \times \text{neural area/intrafascicular area}$) and nerve fiber density (fiber number/ mm^2) in cross-sections acquired 3-5 mm distal to the interposed graft/conduit. Ultrathin sections of nerve tissue were additionally cut and stained with uranyl acetate and lead citrate for evaluation via electron microscopy (Model 902, Carl Zeiss Inc., Chicago, IL).

Functional Assessment

Sciatic nerve function was assessed by examining the evoked motor response in reinnervated EDL muscle upon electrical stimulation of the repaired sciatic nerve. Animals were immobilized in an automated functional assessment station (FASt System, Red Rock Laboratories, St. Louis, MO) where the distal portion of the EDL muscle was fixed to a 5 N load cell. Cathodal, monophasic electrical impulses (duration=200 ms, frequency=single–200 Hz, burst width=300 ms, amplitude=0-1000 μ A) were applied to the sciatic nerve proximal to the interposed nerve graft/conduit via silver wire electrodes, while resulting force production in the EDL was recorded using custom data acquisition software (RRL V. 1.0, Red Rock Laboratories).

Elicited twitch contractions were utilized to determine the optimal stimulus amplitude (V_o) and optimal muscle length (L_o) for isometric force production in the EDL muscle. All subsequent isometric force measurements were made at V_o and L_o . Single twitch contractions were recorded, and maximum twitch force (F_t) was calculated. Tetanic contractions were recorded at increasing frequencies of stimulation (5–200 Hz), allowing two minute intervals between stimuli to prevent muscle fatigue. Maximum isometric tetanic force (F_o) was automatically calculated from the resulting sets of recorded force traces.

Physiological cross-sectional area (PCSA) of the EDL muscle was calculated using the following equation²⁶:

$$PCSA = (M \times \cos\theta) / (\rho \times L_o \times 0.44)$$

where PCSA is the physiological muscle cross-sectional area (cm^2), M the EDL muscle mass (g), $\cos \theta$ the angle of pennation of the EDL muscle ($\sim 0^\circ$), ρ the density of mammalian skeletal muscle (1.06 g/cm^3), L_o the optimal muscle length (cm), and 0.44 the ratio of fiber length to muscle length (L_f/L_m) in rat EDL muscle. Maximum specific isometric force was calculated as the maximum isometric force normalized to muscle PCSA. Values were normalized to those of unoperative control ($n = 4$) EDL muscles to examine the degree of motor recovery experienced in reinnervated musculature. Following assessment, both denervated/reinnervated and unoperative EDL muscles were harvested and weighed. Muscle mass recorded for denervated/reinnervated EDL muscles was normalized to that of the unoperative control EDL muscles to quantify the relative degree of muscle atrophy experienced distal to repaired sciatic nerves.

Statistical Analysis

Data was analyzed with SigmaStat version 3.5 (Systat Software Inc., San Jose, CA). Multiple groups were compared with a one-way analysis of variance (ANOVA) if conditions of normality (assessed with the Kolmogorov-Smirnov normality test) and equal variance (assessed with the Levene Median test) were met. If either ANOVA returned a statistically significant p value, a post-hoc Student-Newman-Keuls test was used to isolate significant differences among the data with correction for multiple comparisons. Significance was set at $\alpha=0.05$ ($p<0.05$). All results are reported as mean \pm standard deviation.

Results

Nerve Graft Harvest

The differential capability of processed nerve allografts to support functional nerve regeneration across a critical 14 mm nerve defect was assessed *in vivo* using a rat sciatic nerve model. Explantation of recipient nerves repaired with fresh nerve isograft and processed nerve allografts (Groups I-IV) six and 16 weeks post-operatively revealed successful integration of all nerve grafts into the host nerve. Proximal and distal ends of all interposed nerve grafts remained coapted to the proximal and distal stump of the transected recipient nerve in all animals. At the terminal time point, all interposed nerve grafts were observed to be glossy white and opaque, with little evidence of rejection or degeneration. In addition, no evidence of neuroma formation was observed at either suture line in both the nerve isograft and processed allograft groups or at any other position along the length of interposed grafts. In contrast, explantation of recipient nerves repaired with silicone nerve guidance conduits (Group V) revealed little to no neural tissue within the implanted conduits. Proximal and distal nerve stumps of the host nerve remained secured inside either end of the implanted conduit in all cases, yet no appreciable nerve cable was observed bridging the two nerve stumps. Host nerve tissue distal to the interposed silicone conduit appeared atrophic and translucent, suggesting a general absence of healthy myelinated axons.

Histomorphometry

Cross sections of recipient nerves obtained 3-5 mm distal to the repaired nerve defect were examined quantitatively to assess the population of axons that successfully crossed each interposed nerve graft and conduit. Six week post-operatively, recipient nerves repaired with fresh nerve isograft revealed numerous myelinated axons (6331 ± 2251) crossing the graft (Fig. 1A, $n = 8$ for each group). Detergent-processed nerve allografts promoted a similar degree of axonal regeneration across the defect, as nerve segments distal to the allograft also demonstrated a large population of myelinated axons (5333 ± 3613). In contrast, AxoGen®-processed nerve allografts and cold-preserved nerve allografts demonstrated significantly fewer myelinated axons crossing the grafts and extending into distal segments of the recipient nerve than both the fresh nerve isografts and the detergent-processed allografts (2717 ± 2468 , 1597 ± 1193). There was no statistical difference in the total number of nerve fibers regenerating through the commercially available AxoGen®-processed allografts and the cold-preserved allografts. All four nerve grafts were observed to promote significantly greater numbers of regenerating nerve fibers than empty silicone nerve guidance conduits as segments of the recipient nerve located distal to interposed conduits revealed no myelinated axons (0). Comparison of quantitative measurements of percent neural tissue and nerve fiber density distal to the interposed nerve grafts and conduits mirrored these findings (Fig. 1B, 1C). The absence of nerve fibers in recipient nerve distal to interposed silicone conduits precluded further histomorphometric assessment.

Assessment of mean nerve fiber width provided an accurate measure of the maturity of myelinated axons successfully regenerating through implanted nerve grafts and conduits. Nerve fibers regenerating through interposed fresh nerve isografts were observed to be significantly thicker ($2.902 \pm 0.168 \mu\text{m}$) than nerve fibers crossing all other grafts and conduits. (Fig. 1D) No statistical difference was observed in the maturity of myelinated axons that successfully crossed interposed detergent-processed, AxoGen®-processed, and cold-preserved nerve allografts ($2.015 \pm 1.252 \mu\text{m}$, $2.784 \pm 0.158 \mu\text{m}$, $2.644 \pm 0.282 \mu\text{m}$, respectively). Further comparison of fiber width distributions obtained distal to implanted nerve grafts did not reveal any significant differences in the percentage of regenerating axons of a specific diameter (data not shown).

Quantitative measures of percent debris within the nerve cross-sections were additionally examined. Despite the observance of less debris in recipient nerve distal to fresh nerve isografts, no significant differences were observed between groups that employed nerve isografts, processed nerve allografts, or silicone conduits. (Fig. 1E)

Representative histological sections acquired distal to the repaired nerve defect in each group are shown in Figure 2.

Electron Microscopy

Electron micrographs of nerve tissue acquired distal to the repaired nerve defect were examined to assess the ultrastructure of nerve fibers successfully regenerating through the interposed nerve grafts and conduits. Viable nerve fibers displaying normal patterns of myelination were observed in nerve segments distal to all implanted nerve grafts (Fig. 3A-3D). Numerous un-myelinated axons were also observed distal to interposed nerve grafts. Additionally, immune-associated infiltrate was not observed in the connected distal stump. The lack of infiltrate suggests the absence of a significant immune response after 6 weeks, which is consistent with our previous data. Nerve tissue obtained distal to implanted silicone nerve guidance conduits did not reveal any viable myelinated or unmyelinated axons (Fig. 3E). Additionally, nerve tissue distal to interposed conduits demonstrated numerous signs of progressive Wallerian degeneration and disorganization of the extracellular matrix.

Functional Assessment

Measurement of evoked force production in the EDL muscle upon electrical stimulation of repaired sciatic nerve was performed to assess the degree to which regenerating motor axons crossed the imposed defect and reinnervated distal motor targets. Maximum isometric tetanic force measurements obtained 16 weeks post-operatively demonstrated that EDL muscles innervated by sciatic nerves repaired with fresh nerve isografts experienced the greatest degree of functional recovery (Fig. 4A). Isograft repair of the imposed sciatic nerve defect was observed to restore $45.91 \pm 7.11 \%$ of unoperative control force production ($3.776 \pm 0.289 \text{ N}$). EDL muscles innervated by sciatic nerves repaired with detergent-processed nerve allografts exhibited decreased motor recovery ($37.45 \pm 8.65 \%$ of unoperative control force production) compared to nerve isografts, though no significant difference was observed. Repair of sciatic nerve defects with AxoGen®-processed and cold-preserved nerve allografts both resulted in a significant decrease in force production in reinnervated EDL muscles ($22.25 \pm 16.87 \%$, $23.73 \pm 8.58 \%$ of unoperative control force production) compared to nerve isografts. Differences in force production observed between experimental groups that employed processed nerve allografts were not statistically significant. In contrast, EDL muscles innervated by sciatic nerves repaired with empty silicone conduits did not produce any force upon electrical stimulation of the proximal nerve stump (0.0% of unoperative control force production). Maximum isometric twitch force

measurements were observed to follow a similar trend to maximum isometric tetanic force measurements (data not shown). Representative twitch and tetanic force traces for each experimental group are shown in Figure 5.

Wet EDL muscle mass was measured following evoked muscle force testing to assess net muscle atrophy resulting from denervation/reinnervation. EDL muscles innervated by sciatic nerves repaired with fresh nerve isograft experienced the lowest amount of net muscle atrophy 16 weeks post-operatively, retaining 83.75 ± 3.86 % of unoperative control muscle mass (0.204 ± 0.019 g) (Fig. 4B). Repair of sciatic nerve defects with detergent-processed and cold-preserved nerve allografts resulted in increased EDL muscle atrophy (71.95 ± 11.50 %, 73.13 ± 14.43 % of unoperative control muscle mass) compared to nerve isografts. EDL muscles innervated by sciatic nerves repaired with AxoGen®-processed nerve allografts demonstrated the greatest muscle atrophy (57.57 ± 27.22 % of unoperative control muscle mass) of any nerve allograft group. No significant differences in EDL muscle mass were observed between experimental groups that employed nerve isografts or processed nerve allografts. Repair of sciatic nerve defects with empty silicone conduits resulted in a dramatic increase in EDL muscle atrophy (17.59 ± 5.68 % of unoperative control muscle mass). EDL muscle atrophy observed following silicone conduit implantation was significantly increased compared to all other experimental groups.

Calculation of maximum specific force production provided a metric of functional capacity independent of muscle atrophy. Similar to maximum isometric tetanic force measurements, EDL muscles innervated by sciatic nerves repaired with fresh nerve isografts demonstrated the greatest specific force production of any experimental group (Fig. 4C). Isograft repair of the imposed sciatic nerve defect was observed to restore 55.13 ± 8.16 % of unoperative control specific force production (34.35 ± 2.17 N/cm²). EDL muscles innervated by sciatic nerves repaired with detergent-processed nerve allografts demonstrated levels of specific force production similar to nerve isograft (50.39 ± 10.74 % of unoperative control specific force production). Use of AxoGen®-processed nerve grafts and cold-preserved nerve grafts both resulted in decreased specific force production (33.31 ± 24.18 %, 32.13 ± 8.55 % of unoperative control specific force production) compared to nerve isografts and detergent-processed nerve allografts. EDL muscles innervated by sciatic nerves repaired with either AxoGen®-processed nerve grafts or cold-preserved nerve grafts demonstrated a significant decrease in specific force production compared to nerve isografts. Repair of sciatic nerve defects with empty silicone conduits did not result in any appreciable recovery of specific force production (0.0 % of unoperative control specific force production), as all EDL muscles appeared to be denervated at the terminal time point.

Discussion

The recent success of acellular nerve allografts in investigational studies and clinical reports suggests an impending paradigm shift in clinical management of short gap, small diameter nerve injuries. In our current clinical practice, commercially available acellular nerve grafts have largely replaced nerve conduits as the preferred alternative to nerve autografts for surgical management of short gap injuries in noncritical, small diameter peripheral nerve defects. The basis for this shift lies in the superior regenerative capacity of processed acellular allografts compared to available nerve conduits; this was highlighted in a recent study in which commercially available processed nerve allograft significantly outperformed commercially available conduits.²³ The presence of native extracellular matrix and intact SC basal laminae within acellularized grafts supported greater numbers of regenerating axons and more successful guidance of regenerating axons compared to empty conduits.^{23,27}

Despite the promise of acellular nerve allografts as an effective nerve substitute, experimental studies to date have only investigated small diameter small gap defects. The currently available acellular nerve grafts possess reduced regenerative capacities compared to fresh nerve isograft and will only support nerve regeneration over limited distances.²³ Comprehensive investigations capable of identifying the limitations of contemporary acellular nerve grafts have yet to occur. One of the primary impediments that limits such studies remains the identification of a low-cost acellular nerve graft that is both comparable to commercially available acellular nerve grafts and conducive to laboratory use. Our study was designed to comparatively assess the regenerative capacities of three established acellular nerve graft models, examine the effect of processing technique on nerve graft efficacy, and to identify an investigational surrogate to commercially available Avance® processed nerve allografts.

Cold-preserved, detergent-processed, and AxoGen®-processed nerve allografts represent the most prevalent and widely studied acellular nerve graft models. Despite arising from a similar appraisal of the molecular bases of nerve graft immunogenicity, each model employs significantly different methods of processing donor nerve tissue.^{14,13,15,24,28} Specific decellularization techniques applied within each model largely determine both the percentage of antigenic cellular constituents removed from the donor nerve, and the quality of preserved nerve extracellular matrix. A lack of comparative studies within prior investigations has prevented direct examination of the effect of processing on axonal regeneration. In this study, differentially processed acellular nerve grafts were directly compared *in vivo* to determine the effect of processing techniques on nerve regeneration.

The regenerative capacity of acellular nerve grafts and empty nerve conduits was evaluated by assessing axonal regeneration and functional recovery following nerve repair. Histomorphometric analysis, a well established technique useful in quantifying pertinent ultra-structural characteristics of nerve tissue, was utilized to examine acute axonal regeneration through implanted nerve grafts and conduits.²⁵ Measurement of evoked force production in reinnervated distal musculature was simultaneously utilized to evaluate the chronic functional sequelae of nerve regeneration through implanted grafts/conduits. Close correlation was observed between histomorphometric and electrophysiological assays throughout the study. Successful axonal regeneration through implanted grafts/conduits largely predicted chronic functional recovery following nerve repair, suggesting the regenerating axons maintained the ability to functionally reinnervate distal end organs. Examination of both metrics revealed that acellular nerve allografts (cold-preserved, detergent-processed, and AxoGen®-processed nerve allografts) supported significant increases in functional nerve regeneration compared to empty silicone nerve guidance conduits. Poor regeneration through empty conduits was expected due to the poor regenerative micro-environment provided by silicone conduits and the critical length of the nerve defects. Specifically, silicone conduits have previously been shown to be unable to support axonal regeneration across nerve defects greater than 10 mm in length.^{29,30} The lack of successful nerve regeneration confirmed silicone nerve conduits as an effective negative control and demonstrated that axonal regeneration observed in this study was not influenced by spontaneous regeneration (i.e. “blow-through”) that is commonly observed in rodent models of nerve injury/repair.⁶

Evaluation of axonal regeneration and functional recovery through acellular nerve grafts revealed that processing technique modulates nerve graft efficacy *in vivo*. In this study, detergent-processed nerve allografts were observed to support superior nerve regeneration and functional recovery compared to both cold-preserved and AxoGen®-processed nerve allografts. Increased nerve regeneration through detergent-processed nerve allografts may result from more successful removal of cellular debris and preservation of native nerve

micro-structure within donor nerve tissue. Hudson *et al.* previously described the superiority of the optimized detergent-processing technique over alternative methods of nerve decellularization.¹⁴ Yet, AxoGen®-processed nerve allografts, which are known to employ similar methods of nerve decellularization, did not demonstrate equivalent levels of nerve regeneration in our study.

Alternatively, improved nerve regeneration through detergent-processed nerve grafts may be a result of shorter lengths of time between explantation of donor nerve and implantation of processed nerve. The detergent-processing technique employed in the current study required four days of post-processing prior to successful decellularization of donor nerve tissue, where AxoGen®-processing and cold-preservation techniques require longer lengths of time. Extended processing times have been shown to adversely affect the integrity of the endoneurial microstructure within nerve grafts.¹⁴ Inferior preservation of nerve ECM in grafts that undergo lengthy processing techniques may yield poor regenerative support for regenerating axons extending through the implanted graft and result in poor functional recovery post-operatively.

Shorter post-processing times may also result in greater retention or preservation of soluble and/or bound growth factors within the decellularized nerve tissue.^{31,32} Prior studies have demonstrated that growth factors such as vascular endothelial cell growth factor (VEGF), basic fibroblast growth factor (bFGF), and transforming growth factor beta (TGF-β) are all released from ECM during active reorganization of the ECM microstructure.^{33,31,34-36} Prolonged chemical or physical alteration of nerve tissue may therefore have increased the removal of embedded neurotrophic cues and progressively decreased the neuroregenerative potential of processed grafts. Extending processing techniques, such as cold-preservation and AxoGen®-processing, may also directly reduce the activity of these neuroregenerative cues through progressive chemical denaturation. Comprehensive, comparative analysis of the micro-structural composition of processed nerve grafts will be required to examine the synergistic effects processing time and processing technique have on preserving the natural neuroregenerative capacity of native nerve ECM.

In this study, AxoGen®-processed nerve grafts were unexpectedly observed to facilitate lesser degrees of functional nerve regeneration compared to detergent-processed nerve allografts. Despite our use of a similar method of nerve decellularization developed by Hudson *et al.*¹⁴, detergent-processed nerve allografts supported increased axonal regeneration compared to AxoGen®-processed nerve allografts. Reduced regenerative capacity of AxoGen®-processed nerve grafts may result from optimization of the AxoGen® decellularization techniques for use with human, rather than rodent, nerve tissue. This species difference needs to be considered in consideration of the current results. Previous tissue processing techniques designed to maintain extracellular matrix integrity have been demonstrated to have significantly different effects on tissue originating from separate species.^{37,38} Despite the fact that the detergent-processed and AxoGen®-processed grafts both utilize SB-10, SB-16, and Triton X-200 detergents in the decellularization of donor nerve tissue, differences in washing time could have differential affects on the integrity of the ECM within donor nerves.

Additional processing steps utilized in the AxoGen® processing technique, including gamma irradiation and flash-freezing, may also affect resulting acellular nerve graft material. While the effect of gamma irradiation on nerve microstructure has not been studied in depth, protocols using repeated episodes of freezing/thawing have been shown to have a significant negative effect on endoneurial microstructure.^{39,40} Due to the proprietary nature of AxoGen® decellularization techniques, little information exists as to what additional techniques are applied to each decellularized nerve graft. Despite this lack of knowledge,

small differences in the processing technique utilized to prepare AxoGen®-processed and detergent-processed nerve grafts may contribute to the reduced regenerative capacity observed in AxoGen®-processed grafts.

Application of chondroitinase ABC (ChABC) to donor nerve tissue during preparation of AxoGen®-processed acellular nerve grafts may also contribute to the differences observed between AxoGen®-processed and detergent-processed nerve allografts. CSPGs are extracellular matrix molecules that decrease the ability of axons of the central and peripheral nervous system to regenerate following injury. In the peripheral nervous system, CSPG are present in the distal stump after nerve injury⁴¹⁻⁴³ and are removed during normal Wallerian degeneration by Schwann cells and macrophages.^{44,45} Prior studies demonstrate that treatment with ChABC effectively removes CSPG from donor nerve tissue and enhances nerve regeneration through resulting acellular nerve grafts.²¹ Following decellularization AxoGen®-processed nerve allografts are known to undergo ChABC treatment to improve axonal regeneration through resulting graft material. Despite numerous studies investigating the positive effect of ChABC on axonal regeneration, ChABC treatment may have a detrimental effect on axonal regeneration in some instances. Excessive removal of CSPG from peripheral nerve tissue may eliminate natural inhibitory cues which are utilized to guide extending neurites.⁴⁶ Additionally, the enzymatic activity of ChABC may have a negative effect on the integrity of the ECM within the donor nerve. However, several studies that evaluate the effect of ChABC treatment on the regenerative capacity of peripheral nerve suggest no negative effects of ChABC treatment on nerve regeneration.^{47,21,48,49}

The AxoGen®-processed allografts used in this study were derived from Brown Norway rats, while the detergent-processed and cold-preserved allografts were derived from Sprague Dawley rats. Despite the fact that both rat strains (Brown Norway and Sprague Dawley) used for donor allografts have a major histocompatibility complex mismatch with Lewis rats, a difference in immunological response between donor strains could have contributed to regenerative differences observed between AxoGen®-processed and detergent-processed nerve allografts. Investigations have shown that, while differences in major or minor histocompatibility complex mismatch can result in difference in regeneration across a nerve allograft⁵⁰, different donor strains that are both major histocompatibility complex mismatch for the recipient facilitate similar neural regeneration.⁵¹ While there is no guarantee that immunological differences between the two donor strains contributed to the differences in axonal regeneration seen in the grafts, the added factor that the cellular components of each donor graft were removed decreases the likelihood of an effect. Allograft processing with both of these procedures has been shown to reduce the immune response to an allograft to the level of an isograft.^{23,20} These data suggest that it is unlikely that immunological differences significantly affected regeneration across each graft. In addition to immunologic differences, genetic differences in allograft architecture or extracellular matrix composition may also have affected nerve regeneration. Previously we have compared several nerve regeneration paradigms (crush, transection, and conduit repair) across five rat strains (Sprague Dawley, ACI, Wistar-Furth, Lewis, and Brown Norway). Upon examination of axonal regeneration using histomorphometry and function recovery using walking track analysis at two different end points (6 and 13 weeks), no statistically significant differences between strains were noted, regardless of endpoint evaluation. The final conclusion from this study was that uniform conclusions about nerve regeneration in the rat may be drawn regardless of strain used.⁵² Similarly, it is not likely that any differences in axonal regeneration were minimally affected by genetic differences between donor allografts.

Comparatively, cold-preserved nerve allografts exhibited comparable performance to commercially available AxoGen®-processed nerve grafts. Cold-preserved allografts and AxoGen®-processed nerve allografts supported statistically indistinguishable levels of

axonal regeneration and functional recovery *in vivo*. The similarity in total number of regenerating fibers and functional output of cold-preserved and AxoGen®-processed nerve grafts suggests that, within the context of rodent models of nerve injury and repair, cold preserved nerve allografts offer a suitable substitute for commercially available AxoGen® processed nerve allografts. The results of this study therefore suggest that cold-preserved nerve grafts may be utilized in future research studies to model the behavior of commercially-available acellular nerve grafts. Utilization of cold-preserved nerve grafts is anticipated to eliminate the prohibitive cost and limited distribution associated with AxoGen®-processed nerve allografts and facilitate increased numbers of studies that examine the preparation and implementation of acellular nerve allografts.

Further evaluation of implanted acellular nerve allografts revealed that only detergent-processed nerve allograft matched the regenerative capacity of fresh nerve isograft. Unlike cold-preserved and AxoGen®-processed nerve allografts, detergent-processed nerve allografts were statistically indistinguishable from nerve isograft in supporting axonal regeneration and functional recovery *in vivo*. The superior performance of detergent-processed nerve grafts suggests that this simple decellularization technique may merit clinical implementation. However, it should be emphasized that the AxoGen® Avance® is the only U.S. Food and Drug Administration approved acellular graft for clinical peripheral nerve repair. Increasingly rigorous trials will still be needed to fully characterize the potential of detergent-processed allografts to facilitate functional nerve regeneration across larger nerve defects (>30 mm) and longer nerve diameters. Prior studies confirm that larger peripheral nerve defects pose an increased challenge to nerve substitutes, as the implanted substitute must support axonal regeneration independent of both the proximal and distal nerve stumps.²³ While the detergent-processed graft performed similarly to the isograft in the 14mm nerve gap model, detergent-processed grafts are not expected to match the ability of fresh nerve isograft to promote functional nerve regeneration across larger, clinically relevant nerve defects (> 30mm).⁵³ The anticipated failure of all processed nerve allografts at this critical length highlights the need for further investigation and development of more effective nerve substitutes capable of promoting functional nerve regeneration at or above the level of fresh nerve isograft.

This study provides further evidence that different processing techniques used to remove the cellular components of acellular nerve allografts affect the regeneration of axons through the graft. For the first time, this effect on axonal regeneration has been shown to translate into differences in functional recovery. Based on our results and the results of others²⁰, we hypothesize that these differences are symptomatic of the variable preservation of endoneurial microstructure provided by each processing technique. Further, we demonstrated that detergent-processed grafts, optimized for rats, outperformed AxoGen®-processed grafts, optimized for humans, despite the use of similar detergents to process the graft. This suggests that for clinical use each processing technique must be optimized for human nerve. If the detergent-processing employed in this study were compared with AxoGen®-processed human grafts for human nerve regeneration, we predict that we would see the inverse of our results (specifically that AxoGen®-processed human grafts would outperform the detergent-processed human grafts). This final point has significance in a clinical setting where the use of commercially available acellular allografts or conduits are cost prohibitive, and the use of custom processed allografts is not prohibited by regulation. In this setting where a physician can prepare his/her own acellular nerve allografts for clinical use, it is imperative to understand that processing techniques described in the literature as effective in rodents will likely need to be optimized for human nerve.

Further, it establishes a low cost processing technique (cold-preservation) that mimics the regenerative performance of the clinically available acellular nerve allograft. Investigations

into the limit of axonal regeneration in acellular nerve allografts will help define the clinical limitations and limit negative outcomes. An example of potential negative outcomes is the over-enthusiasm for nerve conduit use that has resulted in clinical failures in large diameter nerves and long gap injuries⁵⁴. We have shown the superiority of acellularized nerve allografts over empty conduits²³ and now anticipate similar expansion of this autograft substitute with potential deleterious results if the length size parameters are not clearly defined in the laboratory.

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Abbreviations

SC	Schwann cell
ECM	extracellular matrix
UW	University of Wisconsin
SB-10	sulfobetaine-10
SB-16	Triton X-200 / sulfobetaine-16
CSPG	chondroitin sulfate proteoglycan
EDL	extensor digitorum longus
PBS	sodium solution
Vo	optimal stimulus amplitude
Lo	optimal muscle length
Ft	maximum twitch force
Fo	Maximum isometric tetanic force
PCSA	Physiological cross-sectional area
ChABC	chondroitinase ABC

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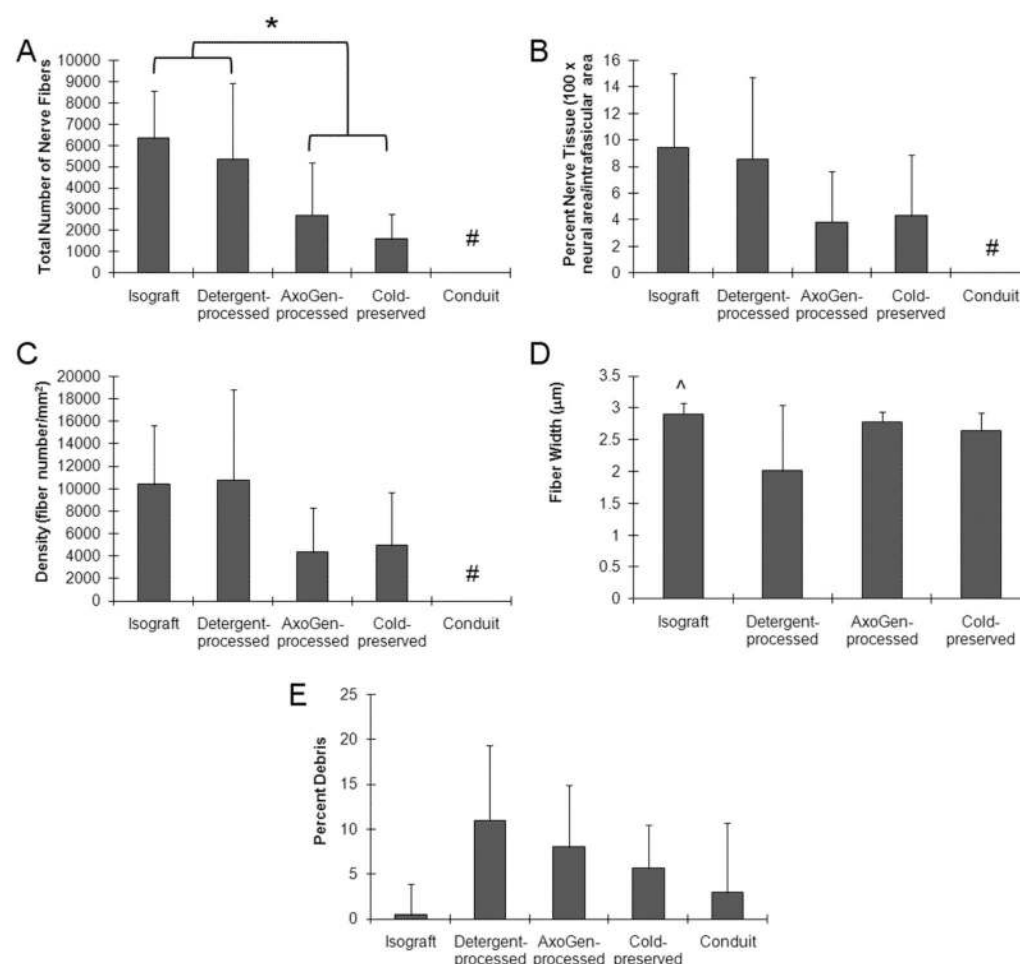


Figure 1.

Histomorphometric findings reveal varying degrees of axonal regeneration through fresh nerve isografts, processed nerve allografts, and nerve guidance conduits 6 weeks post-operatively. (A) Fiber counts demonstrate significantly greater numbers of nerve fibers distal to implanted isografts and detergent-processed allografts than AxoGen®-processed allografts and cold-preserved allografts. (B, C) Calculation of percent nerve tissue and nerve fiber density show increased amount of neural tissue distal to implanted isografts and detergent-processed allografts than AxoGen®-processed allografts and cold-preserved allografts, though no statistical differences were observed. (D) Fiber width measurements demonstrate similar degrees of fiber maturation amongst all implanted nerve allografts. Silicone nerve guidance conduits 14 mm in length did not support successful axonal regeneration, precluding histomorphometric analysis. (E) No significant differences were observed in the amount of debris present in the nerve distal to the implanted grafts/conduits. Data represents the mean \pm standard deviation; * indicates statistical significance ($p < 0.05$); # indicates $p < 0.05$ vs Isograft, Detergent-processed, AxoGen®-processed and Cold-preserved; ^ indicates $p < 0.05$ vs Detergent-processed, AxoGen®-processed, Cold-preserved and Conduit.

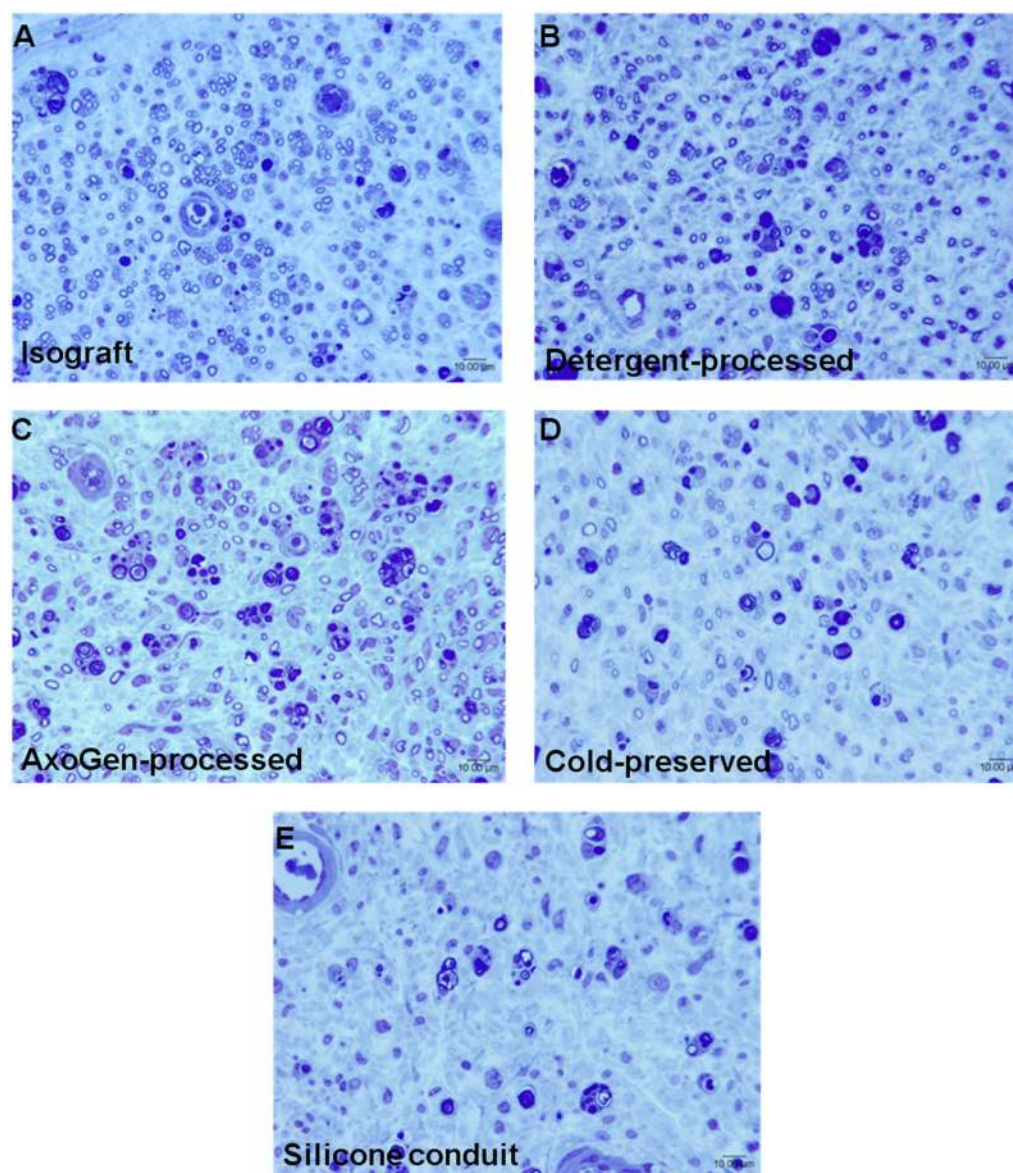


Figure 2.

Representative histological sections demonstrate populations of axons successfully regenerating through fresh nerve isografts, processed nerve allografts, and nerve guidance conduits 6 weeks post-operatively. Sections acquired 3-5 mm distal to implanted nerve isografts (A) and detergent-processed allografts (B) show numerous myelinated axons loosely organized into regenerating units. Sections acquired distal to AxoGen®-processed nerve allografts (C) and cold-preserved nerve allografts (D) show few myelinated axons successfully innervating the host nerve distal to the repair site. Host nerve tissue distal to implanted nerve guidance conduits (E) demonstrate no healthy, myelinated axons.

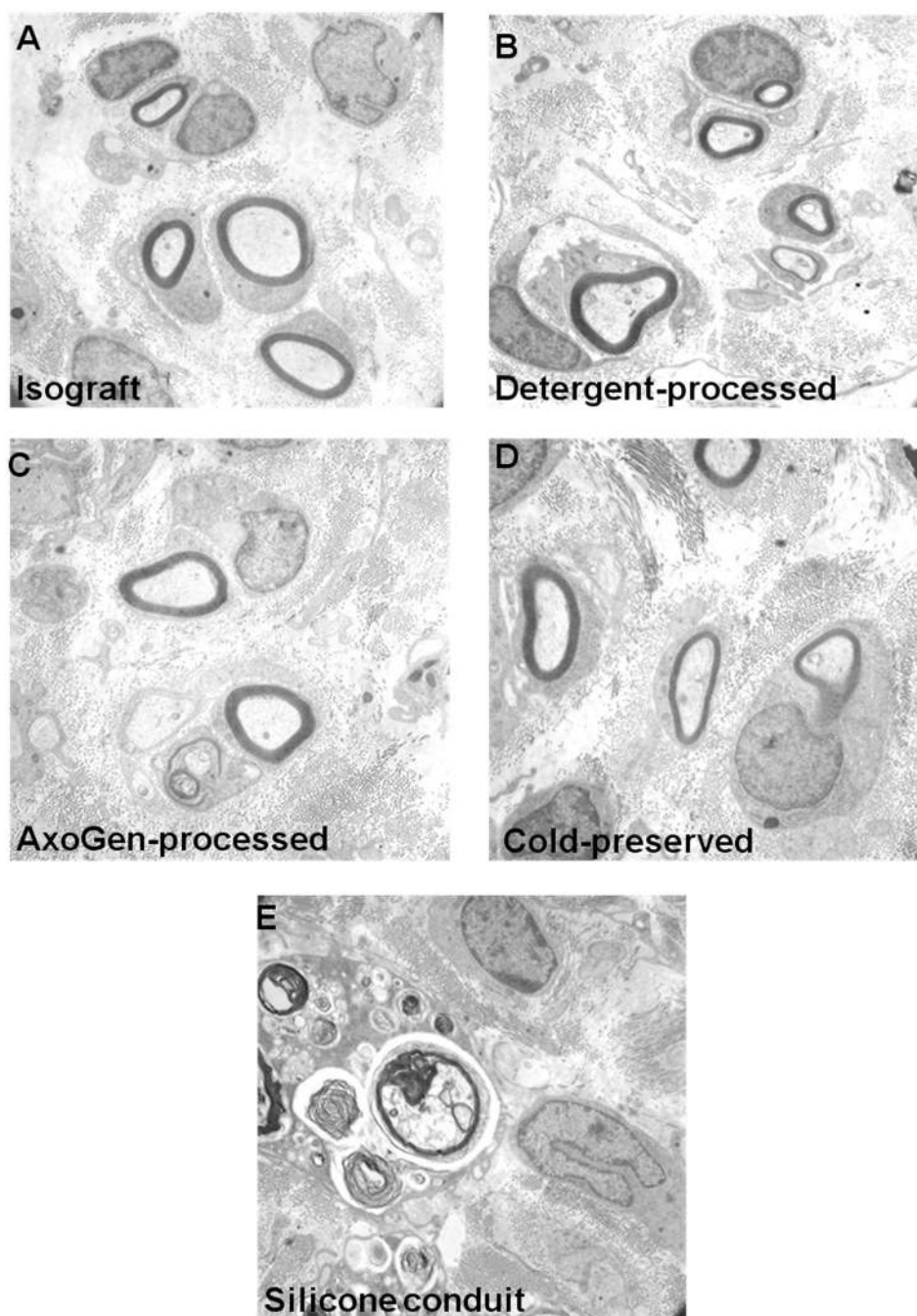


Figure 3.

Representative electron micrographs reveal unmyelinated and remyelinated axons regenerating through fresh nerve isografts, processed nerve allografts, and nerve guidance conduits 6 weeks post-operatively. Micrographs acquired distal to implanted nerve isografts (A), detergent-processed allografts (4360 \times magnification) (B), AxoGen[®]-processed nerve allografts (4360 \times magnification) (C), and cold-preserved nerve allografts (6400 \times magnification) (D) show normal remyelination of regenerating axons and multiple unmyelinated axons within the host nerve (4360 \times magnification). Micrographs acquired distal to implanted nerve guidance conduits (E) demonstrate degenerating axons, neural debris, and an absence of healthy, regenerating axons (4360 \times magnification).

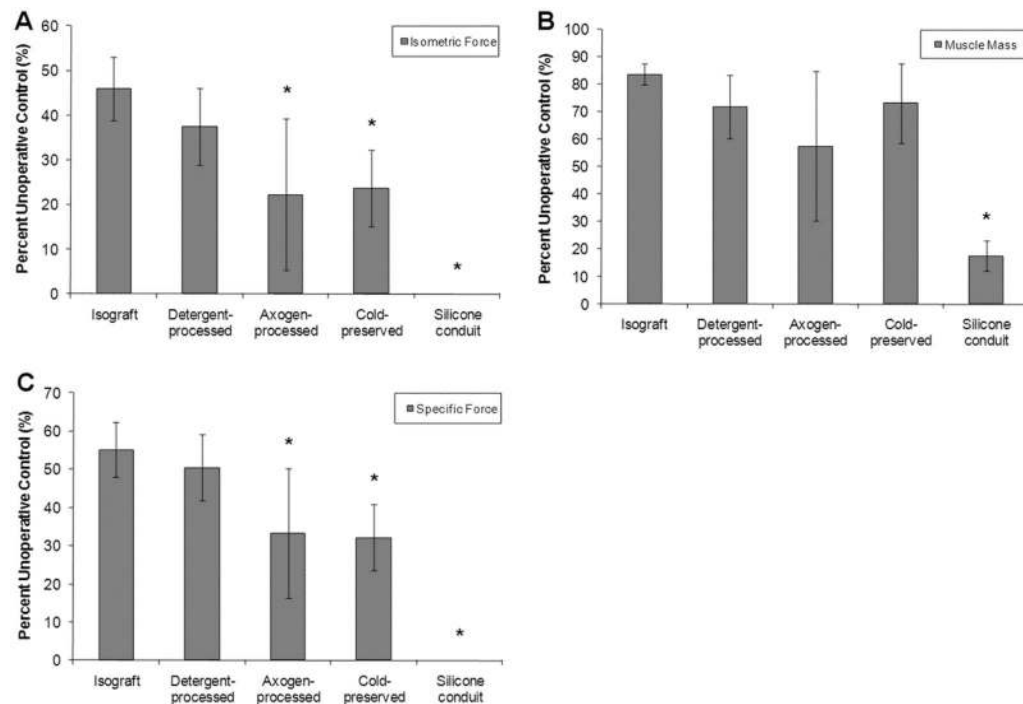


Figure 4.

Evoked muscle force measurements reveal differential motor recovery in distal musculature 16 weeks after implantation of fresh nerve isografts, processed nerve allografts, and nerve guidance conduits. (A) Measurements of maximum isometric force production in EDL muscle innervated by repaired sciatic nerve demonstrate the AxoGen®-processed nerve allografts and cold-preserved nerve allografts support significantly lower degrees of motor recovery compared to fresh nerve isografts. In comparison, silicone nerve guidance conduits did not support any functional motor recovery in distal musculature. (B) Assessment of EDL muscle mass shows that muscles innervated by sciatic nerves repaired with either processed nerve allografts experienced similar degrees of atrophy. EDL muscles innervated by sciatic nerves repaired with AxoGen®-processed nerve allografts did exhibit greater degrees of atrophy, though results were not statistically significant. (C) Calculation of maximum specific force production in reinnervated EDL muscle reveals that, upon correction for differences in muscle atrophy, AxoGen®-processed nerve allografts and cold-preserved nerve allografts still support significantly lower degrees of motor recovery compared to fresh nerve isografts. Data represents the mean \pm standard deviation; * indicates statistical significance ($p < 0.05$) compared to nerve isograft.

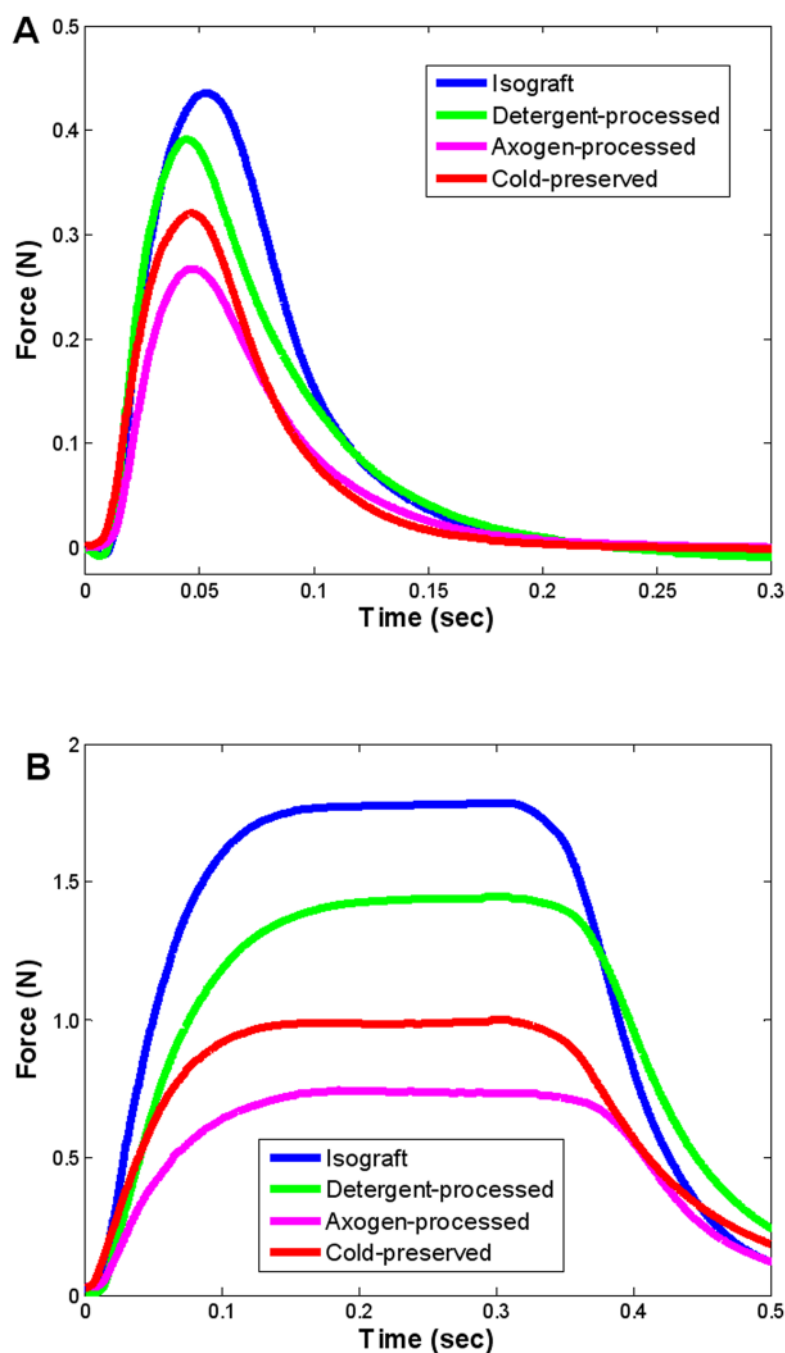


Figure 5.

Representative force recordings obtained from EDL muscle innervated by repaired sciatic nerve show differential recovery of motor function 16 weeks after implantation of fresh nerve isografts, processed nerve allografts, and nerve guidance conduits. Comparison of evoked twitch (A) and tetanic (B) responses demonstrate improved force production in muscles innervated by nerve repaired with nerve isografts and detergent-processed nerve allograft. Observation of normal tetanic responses in EDL muscle innervated by sciatic nerve repaired with both nerve isografts and processed allografts confirm normal function of regenerated motor axons and corresponding motor units.