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Comparing Processed Nerve Allografts and Assessing their Capacity to Retain and Release Nerve Growth Factor

Alonda C. Pollins, MLI^{1,*}, Richard B. Boyer, MD, PhD^{1,2,*}, Marlieke Nussenbaum, MD¹, and Wesley P. Thayer, MD, PhD^{1,2}

¹Department of Plastic Surgery, Vanderbilt University Medical Center, Nashville, TN, USA, 37232

²Department of Biomedical Engineering, Vanderbilt University, Nashville, TN, USA, 37232

Abstract

Peripheral nerve gap injuries continue to present a clinical challenge to today's surgeons. One method of surgical repair, implantation of acellular allografts, has been developed with the aim of bridging the gap with a cadaveric graft following removal of its cellular components, thereby accelerating axonal regeneration and eliminating the need for immunosuppression in recipient patients. While decellularizing allografts reduces rates of graft rejection, the same chemical processing modifies the neural microenvironment, removing neurotrophic factors and modifying the complex extracellular matrix. In this study we explore three common methods for producing acellular allografts. Extracellular matrix content remaining after processing was investigated, and was found to be highly dependent on the decellularization method. In addition, scanning electron micrographs were obtained to evaluate the structural effects of the decellularization methods. Though the content and structure of these processed allografts will contribute to their effectiveness as nerve gap repair candidates, we demonstrate that it also affects their capacity to be supplemented/pre-loaded with the prototypical neurotrophin, nerve growth factor (NGF), essential to neuronal regeneration. While all allografts had some capacity for retaining NGF in the first 24 hours, only Sondell-processed grafts retained NGF over the entire experimental period of 21 days. Future studies will include validating these processed and supplemented allografts as viable alternatives to traditional autograft nerve gap repair.

Keywords

allograft; acellular nerve graft; peripheral nerve repair; nerve gap repair; nerve growth factor; neurotrophin; nerve regeneration

Introduction

Approximately 1–3% of all trauma patients worldwide suffer from associated significant peripheral nerve injuries (1, 2). The ideal treatment for repair of peripheral nerve injury is

Corresponding Author: Wesley P. Thayer, Department of Plastic Surgery, Vanderbilt University Medical Center, 1161 21st Ave S., MCN D4207, Nashville, TN 37232-2345, Phone: 615-936-3759, Fax: 615-936-0167, wesley.thayer@vanderbilt.edu.

*These authors contributed equally.

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tensionless primary repair, though often times a segmental nerve defect is present, in which case the gold standard therapy is autologous sensory cable nerve grafting (3). However, autograft is not always achievable (4). Alternative repair strategies have been studied since the 1980s and include artificial conduits made of various materials, as well as allografts (5–7). There is relatively strong supportive evidence for conduit effectiveness in small nerve gaps, with the best results in gaps less than 20 mm in small diameter nerves (8). Using nerve allograft has many advantages similar to nerve conduits and may additionally provide internal architecture supporting Schwann cell and axon migration (9), which some have shown to play an important role (10). However, the immunogenicity of non-processed human nerve tissue requires the use of immunosuppressive agents, and the toxicity of such agents has hindered routine use of donated human allografts (11, 12).

Advances in tissue processing have overcome the problem of immunological rejection with the development of detergents and protocols capable of decellularizing and removing immunogenic components from nerve allograft, removing the need for immunosuppression (13). Additionally, these techniques can remove inhibitors of axonal outgrowth such as chondroitin sulfate proteoglycans (CSPGs) (14). Unfortunately, in the process of decellularizing the nerve allograft there is also a loss of Schwann cells and their products, such as neurotrophic factors, one being nerve growth factor (NGF) (8). However, nerve processing can be optimized to retain Extracellular Matrix (ECM) proteins and preserve nerve architecture (8).

In this study, we investigated 3 existing processing methods for nerve decellularization. We hypothesize that the method of decellularization will affect both the preservation of ECM protein structure and tissue permeability. Retention and subsequent release of the neurotrophin NGF may also be impacted by the processing method. The content and structure of the processed nerves were assessed, as was their ability to retain and then provide sustained release of preloaded NGF.

Materials and Methods

Ethics

All experimental procedures were approved by and performed in accordance with the standards set forth by the Institutional Animal Care and Use Committee at Vanderbilt University Medical Center.

Donor nerve harvesting and nerve graft processing

Sprague Dawley donor rats were 12 weeks old and weighed 250 to 300g at the start of the experiment. Animals were anesthetized with inhaled isoflurane and then sacrificed via intracardiac injection of Euthasol (pentobarbital sodium, Virbac AH, Fort Worth, TX). Using aseptic technique, the rats were prepped and an incision was made parallel just caudal to the femur. The sciatic nerve was exposed and dissected free of any perineural tissue, then sharply excised, retaining as much length as possible. Nerves were trimmed to 2cm lengths, washed 3X with PBS and stored in RPMI 1640 medium (Life Technologies) at 4 °C until processing.

Nerve segments were decellularized with either the Sondell technique (15), the Hudson optimized acellular technique (13), or Freeze-thaw cycling (7, 16). Table 1 lists the protocols for Sondell and Hudson nerve decellularization. Freeze-thaw cycling was performed with liquid nitrogen submersion for two minutes and subsequent thawing at 37 °C for two minutes, repeated in triplicate. After 3 washes with PBS, processed nerves grafts were stored in PBS solution at 4 °C.

Quantification of Myelin, Collagen and GAGs

Triplicate samples of each treatment were used for quantification of myelin, collagen and glycosaminoglycans (GAG) content. Each 2cm length of processed nerve was divided into 2 separate 1 cm pieces, one for histology and the other for total protein isolation.

Histology samples were fixed in 10% neutral buffered formalin and embedded in paraffin (FFPE) for histomorphometric analysis via Gomori's Trichrome. In brief, FFPE tissues were sectioned at 5 µm, placed on slides and warmed overnight at 60°C. Slides were deparaffinized, rehydrated, and stained for Gomori's Trichrome stain. Digital images of slides were acquired with a Nikon AZ100 M microscope at 10X magnification and collagen and myelin content was assessed using ImageJ.

Total protein was isolated by lysing nerves in protein lysis buffer (9.5 M Urea/4% 3-[(3-cholamidopropyl dimethylammonio)-1 propanesulfonate (CHAPS)/Roche Protease Inhibitor Cocktail/2.5% tributylphosphine), removing cell debris via centrifugation, and finally quantifying by Bradford Assay using Coomassie Protein Assay Reagent (Thermo Fisher Scientific, Waltham, MA) and albumin standards (Pierce, Rockford, IL). GAG content was quantified with the dimethyl methylene blue (DMMB) assay (1,9 dimethyl-methylene blue, Sigma-Aldrich, St. Louis, MO), using total protein.

Electron microscopy

Scanning electron microscopy was performed in the Vanderbilt Cell Imaging Shared Resource. Axial cross-sections of representative acellular nerve samples were fixed with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). The specimens were post-fixed with 1% OsO₄ solution in 0.1 M cacodylate buffer (pH 7.4) for 2 h, dehydrated through a graded ethanol series in PBS, and sputter coated with 3.5 µm of gold. Quanta 250 ESEM (FEI, Hillsboro, OR) was used to capture surface images at 300× to 3000×.

Growth factor loading of decellularized nerves

NGF-β (>98% pure as determined by RP-HPLC and SDS-PAGE; ProSpec, East Brunswick, NJ) was reconstituted to 100 µg/ml in sterile 18MΩ-cm H₂O and filtered through a 0.22 µm filter. Processed nerve grafts were incubated in NGF-β solution for 16 hours at room temperature under agitation. Concentration of incubation solution was initially evaluated in a dilution series with Freeze-thaw processed nerve grafts and sandwich ELISA (ChemiKine NGF Sandwich ELISA, Millipore, Billerica, MA) of tissue lysates. Optimal incubation concentration of 10 µg/ml was chosen at the saturation point of NGF loading of tissue samples.

Growth factor release assay

Following incubation in NGF solution, loading and release of growth factor was evaluated under infinite sink conditions *in vitro* at 37 °C. NGF release was measured for 21 days following initial treatment. Allografts were washed three times in the first 24 hours and once every 24 hours subsequently with 1ml of Tris-buffered saline per wash. The washes were collected and stored in silanized Eppendorf tubes at -20 °C. After 21 days, the nerves were homogenized and total protein was extracted and quantified as before. Concentrations of NGF in nerve lysates and collected washes were then quantified in triplicate using an NGF sandwich ELISA (ChemiKine NGF Sandwich ELISA, Millipore). Washes from day 7 were retained for use in the bioactivity assay.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 5 for Mac OS X (GraphPad Software; San Diego, CA). For comparison of axon counts, Student's t-test was used to compare specific groups. All p values were two-tailed and significance was determined at $p < 0.01$.

Results

Extracellular matrix properties of acellular nerve allografts

Twelve decellularized nerves were evaluated for their extracellular matrix composition, including myelin, collagen, and glycosaminoglycan content. The presence of connective tissue, cellular debris and myelin was first evaluated with semi-quantitative histology of Gomori's trichrome-stained cross-sections. The presence of myelin was greatest in unprocessed nerve and Freeze-thaw decellularized nerve, with a myelin:collagen ratio of 2.44 ± 0.49 and 1.61 ± 0.24 respectively (Figure 1A).

GAG content is known to inhibit the performance of acellular nerve allografts. Prior to enzymatic treatment of allografts, total GAG content is highest in unprocessed nerves (0.423 ± 0.068 ug/mg) and Freeze-thaw decellularized nerves (0.457 ± 0.073 ug/mg) and significantly reduced in Sondell-processed nerves (0.167 ± 0.036 ug/mg, $p = 0.029$) (Figure 1B).

Organization and porosity of nerve allografts was evaluated with scanning electron microscopy. Sondell-processed allografts demonstrated the best preservation of tubular endoneurium and collagen fibril organization (Fig. 2A–B). Empty endoneurial sheaths are visible with diameters up to 10 μ m with intact surrounding basal lamina (Fig. 2B). Hudson-processed nerve allografts showed high disorganization of collagen fibrils and protrusion of axon bundles from the perineurium (Fig. 2C–D). There were no intact endoneurial tubes or basal lamina, and all pores were below 1 μ m in diameter (Fig. 2D). Freeze-thaw cycled nerve allografts were well organized with intact myelin sheaths and basal lamina tubes (Fig. 2E–F). However, cellular debris also appeared to remain within the endoneurium (Fig. 2F). The debris reduces the effective porosity of the allograft and potentially obstructs neurite outgrowth. Additional SEM images have been provided as Supplemental Figure 1.

Nerve growth factor retention in acellular nerve allografts

Twelve acellular nerve allografts processed by Sondell, Hudson or Freeze-thaw methods were evaluated for nerve growth factor retention in vitro over a period of 21 days under infinite sink conditions (Fig. 3A). Sondell-processed allografts were found to have far superior NGF loading (9.25 ± 1.09 ng/mg) after incubation for 1 hour in storage solution at 37 °C. Hudson and Freeze-thaw processed allografts showed low initial NGF loading after 1 hour in storage solution, 1.81 ± 0.60 ng/mg and 1.30 ± 0.36 ng/mg respectively. In Sondell-processed allografts, approximately half of the loaded NGF is released in 1 d, but the remainder is released slowly over following 3 weeks (0.939 µg/mg/wk, $r^2 = 0.962$, $p < 0.0001$). After 21 d of incubation, Sondell allografts have significantly greater retained NGF (1.55 ± 0.25 ng/mg) compared to Hudson (0.60 ± 0.17 ng/mg, $p = 0.013$) and Freeze-thaw (0.35 ± 0.16 ng/mg, $p = 0.003$) nerve allografts as well as compared to unprocessed (normal) nerve (0.18 ± 0.01 ng/mg, $p = 0.002$) (Fig. 3B).

Discussion

This study has several important findings that could be impactful in the practice of peripheral nerve repair. In our characterization of different methods of decellularization using histology and scanning electron microscopy, we found that all 3 methods produced significantly decreased ratio of myelin:collagen, suggesting clearance of neuronal proteins and preservation of ECM proteins. Sondell treated allografts exhibited the least GAG content. The cleavage of GAG side chains of chondroitin sulfate proteoglycans (CSPGs) is known to attenuate inhibitory activity and promote axon regeneration. Chondroitinase ABC (ChABC) is an enzyme used to cleave GAG side chains from CSPGs and has shown promise as a treatment for central and peripheral nerve lesions (14). Sondell allografts also had the best preservation of extracellular matrix architecture and clearance of cellular debris. This is different from the initial findings by Hudson and coworkers, and may have important implications in the manufacturing of current commercially available acellular nerve allografts. Specifically, disorganized collagen fibers and loss of basal lamina tubes in Hudson allografts may hinder outgrowth and diffusion, and may indicate the loss of important growth factor-binding ECM proteins.

In this study we established the feasibility of affinity-based delivery of neurotrophins within acellular nerve allografts. Similar to previous affinity-based delivery systems, the release of NGF from loaded nerve allografts was found to have an initial burst in the first 24 hours followed by a slow release over 21 days. We found that the extracellular matrix characteristics of Sondell processed nerve allografts showed the greatest NGF binding affinity and release profile. NGF is the prototypical neurotrophic factor that supports survival and regeneration after injury. The positive effects of NGF on the regeneration of nerves after injury has been well documented and demonstrated with *in-vitro* and *in-vivo* models (17–19), however its clinical use has been complicated by our inability to provide sustained delivery of the growth factor. The use of nerve conduits as both a method of surgical repair and as a delivery device for the sustained release of neurotrophic factors to promote axonal growth have been widely investigated over the last several years (20–22). A 2014 study by Martino et al found that certain growth factors were found to bind with high

affinity to several extracellular matrix proteins. Among the growth factors originally screened for domains with 'ECM super-affinity', the neurotrophin family was found to have significant affinity to ECM proteins (23). By leveraging this innate neurotrophic factor affinity to ECM proteins, acellular nerve allografts may be capable of retaining these growth factors for sustained effect. Sondell allografts retained much higher molar ratios of NGF than Hudson and Freeze-thaw allografts, and retained greater than 35% of the initial NGF load at 7 days and greater than 15% at 21 days. The cellular debris and ECM disruption observed in Hudson and Freeze-thaw allografts may contribute to lower NGF diffusion and available binding sites during loading.

Unfortunately, the mechanisms for why Sondell treated allografts displayed such an affinity for NGF loading cannot be elucidated by the methods employed in this study. However, some speculation can be made. While both use Triton to some extent, they differ greatly on the amount and length of treatment and both have other surfactants included in the processing which play a significant role in the final product. Sondell treatment utilizes a relatively high (3%) concentration of Triton as well as 4% sodium deoxycholate, while Hudson treatment uses a relatively low (0.14%) concentration in combination with sulfobetaine 16 (SB-16) and alternating with sulfobetaine 10 (SB-10). While Triton is a non-ionic surfactant, sodium deoxycholate is an anionic surfactant, and sulfobetaine is a zwitterionic surfactant exhibiting properties of both. Triton and sodium deoxycholate have both been shown to retain more structure than other zwitterionic surfactants and eliminate most DNA content. (24–26) However, residues of both Triton and sodium deoxycholate have been identified from the resulting decellularized tissues, while zwitterionic surfactants cleared the tissue more readily. (26) The very different chemistries of each of these surfactants are certain to impact the affinity of the resulting grafts for not only growth factors, such as NGF, but other proteins as well.

One limitation of this study is the *ex vivo* and *in vitro* nature of the study. These processed acellular allografts will still need to be tested *in vivo* to prove their effectiveness as an alternative to autografting. Rat sciatic nerve injury is a well-established research model for peripheral nerve repair, but it cannot be used to accurately reproduce large (>2 cm) nerve gap injuries, and large animal studies, where a larger gap can be studied, are prohibitively expensive. However, the ability to test multiple processing methods/neurotrophin combination *ex vivo* allows for relatively quick elimination of poor performing combinations from further study, thus narrowing our candidates for *in vivo* experimentation.

Future aims for this work include comparison to other processed allografts, including chondroitinase treated allografts. Other neurotrophins, such as brain derived nerve growth factor and ciliary neurotrophic factor, should be tested *ex vivo* to assess their ability to be loaded into and released from these processed allografts. Once candidate processing method/neurotrophin combinations have been selected, they should be tested in the small animal model, and if the results prove promising, large animal testing over longer gaps should be considered.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

A) Myelin-connective tissue ratio calculated from Gomori's trichrome stained cross-sections; ns = no significance, * = $p < 0.01$, ** = $p < 0.001$. B) Glycosaminoglycan content in acellular nerve allografts quantified with DMMB assay.

Figure 2. Scanning electron microscopy of: A) Sondell-processed grafts at low and B) high magnification; C) Hudson-processed grafts at low and D) high magnification; and E) Freeze-thaw cycled grafts at low and F) high magnification. Scale bars: A, C, E = 100µm; B, D, F = 10µm.

Figure 3.

In vitro NGF release assay showing A) retained NGF time course and B) retained NGF at final time point of 21 days.

Table 1

Nerve decellularization protocols adapted from existing literature.

Sondell Protocol [17]			Hudson Protocol [18]	
Step	Solution	Time (h)	Solution	Time (h)
1	46mM (3%) Triton X-100	15	125mM SB-10, 10mM phosphate, 50 mM sodium	15
2	96mM (4%) sodium deoxycholate	24	0.14% Triton X-200, 0.6mM SB-16, 10mM phosphate, 50mM sodium	24
3	DI H ₂ O	1 × 7	50mM phosphate, 100mM sodium	0.5 × 3
4	46mM (3%) Triton X-100	15	125mM SB-10, 10mM phosphate, 50 mM sodium	7
5	96mM (4%) sodium deoxycholate	24	0.14% Triton X-200, 0.6mM SB-16, 10mM phosphate, 50mM sodium	15
6	PBS (pH 7.2) at 4 C	Until use	10mM phosphate, 50mM sodium	Until use