Characterization of a Novel Decellularized Peripheral Nerve Graft

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Introduction

Currently the clinical standard of care for treatment of extensive peripheral nerve damage is the autograft, which consists of a donor nerve from the patient implanted to bridge the site of injury. This technique, although providing a non-immunogenic solution to guide in nerve regeneration, is limited by several constraints including donor site morbidity. To address these concerns, our lab has developed a novel native nerve graft rendered acellular by chemical treatment[1]. In contrast with previous chemical decellularization techniques, our protocol is optimized to produce a non-immunogenic universal tissue implant without sacrificing extracellular matrix integrity. The result, known as the Optimized Acellular (OA) graft, has the capability to provide a universal solution to surgeons, eliminating the need for the secondary surgery associated with the autograft and allowing for a storable and versatile tissue engineering alternative.

To assess the regenerative capacity of the OA graft, a battery of tests were chosen to verify the safety and efficacy with respect to positive and negative controls. The Lewis rat sciatic nerve model was chosen for its versatility in testing methodologies and lack of autotomy. To address potential concerns with immunoreactivity and molecular remodeling, short-term implants were removed and analyzed using quantitative immunohistochemistry. Long-term functional regenerative capacity was assessed using the sciatic function index (SFI) protocol developed by de Medinaceli[2] and modified by Bain et al[3]. The data in this study was blinded and normalized against several controls to provide an accurate representation of regeneration in the OA graft. Positive controls included the allograft (analogous to the autograft) and surgical realignment of severed ends without graft implantation. Two previous decellularization methods, the Thermal[4] and Sondell[5] protocols, were also compared, though both of these methods cause significant structural damage to the graft.

Experimental Methods

Surgical Procedure Lewis rats were anesthetized using inhaled isoflurane followed by an intaperitoneal injection of 120 mg/kg body weight Ketamine and 15 mg/kg body weight Xylazine. A 5 cm incision was made to expose the sciatic nerve, which was then transected to create a 10 mm gap. The graft of interest was inserted and immobilized using 6 radial 10-0 sutures through the epineurium (3 proximal, 3 distal). The incision was closed using running sutures and the skin was stapled. At the conclusion of the study, nerve grafts were recovered in the same manner and immediately fixed with a 3% glutaraldehyde and 4% paraformaldehyde solution for 30 minutes.

Immunohistochemistry Fixed nerve tissue was dehydrated in an alcohol gradient and immersed in paraffin. Once hardened, 7 µm sections were produced using a microtome. These sections were stained using a primary/secondary antibody system followed by DAB and eosin visualization. Slides were imaged and a digital threshold filter applied to distinguish

stained areas from background. The black areas were quantified for percent coverage and/or total number of stained regions.

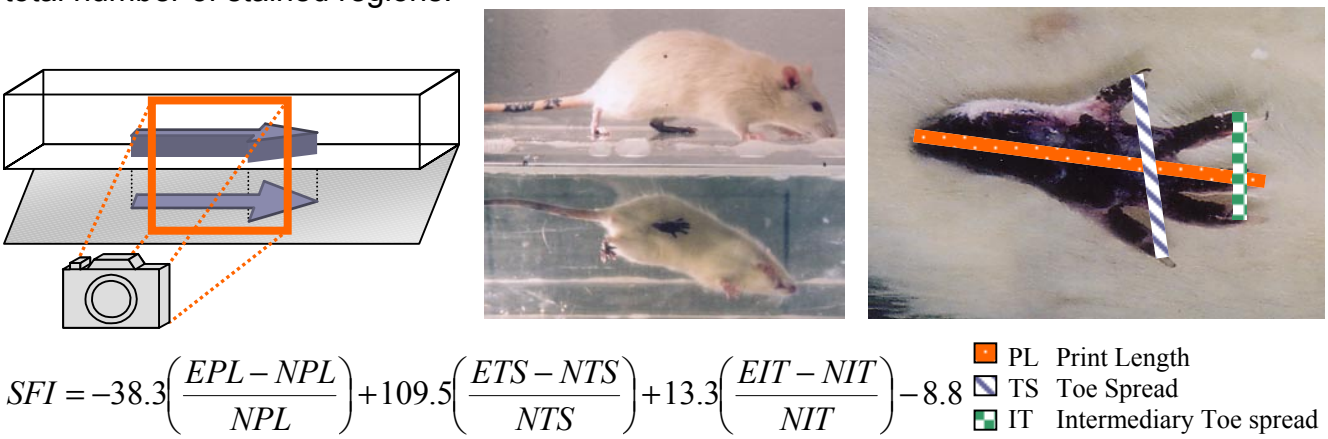

Figure 1: Walking Track Analysis (E is experimental, N is normal paw)

Functional Analysis Animals were briefly exposed to airborne isoflurane while black marker was applied as a contrast agent. Rats were allowed to recover and then placed in a rectangular plexiglass walking track (Figure 1). The rat's normal walking gait was recorded a minimum of three times using a digital video camera. The non-experimental paw served as a positive control for normalization purposes. Individual frames from this video were digitally captured and analyzed using Scion Image for the appropriate parameters used in the sciatic function index calculation (Figure 1).

Results and Discussion

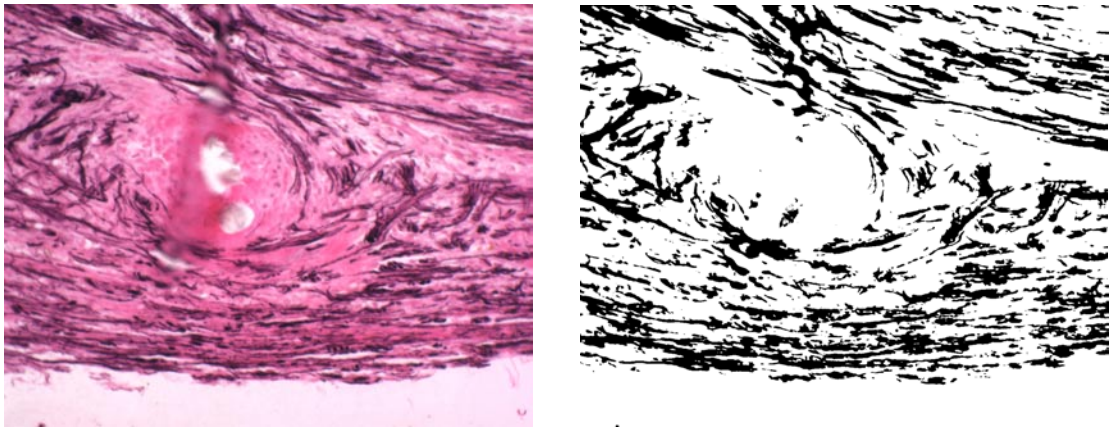

Figure 2: RT97 Neurofilament stain and threshold image

We have developed a novel peripheral nerve graft composed of decellularized native nerve to address the operational limitations intrinsic to the autograft, the current clinical standard of care for peripheral nerve injuries. The OA graft, composed of intact basal lamina and surrounding protein structure, provides an ideal environment for axon regeneration while eliminating the need for a secondary surgery and the lack of an off-the-shelf solution. To confirm the removal of all immunogenic material, immunohistochemistry was used to verify that the OA graft is safe and does not elicit an immune response. The difference in CD8+ coverage (directly proportional to cytotoxic T-cell levels) is statistically insignificant from the allograft, suggesting that the graft is well tolerated immunologically (data not shown).

Following immune response characterization, functional and histological testing provided data to investigate the regenerative capacity of the OA graft. A representative sample of nerve was imaged and a threshold filter applied to distinguish axon stain from background stain (Figure 2). This black area was measured with respect to white and reported as percent coverage. Using this method, axon count (Figure 3) revealed no significant difference between the OA graft and positive control, which suggests that the OA graft supports acceptable axon regeneration. When compared to the previous two decellularization methods, the OA graft exhibits a notably higher axon density and qualitatively. While the functional studies are preliminary, the OA graft also demonstrated a similar average SFI to the surgical realignment control (Figure 4). This suggests that the graft supports regeneration similar to that of current clinical treatments.

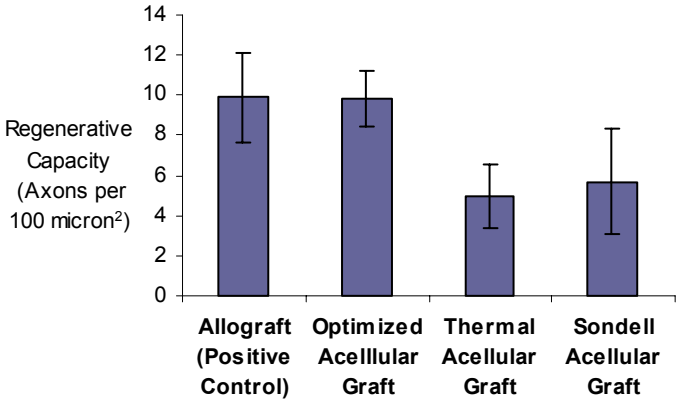

Figure 3: OA Graft Supports Comparable Regeneration to Positive Control

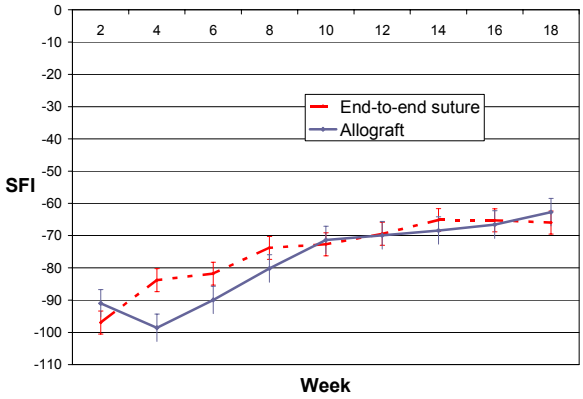

Figure 4: Functional Recovery via Sciatic Function Index

In conclusion, we have produced a nerve graft that addresses many of the limitations of the current treatment. The OA graft exhibits a comparable immune response to the autograft as well as supporting similar regeneration, as measured via axon count microscopically and SFI macroscopically. Further studies are currently underway to confirm these results with a larger test group; these studies will examine long-term effects up to one year following injury. These results suggest that the OA graft may be a viable future treatment for peripheral nerve injuries. This system also provides a robust and flexible system for the analysis of the processes involved in regeneration, which will ultimately increase our knowledge of how nerves regenerate and how to better facilitate reinnervation.

References

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