ACKNOWLEDGMENTS

I gratefully acknowledge the Wake Forest Department of Orthopaedic Surgery for their assistance with this work and KeraNetics, the Armed Forces Institute for Regenerative Medicine, and the Errett Fisher Foundation for funding support.
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<th>Description</th>
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<tbody>
<tr>
<td>A/A</td>
<td>Antibiotic/Antimicotic</td>
</tr>
<tr>
<td>AChR</td>
<td>Acetylcholine Receptor</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>APB</td>
<td>Abductor Pollicis Brevis</td>
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<tr>
<td>BDNF</td>
<td>Brain Derived Neurotrophic Factor</td>
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<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
</tr>
<tr>
<td>CMAP</td>
<td>Compound Motor Action Potential</td>
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<tr>
<td>CNS</td>
<td>Central Nervous System</td>
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<tr>
<td>CSA</td>
<td>Cross-sectional Area</td>
</tr>
<tr>
<td>DAPI</td>
<td>Diamidino-2-Phenylindole</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DRG</td>
<td>Dorsal Root Ganglia</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>EDL</td>
<td>Extensor Digitorum Longus</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked Immunosorbant Assay</td>
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<tr>
<td>ESC</td>
<td>Embryonic Stem Cell</td>
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<td>EtOH</td>
<td>Ethanol</td>
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<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
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<tr>
<td>FGF</td>
<td>Fibroblast Growth Factor</td>
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<tr>
<td>FGF-2</td>
<td>Basic Fibroblast Growth Factor</td>
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<td>HHK</td>
<td>Human Hair Keratin</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>HRP</td>
<td>Horseradish Peroxidase</td>
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<td>IGF</td>
<td>Insulin-like Growth Factor</td>
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<td>KOS</td>
<td>Human Hair Keratin</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>MAP</td>
<td>Muscle Action Potential</td>
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<tr>
<td>MAT</td>
<td>Matrigel</td>
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<tr>
<td>MBP</td>
<td>Myelin Basic Protein</td>
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<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
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<tr>
<td>MSC</td>
<td>Mesenchymal Stromal Cell</td>
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<tr>
<td>NCV</td>
<td>Nerve Conduction Velocity</td>
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<tr>
<td>NF</td>
<td>Neurofilament</td>
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<tr>
<td>NGF</td>
<td>Nerve Growth Factor</td>
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<tr>
<td>NHP</td>
<td>Non-Human Primate</td>
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<tr>
<td>NIH</td>
<td>National Institutes of Health</td>
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<tr>
<td>NMJ</td>
<td>Neuromuscular Junction</td>
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<tr>
<td>NN</td>
<td>Necrotic Nerve</td>
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<tr>
<td>NNC</td>
<td>Necrotic Neuronal Cell</td>
</tr>
<tr>
<td>NP</td>
<td>Neural Precursor</td>
</tr>
<tr>
<td>NTR</td>
<td>Neurotrophin Receptor</td>
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<tr>
<td>OD</td>
<td>Optical Density</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PD</td>
<td>Proximal-Distal</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
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<tr>
<td>PGA</td>
<td>Polyglycolic Acid</td>
</tr>
<tr>
<td>PHB</td>
<td>Polyhydroxybutyrate</td>
</tr>
<tr>
<td>PL</td>
<td>Phospholipase</td>
</tr>
<tr>
<td>PLC</td>
<td>Poly(l-lactide-co-caprolactone)</td>
</tr>
<tr>
<td>PO</td>
<td>Proximal-Open</td>
</tr>
<tr>
<td>PNI</td>
<td>Peripheral Nerve Injury</td>
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<tr>
<td>PNS</td>
<td>Peripheral Nervous System</td>
</tr>
<tr>
<td>RFU</td>
<td>Relative Fluorescent Unit</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>RT</td>
<td>Room Temperature</td>
</tr>
<tr>
<td>SAL</td>
<td>Saline</td>
</tr>
<tr>
<td>SC</td>
<td>Schwann Cell</td>
</tr>
<tr>
<td>TBS/T</td>
<td>Tris Buffered Saline/Tween 20</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming Growth Factor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like Receptor</td>
</tr>
<tr>
<td>TMB</td>
<td>Tetramethylbenzidine</td>
</tr>
<tr>
<td>VACHT</td>
<td>Vesicular Acetylcholine Transporter</td>
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ABSTRACT

Peripheral nerve injury is a relevant clinical concern. Although surgical management of these injuries has improved over time, the current “gold standard” for nerve transection injury, autograft, has many limitations. Nerve conduits are used as an alternative to autograft surgery but they are currently recommended for the repair of small defects (≤3cm) in small diameter sensory nerves only. Use of filler materials inside the conduit have been shown to increase the defect size in which conduits can be effective and can result in greater functional recovery in animal models.

This work uses a novel conduit filler derived from a human hair keratin biomaterial hydrogel with the goal of promoting the clinical translation of an effective alternative to autograft surgery. The work consists of 3 aims based upon the hypothesis that keratin hydrogel enhances peripheral nerve regeneration by early cellular interactions in the injured peripheral nervous system: 1) To demonstrate the efficacy of the keratin hydrogel in a clinically relevant non-human primate model 2) To investigate the keratin hydrogel’s effect on inflammatory and regenerative cells in the injured peripheral nervous system using a rat sciatic nerve study 3) To characterize keratin’s influence on cellular interactions using a simplified culture system.

Methods for this work include nerve conduction studies by electrophysiology, functional testing, histology and histomorphometry of nerve and muscle tissue, immunogenicity testing, isolation and culture of primary Schwann cells, and in vitro proliferation, migration, and cytokine expression assays.
Electrophysiological and histological outcome measures in the non-human primate study showed that keratin hydrogel significantly enhanced median nerve regeneration over 12 months compared to a saline control. The rat study and in vitro assays provided evidence that keratin hydrogel influences peripheral nervous system cellular response to injury and that this altered cellular response may contribute to enhancement of peripheral nerve regeneration. Future work based on the studies in this document may ultimately result in a clinically available alternative to autograft surgery for peripheral nerve injury patients.
Title: Peripheral Nerve Regeneration, Research Trends and Applications for Clinical Nerve Repair

Introduction

Traumatic peripheral nerve injury (PNI) is most often caused by motor vehicle crashes among civilians and combat blast injury among military servicemen (Campbell, 2008). The patient population is typically young males presenting with upper extremity injuries and there is high likelihood of disability resulting in work loss and diminished quality of life (Lundborg and Rosen, 2007; Novak et al., 2011). The level of disability resulting from these injuries is affected by many factors including injury severity, patient age, injury location, and the presence of scar tissue, and can involve physical impairment such as motor and sensory dysfunction, neuropathic pain, and psychosocial factors such as pain catastrophizing, anxiety, and depression (Fu and Gordon, 1997; Ray and Mackinnon, 2010; Novak et al., 2011).

PNI is evaluated on 2 classification scales: the Seddon scale and the Sunderland scale. The Seddon scale divides nerve injuries into 3 types: neurapraxia, in which axon continuity is preserved, axonotmesis, with loss of axon continuity but preservation of connective tissue, and neurotmesis, which is disruption of the entire nerve and may include complete transection (Seddon, 1972). Sunderland later developed a numerical scale on which neurapraxia is equivalent to a level 1 while axonotmesis is divided into 3 levels of severity ranging from 2 to 4. Complete nerve disruption or neurotmesis is equivalent to a Sunderland level 5 (Sunderland, 1991). Minor PNI are capable of
spontaneous regeneration and functional recovery while more severe injuries require surgical intervention with varying prognoses (Lee and Wolfe, 2000).

Peripheral nerves are made up of an outer layer of connective tissue called the epineurium which encircles the nerve tissue and runs between bundles of axons called the fascicles. Each fascicle is surrounded by the perineurium, connective tissue which provides flexible strength to the nerve. Individual axons in the fascicles are surrounded by a protective layer of connective tissue called the endoneurium (Lee and Wolfe, 2000). Each peripheral nerve has its own blood supply with blood vessels running longitudinally through the connective tissue and transverse branches connecting them in a complex network (Ramage, 1927). Large diameter axons are surrounded by a myelin sheath which serves to insulate the axon and promotes rapid saltatory conduction of electrical impulses called action potentials. Each myelin sheath is provided by a single Schwann cell (SC) which wraps itself around the axon. Spaces between the myelin sheaths are called nodes of Ranvier and contain ion channels which allow the propagation of an action potential down the axon to its terminal. Small diameter unmyelinated axons also produce action potentials and they are wrapped by nonmyelinating SC. Therefore, every axon in the peripheral nervous system (PNS) is surrounded by a tube of SC basal lamina (Ide, 1996). The other major cell types in peripheral nerves are fibroblasts and macrophages which reside in the endoneurium (Causey and Barton, 1959) and endothelial cells which make up the blood vessels and provide the blood nerve barrier (Lee and Wolfe, 2000).

A distinct difference between the central nervous system (CNS) and PNS is the ability of the PNS to regenerate following injury. The CNS consists of a mesh-like network of glial cells with no basal lamina surrounding the axons (Schwab and Caroni,
The extracellular matrix (ECM) of the CNS is comprised of proteoglycans, hyaluronan, and tenascin and has been shown to be nonpermissive for neurite outgrowth *in vitro*. Digestion of proteoglycans in the glial scar formed after CNS injury has been shown to promote axonal regrowth *in vivo* (Zimmermann and Dours-Zimmermann, 2008). The basal lamina of SC in the PNS is thought to be the primary effector of peripheral nerve regeneration. Adhesion molecules in this specialized ECM such as collagen, entactin, fibronectin, and laminin contain SC binding sites and contribute to the formation of a natural conduit that facilitates axonal regeneration under optimal conditions (Ide, 1983; Ide et al., 1983). SC have an essential role not only in normal peripheral nerve function, but also in regeneration after injury (Mirsky and Jessen, 1999).

Peripheral nerve transection injury catalyzes a series of events beginning with the retrograde degradation of the distal nerve end, which is referred to as Wallerian degeneration, and the membrane resealing of the proximal nerve end and both of these processes are controlled by calcium ion ($\text{Ca}^{2+}$) influx into the axoplasm. On the distal side, $\text{Ca}^{2+}$ activates calpain, a protease needed for axonal degeneration, and the axonal cytoskeleton and cell membrane disintegrate and the myelin sheath degrades (Gaudet et al., 2011). The endoneurial macrophages and SCs proliferate, activate, and begin myelin phagocytosis. The phagocytic cell population is later increased by the recruitment of monocytes from the bloodstream which infiltrate the injured nerve tissue via the disrupted blood nerve barrier and myelin sheath (Gaudet et al., 2011). The remaining distal structure following the degradation of the axon cytoskeleton and myelin phagocytosis is a SC band of Büngner or SC column, a structure which resembles a
hollow tube consisting of the neurolemma in apposition to the basal lamina (Tetzlaff et al., 1989). Adhesion to the basal lamina molecules in the SC column causes SC migration, proliferation, and secretion of growth factors required for guiding axon sprouts from the proximal nerve stump. The adhesion molecules that facilitate axon-SC binding have been identified as immunoglobulins such as L1, N-CAM, and P0 and Ca\(^{2+}\) dependent molecules such as N-cadherin. During neural development, L1 and N-CAM are found on both axon and SC cell membranes. Following denervation, these molecules are upregulated on the SC membranes allowing them to bind together to form bands of Büngner and on regenerating axon cell membranes, allowing them to elongate through the SC column (Ide, 1996; Mirsky and Jessen, 1999). N-cadherins on the SC and regenerating axons also stimulate axon outgrowth by allowing cell-cell adhesion of these molecules (Ide, 1996). L1, N-CAM, and N-cadherin have been shown to promote neurite outgrowth by promoting tyrosine kinase phosphorylation of the fibroblast growth factor (FGF) receptor in neurons (Skaper et al., 2001). Denervated SC express elevated levels of neurotropic factors such as nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), insulin-like growth factor (IGF) and FGF (Ide, 1996; Mirsky and Jessen, 1999). The exogenous application of these growth factors has been shown to enhance peripheral nerve regeneration in many studies (Chen et al., 2006).

The proximal end of a transected nerve undergoes membrane disruption and influx of high concentrations of extracellular ions such as Ca\(^{2+}\) and sodium (Na\(^{+}\)) causes axon depolarization. Ca\(^{2+}\) activates proteases calpain and phospholipase A2, which can mediate rapid membrane resealing and occurs 1 to 2 nodes of Ranvier proximal to the lesion site (Shim and Ming, 2009). In the cell body, the soma enlarges and the nucleus
migrates to the periphery signifying a switch from neurotransmitter synthesis to structural protein synthesis (Lee and Wolfe, 2000). Following distal Wallerian degeneration, SCs in the SC columns secrete neurotrophic factors such as FGF that guide sprouting axons from the proximal end. Regenerating axon sprouts can begin to emerge from the most proximal nodes of Ranvier within hours after injury. This early event is controlled by local protein synthesis and therefore independent of the cell body, while later axon extension is controlled by the rate of anterograde transport of growth components from the cell body (Ide, 1996). The growth cones of regenerating axons can grow across the gap between the nerve ends and enter the SC columns eventually reaching the distal targets (McQuarrie, 1985). A study by Parrinello et al. showed that regenerating axons migrated closely behind dedifferentiated SCs and fibroblasts within days following sciatic nerve crush injury in rats (Parrinello et al., 2010).

There are 3 main techniques for surgical repair of peripheral nerve lesions in which tension-free end-to-end re-anastomosis is not possible. The techniques are autograft, which is considered the clinical “gold standard”, acellular allografts, and nerve conduits. Many preclinical studies have been conducted to improve outcomes for the two latter techniques because autograft has many limitations, including of the lack of available donor tissue, donor site complications, and multiple surgeries.

**Autografts**

In many nerve transection cases, re-anastomosis of the two nerve ends would create excessive tension that is deleterious for successful nerve regeneration, functional recovery and may result in neuroma formation. If scar tissue is present, the surgeon will
resect the proximal and distal nerve ends until normal fascicular architecture is visible, creating a gap in the tissue (Ray and Mackinnon, 2010). The clinical “gold standard” for tension-free repair of nerve gaps is the autograft (Matsuyama et al., 2000). The most commonly used nerve is the sural nerve, a sensory nerve in the lower leg, although sensory nerves in the forearm and hand including the lateral or medial antebrachial cutaneous nerves, the dorsal cutaneous branch of the ulnar nerve, or the superficial branch of the radial nerve may be utilized (Lee and Wolfe, 2000; Matsuyama et al., 2000). There are different strategies for autograft repairs depending on the type of nerve to be repaired and the injury location. Small diameter nerves may utilize a single graft segment while larger diameter nerves may require multiple small diameter grafts if the fascicular architecture can be identified. The group fascicular repair technique may be performed using cable grafts to repair individual corresponding fascicles and is especially important for repair of mixed motor nerves because it improves the chances of specific target reinnervation (Matsuyama et al., 2000; Dahlin, 2008). There are commonly reported complications for patients that undergo autograft harvest surgeries. In one report of patients that underwent sural nerve harvest, 44% of patients complained of sensory deficiency, 42% of calf tenderness, and 16% displayed neuroma formation and intolerable pain (Staniforth and Fisher, 1978) while another study showed only 6.1% of patients presenting with symptoms due to neuroma (Ortiguela et al., 1987). A more recent study of 38 patients reported nerve impairment symptoms in 17.5% of patients, the most common being a shock-like or stabbing pain (Martins et al., 2012). Although function outcomes are generally good with the use of autograft and techniques for harvesting the
sural nerve have improved over time, these complication rates have provided motivation in the search for alternative technologies.

**Acellular Grafts**

The vital function of the basal lamina in peripheral nerve regeneration can be examined by the use of acellular nerve grafts. The method for creating an autologous acellular nerve graft was illustrated in the early 1980’s in a mouse sciatic nerve model. A 6 to 7mm portion of tissue was excised and subjected to repeated freeze-thaw treatment to disrupt the cellular material. The tissue portion was then sutured into the original defect site, creating tubes of basal lamina that supported the regenerating axons, thereby suggesting that the basal lamina forms a functional scaffold for regeneration (Ide et al., 1983). This decellularization technique was also tested in a non-human primate (NHP) ulnar nerve model. Acellular and cellular allografts were prepared by transecting a funiculus of the ulnar nerve and attaching its proximal and distal ends to the neighboring muscle one week before removal. This time period before grafting was thought to allow the phagocytosis of myelin and cytoskeletal debris by macrophages and SCs. The acellular nerve segments were then prepared by the freeze-thaw method and transplanted into the host. Histological analysis at 20 weeks showed that regenerated axons in the acellular grafts grew up to 7cm into the host nerve compared to the allograft controls which only grew into the proximal end of the graft (Tajima et al., 1991).

Chemical extraction of nerves has been shown to create a better functional acellular basal lamina scaffold without the residual cell debris left by the freeze-thaw method (Dumont and Hentz, 1997). The removal of all cell debris using a chemical process may
eliminate the possibility of an immune response that could occur during heterologous use. Sondell et al. used rat sciatic nerves treated with chemical detergents that were immunostained for myelin and S100, confirming that no cell debris remained. These treated nerves were then used as heterologous allografts. Resulting data showed that these acellular allografts facilitated an environment for regeneration. At 20 days, immunostaining indicated no signs of rejection as well as infiltration of the nerve graft by SC and blood vessels from both the proximal and distal nerve ends. Invasion of macrophages was noted but to a lesser extent than autologous cellular grafts (Sondell et al., 1998).

Histology is an important indicator of success following a nerve graft surgery in animal models, but functional recovery is the primary goal for translational research. Dumont et al. compared acellular peroneal nerve grafts to isografts in rats and showed that the acellular nerve grafts had limited functional recovery, lesser target muscle weight, and decreased myelin area and fiber width after 3 months (Dumont and Hentz, 1997). Later studies in a rat peroneal nerve model found that acellular isografts were capable of supporting nerve regeneration in a 2cm gap using walking track analysis every 3 weeks, and histology and extensor digitorum longus (EDL) muscle contractile function as outcome measures at 15 weeks, although a 4cm gap in the same study showed no functional or histological recovery, indicating that there is a critical defect length for successful acellular graft repair (Haase et al., 2003). Another study by Zhong et al. compared both acellular nerve allografts and fresh autografts to fresh allografts in a canine sciatic nerve model. Functional locomotor recovery of a 5cm sciatic nerve gap by 5 months was only shown in the acellular allografts and fresh autografts.
Histomorphometry data indicated the acellular allografts allowed regenerating fibers to grow through the graft and reinnervate the target muscle (Zhong et al., 2007). Because autografts result in donor site morbidity, the use of acellular allografts may show promise as a clinical alternative. An FDA approved human cadaver acellular graft sold under the trade name Avance® was used in a recently published multicenter clinical trial and showed 87% positive functional outcomes in 132 nerve injuries in gaps ranging from 5mm to 50mm (Brooks et al., 2012).

Nerve conduits

Another technique used in peripheral nerve repair is entubulation of the nerve ends with a nerve conduit. The first conduits were constructed of silicone-polymer tubing and showed regeneration across small (6mm and 10mm) gaps in the rat sciatic nerve. Lundborg et al. used a proximal-distal (PD) group in which both the proximal and distal nerve ends were sutured into the conduit and a proximal-open (PO) group in which only the proximal nerve end was included. Histological evaluation at 4 weeks showed that axonal regeneration in the 10mm gaps had only occurred in the PD group suggesting the presence of factors that support nerve growth in the distal nerve end and the limitation of gap length on nerve regeneration (Lundborg et al., 1982). Further testing of the silicone tubes in a 10mm rat sciatic nerve model by Le Beau et al. and Merle et al. showed regeneration despite abnormal effects of nerve compression due to polymer swelling during material degradation (Le Beau et al., 1988; Merle et al., 1989). The conduits constructed of silicone tubing have also been
instrumental in the study of peripheral nerve regeneration *in vitro*. The sequence of events that occur from entubulation repair through the reinnervation of the distal nerve end is now well characterized (Heath and Rutkowski, 1998). Immediately following repair, the transected nerve ends secrete a fluid containing the structural proteins fibronectin and fibrinogen, as well as various neurotrophic factors. Within days, fibrinogen polymerizes with fibronectin creating an acellular fibrin matrix that supports cell migration and later axonal regeneration from the proximal nerve stump (Heath and Rutkowski, 1998; Lee and Wolfe, 2000; Chen et al., 2006).

Modern alternatives to silicone, a non-degradable material used for early conduit construction, are biodegradable synthetic materials such as poly-glycolic acid (PGA), poly(organo)phosphazine, and poly(l-lactide-co-caprolactone) (PLC) and endogenous ECM proteins such as collagen type I (Heath and Rutkowski, 1998; Deal et al., 2012). PLC conduits were studied by den Dunnen et al. using a 10mm rat sciatic nerve model. Morphometric analysis at 3 weeks showed axonal regrowth and myelination (similar results in the previously mentioned silicone-polymer conduit studies took 10 weeks to obtain). There were no signs of abnormalities due to constriction such as those noted in the silicone-polymer conduit studies, yet the proximal and distal nerve ends were significantly different from controls when axon diameter and myelinated axon number was evaluated. It was hypothesized that the decreased time and increased quality of regeneration compared to silicone-polymer conduits could have been due to the permeability of the biodegradable material, which would allow the diffusion of growth factors and nutrients from the external space (den Dunnen et al., 1996).
Collagen type I conduits stabilized with a formaldehyde gas crosslinking procedure have also shown encouraging results for peripheral nerve regeneration in both a 4mm rat sciatic nerve gap and a 4mm NHP median nerve gap. Electrophysiological analysis of evoked muscle action potentials (MAP) in the rat gastrocnemius muscle showed similar regeneration for collagen conduits and autografts. Evoked MAP in the NHP abductor pollicis brevis (APB) muscle also showed similar innervation of the distal stump for the collagen conduits and autograft controls (Archibald et al., 1991). A study by Dellon et al. showed a successful reconstruction of up to 3cm in a NHP ulnar nerve model for both PGA conduits and autografts, further suggesting that conduits can be used as an alternative to nerve autografts (Dellon and Mackinnon, 1988).

Successful animal studies of nerve conduits have prompted clinical trials and FDA approval for PGA conduits (sold as Neurotube or Neuregen) and collagen type I conduits (sold as NeuraGen) (Belkas et al., 2004). The majority of clinical studies of conduits are for repairs of small defects (≤ 3cm) in small diameter sensory nerves and show ≥ 75% of patients with positive functional outcomes (Taras et al., 2005; Schlosshauer et al., 2006; Lohmeyer et al., 2009; Taras et al., 2011; Deal et al., 2012). A recent case study of a 53 year-old female who underwent carpal tunnel decompression surgery and presented with dysesthesias and decreased function in her thumb illustrates the clinical effectiveness of conduit repair. Pre-surgical testing revealed denervation and atrophy of both abductor and opponens pollicis muscles. Exploratory surgery identified median nerve damage caused by a neuroma and a 4cm gap repair was conducted by inserting a small section of healthy proximal median nerve into the medial ends of two 2 cm PGA conduits and suturing the lateral ends to the proximal and distal stumps. The
patient regained full function of her thumb and her dysesthesias ceased (Hung and Dellon, 2008). The small segment of healthy nerve served as a source of SC, which indicates that conduit fillers can enhance regeneration with conduits.

**Hydrogels and Cell Seeding**

Luminal fillers consisting of ECM components, neurotrophic factors, and cells can improve the quality of nerve regeneration as well as increase the gap length for nerve repair with conduits (Jenq and Coggeshall, 1987; Heath and Rutkowski, 1998). This concept was investigated by Shirley et al. using a rat peroneal nerve transection model. Recombinant BDNF or phosphate buffered saline (PBS) was injected into a 5mm silicone elastomer channel and sutured in place to the proximal and distal nerve ends creating a 3mm gap. Gait analysis was performed at 2 week intervals to evaluate functional recovery and no significant differences between the two groups were observed. It was hypothesized that the BDNF may diffuse from the channel too early and a time release method might yield positive results (Shirley et al., 1996). Because SC are known to secrete neurotropic factors such as BDNF, SC transplants suspended in a gel matrix were investigated for use as conduit fillers. Early studies by Guenard et al. compared the use of heterologous and syngeneic SC suspended in Matrigel to facilitate rat sciatic nerve repair through acrylonitrile vinylchloride copolymer conduits. Study results showed that rejection of the heterologous SC impeded regeneration almost completely while syngeneic cells aided nerve repair with an increased number of myelinated axons and larger cross-sectional surface area of the regenerated nerve tissue after 3 weeks (Guenard et al., 1992). A later study by Mosahebi et al. disputed these results when comparing syngeneic and allogeneic neonatal SC suspended in a fibronectin and alginate gel matrix.
This matrix was injected into a polyhydroxybutyrate (PHB) conduit and used for a 10mm rat sciatic nerve gap repair. Suprisingly, the allogeneic SC promoted regeneration despite rejection and there was no histological difference between the allogeneic and syngeneic groups at 3 weeks. Both groups had a significant enhancement of regeneration compared to controls with no cells. These results suggest the possibility of an “off the shelf” approach to cell therapy that may eliminate the delay in harvest and culture of autologous SC for this type of treatment since time is a critical factor for a successful peripheral nerve repair (Mosahebi et al., 2002). A later study by Mosahebi et al. illustrated the importance of fibronectin in the alginate gel seeded with syngeneic SC using the same experimental model. Treatment groups included alginate gel only, alginate gel + SCs, alginate gel + fibronectin, and alginate gel + fibronectin + SCs and only the last group showed a significant increase in axonal sprout number at 6 weeks compared to empty conduit controls (Mosahebi et al., 2003).

A novel approach to engineering a scaffold for use as conduit luminal filler is the oxidation of keratin proteins in human hair fibers to create a biomaterial hydrogel. This keratin hydrogel was used as conduit filler in silicone conduits and compared to saline-filled conduits and sural nerve autografts in a 4mm mouse tibial nerve repair. Outcome measures at 6 weeks included electrophysiology, muscle force generation, histology, and histomorphometry. The keratin group showed enhanced electrophysiological results including significantly lower values for nerve conduction latency compared to the autograft and saline groups. The recovery of compound motor action potential (CMAP) was significantly higher for the keratin group compared to the saline group and higher than the autograft group. Histomorphometry of the regenerated nerves revealed
significantly higher blood vessel cross-sectional area in the keratin nerves compared to the saline and autograft groups (Sierpinski et al., 2008). Long-term results of this study showed that the nerve conduction latency remained significantly lower for the keratin group compared to the other two groups at 3 months. At 6 months, the nerve conduction latency and CMAP amplitude for the keratin and autograft was equivalent though the keratin nerves had significantly greater axon density (Apel et al., 2008).

Following the mouse studies, the keratin hydrogel conduit filler was then compared to sural nerve autograft and saline-filled conduits in a rabbit 2cm tibial nerve injury model using NeuraGen® type I collagen conduits instead of silicone. At 3 months, electrophysiological evaluation revealed that the keratin group had significantly lower nerve conduction latency and significantly higher CMAP amplitude than the autograft and saline groups. Histomorphometry data showed a trend toward larger nerve area and more total axons per nerve in the keratin group, although these differences were not statistically significant (Hill et al., 2011).

Keratin hydrogel is unique in its application as a conduit filler because unlike ECM proteins, it is resistant to proteolytic degradation (Kaluzewska et al., 1991). Biocompatibility studies have shown that the keratin biomaterial degrades in mouse subcutaneous implants within 8 weeks (de Guzman et al., 2011). These characteristics allow the keratin hydrogel to act as a scaffold in the conduit during early cellular infiltration but not to persist and inhibit axonal regeneration and remyelination. The mouse and rabbit studies illustrated that a keratin hydrogel-filled conduit may be as effective as the clinical “gold standard” without the drawbacks of multiple surgeries, donor site morbidity and loss of function.
Schwann cell and Stem cell therapies

Some preclinical research is focused on the use of embryonic stem cell (ESC) therapies for neurosurgical applications. In a study by Cui et al., a 10mm rat sciatic nerve axotomy was induced by transecting the nerve and re-attaching the epineurium to serve as a natural conduit for repair. ESC were differentiated into ESC neural precursors (ESC-NP) and the cells were injected into the epineurial sheath using culture medium as a control. At 3 months, the ESC transplant facilitated regeneration yielding fiber diameters close to uninjured levels. Histological evaluation revealed that the ESC may have differentiated into Schwann-like cells that enhanced regeneration of the axons. Electrophysiological testing of CMAP amplitude showed that the transplanted nerves amplitude values was close to their pre-axotomy levels while the medium controls had no measurable CMAP. The use of ESC for clinical therapy could provide an alternative to SC transplantation given that ESC have a more rapid proliferation rate. However, the use of ESC is controversial because of ethical concerns about the use of human embryos, the possibility of transplant rejection, and the risk of tumorigenesis (Cui et al., 2008).

Some new approaches for clinical translation of SC therapy have focused on genetically enhancing autologous SC to overexpress neurotropic factors (Haastert and Grothe, 2007). Endogenous basic FGF (FGF-2) and FGF receptors are upregulated during PNI for both the low (18kD) and high molecular weight (21 and 23kD) isoforms (Timmer et al., 2003). In a study by Haastert et al., rat neonatal SC were transfected to overexpress either the 18kD (SC-18) or 21 and 23kD (SC-21/23) isoforms of FGF-2, suspended in a Matrigel scaffold, and transplanted into a silicone conduit to study its effect in a rat 15mm sciatic nerve gap. Analysis of sensory functional recovery using the
reflex withdrawal test showed that SC-21/23 cells showed earliest signs of recovery at 4 weeks. At 12 weeks both SC-18 and SC-21/23 showed signs of sensory functional recovery compared to no recovery for physiological SCs (SC-p). At 16 weeks, the SC-21/23 group had the most myelinated, regenerated axons at distal levels in the conduit. Retrograde labeling confirmed that motor neurons only contributed to the regenerated tissue cables in the SC-18 and SC-p groups at 16 weeks (Haastert et al., 2006b). These findings indicate the need for possible motor neuron-specific tropic factors to achieve motor function recovery. The transfection of rat SC has been improved and successfully translated to human SC (Haastert et al., 2006a; Haastert et al., 2007). This could stimulate clinical testing of autologous SC therapy, although the expansion of human glial cells is time-consuming and donor tissue must be utilized. More recent, SC therapy studies utilized autologous mesenchymal stromal cells (MSC) differentiated into SC in a 10mm NHP median nerve conduit repair. Comparison of the MSC-filled conduits to saline-filled conduits revealed enhanced functional, electrophysiological, and histological outcomes for the MSC group (Wakao et al., 2010). Human clinical trials are needed to determine if differentiated autologous MSC for SC therapies will show promise for PNI patients in the future.

Conclusion

PNI remains a relevant clinical concern. There are numerous causes of PNI including motor vehicle accidents, electrical burn injuries, gunshot wounds, cutting incidents or surgical procedures. A 10 year study showed that 2.8% of 5,777 of trauma patients were diagnosed with PNI and of these patients, half required surgery (Noble et al., 1998). Surgical restoration of peripheral nerves can be difficult especially if end to end repair is
not an option. Sensory nerve autografts remain the most widely used surgical technique to produce a tension-free repair, despite the limitations. Many studies have indicated viable alternatives to autografts and there is an immense need for further exploration of these techniques in the clinic.

The mouse and rabbit tibial nerve studies showed that keratin hydrogel can perform as well as sural nerve autograft and indicated keratin’s potential biological activity in a cell culture system (Apel et al., 2008; Sierpinski et al., 2008; Hill et al., 2011). Biocompatibility studies showed that the keratin biomaterial degrades within 8 weeks of implantation (de Guzman et al., 2011). Based on these study outcomes, the overarching hypothesis for this work is that keratin hydrogel conduit filler enhances peripheral nerve regeneration through early cellular interactions in the injured PNS. A long-term study of functional recovery in a NHP median nerve study was employed to confirm the effectiveness of the keratin biomaterial hydrogel in a clinically relevant model. A rat sciatic nerve injury model was then utilized to identify the early cellular events that occur with the use of keratin hydrogel in the nerve conduit and a cell culture system was then used to further characterize the cellular interactions. The aim of the following work is to promote the clinical translation of an effective alternative to the current “gold standard” for nerve repair by examination of its mechanism of action.

References


CHAPTER 1

Title: A Human Hair Keratin Hydrogel Enhances Median Nerve Regeneration in Non-Human Primates: An Electrophysiological and Histological Study†

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First Author Contribution: Lauren A. Pace provided assistance in the animal surgeries and performed all electrophysiological procedures, functional testing, histology and histomorphometry, and serum ELISA

Note: This manuscript was submitted to Experimental Neurology and any stylistic variations result from the submission requirements of the journal

†This study is taken in part from a dissertation submitted to the Neuroscience Program, Wake Forest University Health Sciences, in partial fulfillment of the requirements for the degree of Doctor of Philosophy
Abstract: A human hair keratin biomaterial hydrogel was evaluated as a nerve conduit luminal filler following median nerve transection injury in 10 *Macaca fascicularis* non-human primates (NHP). A 1cm nerve gap was grafted with a NeuraGen® collagen conduit filled with either saline or keratin hydrogel and nerve regeneration was evaluated functionally and electrophysiologically for a period of 12 months. The keratin hydrogel-grafted nerves showed significant improvement in return of compound motor action potential (CMAP) latency, recovery of baseline nerve conduction velocity (NCV), and higher CMAP amplitude compared to the saline treated nerves. Histological evaluation was performed on retrieved median nerves and abductor pollicis brevis (APB) muscles at 12 months. Nerve histomorphometry showed significantly larger nerve area and higher axon density in the keratin group while axon diameter and myelin thickness were similar between the 2 groups. The keratin APB muscles showed less atrophy, significantly higher myofiber density, and higher percentage of neuromuscular junction (NMJ) innervation than the saline group. This is the first published study to show that an acellular biomaterial hydrogel nerve conduit filler can be used to enhance peripheral nerve regeneration and motor recovery in an NHP model.

Keywords: Peripheral nerve, Regeneration, Hydrogel, Conduit, Median nerve, Keratin biomaterials, Monkey

Introduction

Nerve injury of the upper extremity occurs most frequently in young males as a result of motor vehicle collision. These injuries can result in permanent disability and diminished quality of life (Noble et al., 1998; Lundborg and Rosen, 2007; Novak et al., 2011).
Techniques for surgical management of peripheral nerve transection injuries vary depending on injury severity, although primary end-to-end neurorrhaphy is the preferred treatment. However, if primary repair cannot be performed due to severe local tissue trauma or retraction of the distal or proximal nerve stumps, a graft may be interposed between the two nerve ends in order to attain a tension-free repair (Campbell, 2008). Autologous nerve grafts, most commonly harvested from the sural nerve, have long been considered the “gold standard” for peripheral nerve repair, although they require extensive microsurgical skills, are technically challenging, and result in increased surgical time and donor site morbidity (Schultz et al., 1992).

Alternatives to autograft are currently under investigation in both preclinical studies and clinical trials (Dahlin, 2008; Deal et al., 2012). Hollow tubes, referred to as nerve guides or nerve conduits, have been investigated as an alternative to autograft since 1982 when Lundborg et al. used a silicone tube to bridge a sciatic nerve defect in rats (Lundborg et al., 1982). Because silicone tubes are non-resorbable, later research focused on the development of bioabsorbable conduits, several of which are now available for clinical use. There are conduits constructed of collagen type I (NeuraGen®, Neuroflex™, and NeuroMatrix™), polycaprolactone (Neurolac®), and polyglycolic acid (Neurotube®). The advantage of nerve conduits is their relative ease of surgical placement and elimination of donor site morbidity from autograft harvest. Each conduit type has been extensively studied in animal models (Waitayawinyu et al., 2007; Meek and Coert, 2008; Alluin et al., 2009; Deal et al., 2012). However, there is a paucity of clinical studies showing the effectiveness of nerve conduits. Based on previous reports of primarily small case series, nerve conduits are currently recommended for repair of small gap lengths (≤
(Taras et al., 2005; Schlosshauer et al., 2006; Meek and Coert, 2008; Lohmeyer et al., 2009; Taras et al., 2011; Deal et al., 2012). There are only a few clinical studies examining the effectiveness of bioabsorbable conduits in large mixed motor and sensory nerve gaps and currently, autologous nerve graft or allograft repair are recommended to achieve functional recovery (Taras et al., 2005; Meek and Coert, 2008; Mackinnon, 2011; Deal et al., 2012).

The effectiveness of nerve conduits for repair of mixed motor or sensory nerves has been assessed in an NHP model. Collagen conduits revealed similar functional recovery compared to sural nerve autografts for median nerve injuries in gap sizes ranging from 5 to 50 mm (Archibald et al., 1991; Archibald et al., 1995; Krarup et al., 2002). In order for bioabsorbable conduits to become an alternative treatment method in small and large gaps in mixed motor and sensory nerves, peripheral nerve regeneration through bioabsorbable conduits needs to be improved (Apel et al., 2008; Dodla and Bellamkonda, 2008; Sierpinski et al., 2008).

Prior to nerve repair with a conduit, resection of the injured nerve tissue is performed (Matsuyama et al., 2000). Inside the conduit, the nerve ends exude a fluid which forms a fibrin matrix providing a native scaffold for migrating cells such as Schwann cells (SC) (Chen et al., 2006). Based upon this phenomenon, luminal fillers consisting of extracellular matrix proteins, syngeneic, allogeneic or autologous SC, growth factors and several combinations of the previously listed components have been used in experimental studies in combination with nerve conduits to enhance nerve regeneration. In preclinical experiments, these fillers have shown modest to significant improvement of functional recovery in several animal models (Jenq et al., 1987; Heath and Rutkowski, 1998;
Mosahebi et al., 2002; Mosahebi et al., 2003; Chen et al., 2006). However, relatively few of these studies have progressed down a translational pathway toward clinical trial. In order to present a viable option to autograft, conduit fillers must show improvement over saline-filled conduits in clinically-relevant large animal models.

The present study uses a novel filler material, keratin biomaterial hydrogel, which has been shown in previous studies in mice and rabbits to be more effective or equivalent to sensory nerve autograft (Apel et al., 2008; Sierpinski et al., 2008; Hill et al., 2011). Keratin biomaterial has the distinct advantage of being resistant to proteolytic degradation (Kaluzewska et al., 1991). Therefore, keratin biomaterial persists in the nerve conduit at the repair site and its degradation can be better controlled than other natural protein filler materials. Moreover, keratins have been shown to be more biocompatible than synthetic materials (de Guzman et al., 2011). Importantly, keratin is a structural biomolecule that provides support and sites of attachment for infiltrating cells without interfering with downstream nerve regenerative processes (Sierpinski et al., 2008). The purpose of this study was to investigate peripheral nerve regeneration using a keratin biomaterial hydrogel as a luminal filler inside a bioabsorbable conduit in an NHP nerve injury model. We hypothesized that keratin biomaterial as a conduit luminal filler enhances nerve regeneration leading to improved functional, electrophysiological, and histological outcomes compared to saline as standard filler. This study is the first to test the potential of a keratin biomaterial hydrogel conduit filler in a clinically-relevant model using NHP.
Materials and Methods

Non-human primate injury model

Unilateral (n=4) and bilateral (n=5) median nerve transections and repairs were performed in ten female Macaca fascicularis monkeys. The study was approved by the Wake Forest University Animal Care and Use Committee and conducted according to the NIH Guide for the Care and Use of Laboratory Animals. The animals were sedated with ketamine (15 mg/kg) and acepromazine (0.05 mg/kg), intubated, and anesthesia was maintained with isoflurane (1.5 to 2.0 volume %). All procedures were performed under aseptic conditions. The median nerve was transected 5cm proximal to the middle wrist crease, and a 5 to 6mm portion of the nerve was removed. A 1cm X 2mm ID bovine collagen I nerve conduit (NeuraGen, Integra Life Sciences) was sutured to the proximal nerve end using a 9-0 nylon epineurial suture (Ethicon). In 8 cases, the conduit lumen was filled with a 15% weight/volume (w/v) keratin hydrogel and in 6 cases the conduit lumen was filled with sterile saline. The conduit was then interposed between the nerve ends creating a 10mm gap. The soft tissues overlying the nerve repair were approximated with 5-0 Vicryl suture (Ethicon). The subcuticular layers were approximated with 5-0 Vicryl (Ethicon) and a sub-dermal suturing technique employing 4-0 Vicryl (Ethicon) was used to close the skin incision. A thin film of tissue adhesive (Vetbond, 3M) was placed along the incision line. Buprenorphine (0.01 mg/kg) was administered prior to extubation and every 8 hours for 24 hours for analgesia. The animals were allowed to recover from anesthesia under a heat lamp. All animals were monitored by the veterinary staff on a daily basis and did not display distress or any secondary complications from the surgeries such as self-mutilation or infection throughout the study period.
Preparation of Keratin Hydrogel

Keratin hydrogels were prepared as described previously (Sierpinski et al., 2008; de Guzman et al., 2011). Briefly, human hair was oxidized with a 2% peracetic acid solution and rinsed with water to remove any residual oxidant. Soluble keratins were extracted into tris(hydroxymethyl)aminomethane (Tris) base and deionized water. The extracted solution was then, dialyzed, neutralized, lyophilized, and ground into a fine powder. The lyophilized keratin was sterilized via \( \gamma \)-irradiation at a dose of 10 kGy and aseptically reconstituted in phosphate buffered saline to form a 15% (w/v) hydrogel.

Electrophysiological Procedures

Baseline electrophysiological measurements were obtained prior to surgery and assessments were performed at 4 week intervals up to a period of 24 weeks and then at 12 week intervals up to the final assessment at 52 weeks using a SierraWave electrodiagnostic system (Cadwell Laboratories). Prior to electrophysiological evaluation, all animals were sedated with ketamine (15 mg/kg) and acepromazine (0.05 mg/kg) and deep general anesthesia was maintained with isoflurane (1.5 to 2.0 volume %).

Motor Nerve Conduction Studies

The compound motor action potential (CMAP) was recorded from the abductor pollicis brevis (APB) muscle using either a 6mm tin surface electrode (Rochester Electro-Medical, Inc.) placed 2mm distal to the muscle insertion or a 0.40mm subdermal needle electrode (Cadwell Laboratories) placed within the muscle. The APB is solely innervated by the recurrent branch of the median nerve and commonly affected by median nerve injury. The supramaximal stimulating voltage was determined by gradually increasing the stimulating
current on an uninjured median nerve until there was no increase in CMAP amplitude. This stimulating voltage was then used for all conduction studies. The median nerve was electrically stimulated with a handheld electrical stimulator (Stim Troller™, Cadwell Laboratories) using straight, round-head stimulator probes (Cadwell Laboratories) placed 10 mm apart. CMAP latency was recorded from 2 stimulation electrode distances, the proximal site being proximal to the lesion, with a single 1msec pulse of 20mA and used to calculate the nerve conduction velocity (NCV) by the formula:

\[
NCV = \frac{\text{distance}_2 - \text{distance}_1}{\text{latency}_2 - \text{latency}_1} \text{(in mm)} \quad \text{(Mallik and Weir, 2005)}
\]

**Functional Testing**

To assess functional recovery following denervation of the APB muscle, the animals were trained on a grasping task with a pegboard fitted with wooden dowels at dimensions forcing them to use their fingertips to retrieve small food items (e.g. raisins and nuts) (Spinozzi et al., 2004) with the lateral aspect of the thumb and the index finger prior to surgery. After training and at 8 week intervals after surgery, the animals performed the same task and were recorded with a high definition video camera (HDR-SR7, Sony). The videos were converted to MPEG format and analyzed at still frame replay to visualize the return of thumb abduction function.

**Nerve Histology**

Following a terminal electrophysiological assessment, the nerves were harvested with a 4-0 Vicryl (Ethicon) suture placed into the proximal end for orientation, fixed in 10% neutral buffered formalin for 7 days at 4°C and rinsed in PBS. Segments from both mid-conduit
and 1mm distal to the distal graft suture were post-fixed in 1% osmium tetroxide, dehydrated in increasing concentrations of ethanol and embedded using epoxy resin. Semi-thin sections (0.5µm) were cut using an LKB III Ultramicrotome (LKB Instruments), mounted on slides, and stained with 1% toludine blue.

*Nerve Morphometry*

Photomicrographs were obtained at 25x, 200x, and 1000x magnification using a Zeiss microscope and 630x using a Leica microscope. The 630x and 1000x magnification images were obtained with oil immersion lenses (63x and 100x objectives with numerical apertures of 1.40 and 1.25, respectively). The circumference of the nerves was traced on the 25x images and the total nerve area quantified using ImageJ software (NIH). Axon density and diameter were calculated by automated analysis of 4-5 randomly sampled 200x images using ImagePro 6.3 software (Media Cybernetics Inc). Total axons per nerve was quantified by multiplying the axon density by the nerve area. Myelin thickness was calculated by manual quantification of a minimum of 150 randomly selected axons from 3-5 1000x images using ImagePro 6.3 software. G-ratio, which is the ratio between axon diameter and myelinated fiber diameter, was quantified by manual measurement of 30-50 randomly selected axons from 630x or 1000x images using ImageJ software.

*Muscle Histology*

The APB and remaining thenar eminence muscles were harvested 12 months after surgery following terminal electrophysiology measurements, fresh-frozen in liquid nitrogen, and stored at -80°C. Prior to analysis, the APB muscles were placed into O.C.T. compound (Tissue-Tek, Sakura Finetek) and both 25 and 10 µm cryosections were collected with a
cryostat (CM1950, Leica Microsystems). The 25 µm sections were used to examine neuromuscular junctions (NMJs). The tissue was fixed with 4% paraformaldehyde (PFA), blocked in 5% serum, and immunolabeled with primary antibodies for neurofilament H and L (1:200, Cell Signaling Technology) and vesicular acetylcholine transporter (VAChT) (1:250, Santa Cruz Biotechnologies), FITC-conjugated (anti-mouse, 1:100, Vector Labs) and Alexa Fluor 488 secondary (anti-goat, 1:1000, Invitrogen, Carlsbad, CA) antibodies were used to visualize pre-synaptic structures. Acetylcholine receptors (AChR) were visualized with Alexa Fluor 594-conjugated alpha bungarotoxin (1:500, Life Technologies) (Figure 6E). The 10 µm sections were used to examine cross-sectional fiber area and fiber density (Figure 6C). The tissue was fixed in Bouin’s fixative (Polysciences Inc.) and stained with a Masson’s trichrome staining protocol for collagen fibers.

_Muscle Morphometry_

Photomicrographs of the 10 µm sections were obtained at 200x magnification with a Zeiss microscope. Myofiber cross-sectional area (CSA) and fiber density was calculated by automated analysis using Image Pro 6.3 software. Percent atrophy of the keratin and saline treatment group muscles was calculated using the percent difference between the mean CSA of the uninjured controls and mean CSA of each group. The 25 µm sections were analyzed by a Zeiss LSM 510 confocal microscope. Z stacks showing clusters of labeled AChR were obtained and analyzed with LSM Image Browser software (Carl Zeiss). The presence or absence of labeled axon terminals and VAChT in each motor unit was quantified manually in three Z stacks per muscle and expressed as a percentage of the total AChR.
Antibody Titer

Following the final electrophysiology procedure, 4 of the animals that received keratin hydrogel underwent a bolus injection procedure with sterile keratin solution (0.4mg/kg in a 4 w/v % solution) into the antebrachial vein 12 months after the nerve transection and repair procedure to test the immunogenic potential of the keratin hydrogel. The animals were monitored for signs of anaphylaxis for a period of 1 hour following the injection and were monitored by the veterinary staff on a daily basis for 7 days. Blood samples were then collected in serum-separating tubes (BD Vacutainer) and allowed to stand at 20ºC for 30 minutes before centrifugation to separate the serum. Serum samples from 2 animals that were naïve to keratin were also collected and used as negative controls. The serum protein concentration was quantified with a DC protein assay (Biorad Laboratories). An antigen-down ELISA was developed by coating a microtiter plate with the keratin protein used in the bolus injection procedure as the antigen at 20ºC overnight, using the collected serum as the primary antibody, an HRP-goat anti-human IgG (1:1000, Life Technologies) as secondary antibody and TMB substrate (Thermo Scientific) for detection. The detected signal was compared to a standard curve using an anti-basic hair keratin K81 antibody (Progen Biotechnik). Samples were analyzed at 2 concentrations of 0.5 μg/mL and 1.0 μg/mL and all concentrations were assayed in triplicate.

Statistical Analysis

All data are presented as mean ± standard deviation. For the electrophysiology experiments, a repeated measures ANOVA was performed followed by Bonferroni post hoc tests using GraphPad Prism 4 software (GraphPad). For the ELISA, a two-way
ANOVA with Bonferroni post-hoc tests was performed and for all remaining analyses, a one way ANOVA was performed with Bonferroni post-test using GraphPad 4.0. In all tests, $P < 0.05$ was considered statistically significant.

Results

Motor Conduction Studies

At 16 weeks following surgery, none of the animals showed a detectable return of APB CMAP latency, which was defined as a 100% change from baseline values defined as 0%. At 20 weeks, the keratin group showed a significantly greater percent change in CMAP latency (60.2±25.4%) compared to the saline group with values of (95.3±11.4%, $P < 0.01$). The percent difference in CMAP latency remained lower for the keratin group compared to the saline group until 36 weeks after surgery. At 44 weeks, the CMAP latency was similar in both groups (keratin 39.1±23.6% vs. saline 45.4±9.2% Figure 1A). The recovery of baseline NCV was significantly higher in the keratin group compared to the saline group at 52 weeks (78.5±17.4% and 50.3±20.3%, respectively; Figure 1B). The CMAP amplitude at 52 weeks was not significantly different between the keratin, saline, and uninjured control (16.6±4.0 mV) groups, although the keratin group had higher mean amplitude (11.7±5.3 mV) compared to the saline group (8.7±3.7 mV) (Figure 1C, $P = 0.051$).

Functional Testing

Due to the use of both unilateral and bilateral injuries in order to maintain an uninjured control group, quantifiable differences in the recovery of thumb abduction and thus pinch grasp between the keratin and saline groups by still frame replay of the videos were
difficult to obtain. All of the animals displayed visible recovery of thumb abduction between 20 and 24 weeks after surgery.

*Nerve Morphometry*

Analysis of mid-conduit nerve area showed a larger mean area for the keratin group (2.3±0.87 mm²) compared to the saline group (1.6±0.68 mm²) and native nerve (1.0±0.33 mm²) (Figure 2A). The distal nerve area was significantly larger for the keratin group (2.9±0.9 mm²) compared to the saline group (1.7±0.5 mm², *P* < 0.05, Figure 2B). The keratin nerves were significantly larger than native nerve for both tissue segments analyzed while the saline nerves showed no significant difference. Axon density of the middle and distal nerve segments showed a slightly higher average density in the keratin group (10110±1929 and 9054±1821 axons/mm²), compared to the saline group (6926±1991 and 7663±854 axons/mm²). Native median nerve axon density was also analyzed showing significantly higher values (18166±3897 axons/mm²) compared to the keratin and saline middle and distal values (*P* < 0.0001, Figure 2C&D). The keratin nerves had significantly higher total axons per nerve in the middle and distal tissue segments (22469±5822 and 28134±12409 axons) than both saline (11700±8474 and 12525±2488 axons) and native nerves (9389±2324 axons, *P* < 0.01, Figure 2E&F). Photomicrographs taken of the nerve sections at 630x magnification allowed visualization of decreased axon density, smaller axon diameter, and differences in myelination between the native (Figure 3A), keratin (Figure 3B), and saline nerves (Figure 3C). Axon diameter was analyzed by expressing the obtained values as a histogram, demonstrating the percentage of population at each diameter in µm. The native nerve had a bimodal distribution with peaks at 1 µm and 9 µm representing two distinct axon distributions corresponding to the large motor axons and the
smaller sensory axons, which reflects the mixed function of the median nerve (Figure 4C, F&G). Conversely, the keratin and saline groups had a unimodal distribution for both the middle and distal tissue with both groups showing a peak at 2 µm (Figure 4 A&B, D-G). The myelin thickness of the native nerve also displayed a bimodal distribution with peaks at 0.5 and 1.7 µm (Figure 5C, G&H). Again, the keratin and saline groups had a unimodal distribution for myelin thickness at both middle and distal tissue segments. In the middle nerve segments, the keratin group had a peak at 0.6 µm while the saline group had a slightly higher peak at 0.9 µm (Figure 5A, B&G). In the distal tissue, the keratin nerves showed a peak between 0.4 and 0.7 µm and the saline group showed a slightly higher peak at 0.9 µm (Figure 5D, E&H). Quantification of g-ratio showed no significant differences between the keratin (0.468) and saline (0.435) treatment groups and native nerve controls (0.426, \( P = 0.477 \), Figure 5F).

**Muscle Morphometry**

Analysis of the APB muscle myofiber CSA showed a larger average myofiber size for the uninjured (7676 ± 2045 µm²) and keratin (6776 ± 1973 µm²) treatment groups compared to the saline group (4878±1049 µm², \( P = 0.081 \), Figure 6A). The keratin muscles showed 12.2% atrophy compared to 36.4% atrophy in the saline muscles after a period of 12 months. The myofiber density was significantly higher in the uninjured controls (565.4±122.2 fibers/mm²) and keratin group (542.5±175.9 fibers/mm²) compared to the saline group (220.2±38.1 fibers/mm², \( P = 0.0021 \), Figure 6B). The average percentage of innervated motor end plates was identical between keratin (100%) and control muscles (100%) while the saline group showed a slightly lower value (81.1%, \( P = 0.1396 \), Figure 6D).
Antibody Titer

The ELISA revealed that none of the animals receiving a bolus injection of keratin had a detectable difference in antigen binding activity of the serum compared to the control animals that were keratin naïve. The highest detected value of 5019±2702 ng/mL corresponded to one of the negative controls. Human albumin, which was used as a control to examine the background signal, had the highest signal of all samples, suggesting the probability of non-specific binding of serum albumin to keratin proteins (Figure 6F). The human albumin samples had significantly higher binding than all other samples at both concentrations analyzed ($P < 0.001$). No adverse reaction to the keratin bolus injection was noted.

Discussion

This study hypothesized that the use of keratin biomaterial hydrogel as luminal filler in nerve conduits improves functional, electrophysiological, and histological outcomes following median nerve repair in NHP. The electrophysiological and histological studies showed an earlier onset of regeneration and enhanced outcomes for the keratin nerves compared to the nerves repaired with a saline-filled conduit. It is important to note that the saline-filled conduits were not expected to remain as such. As described by Lundborg (Lundborg, 2005), nerve conduits quickly become filled with exudate from the severed nerve stumps and a fibrin matrix forms to act as a natural scaffold for regeneration. In choosing a 1cm gap for this study, it was considered that this size was small enough that a fibrin matrix would form across the entire length of the defect, but that the proximal and distal stumps would not be so close as to present little challenge to the regenerative process.
Moreover, since keratin biomaterials resist proteolytic degradation (mammals do not produce keratinases) (Kaluzewska et al., 1991) and the natural fibrin matrix does not, a shorter gap ensured that premature “liquefaction” in the saline-filled test group would not give a biased advantage to the keratin-filled test group. Also, a control group using sensory nerve autograft was considered, but this is not a common clinical repair strategy at the 1cm gap distance and would likely not be considered by a skilled surgeon if better nerve conduit filler technologies were commercially available.

The high definition videos taken of the grasping task gave an excellent qualitative assessment of functional recovery in the animals. The differences in CMAP latency and NCV between the keratin and saline nerves were difficult to detect in the videos as 1 cohort underwent unilateral injury and repair while the remaining animals underwent bilateral injury and repair. The unilateral animals preferred their uninjured hand throughout the study and an increase in injured hand use was highly variable. In general, the loss of thumb abduction and subsequent recovery over time was visible for all animals.

Motor conduction studies revealed that the onset of measurable electrophysiological function and reduction of CMAP latency occurred sooner in the nerves that received the keratin hydrogel conduit filler. This suggests that the axons in the keratin nerve group re-innervated their targets in the APB muscle more quickly than those in the saline nerve group. In a previous study, Archibald et al. found that there were no significant differences in reinnervation time of the APB muscle between sural nerve autograft and a saline-filled collagen nerve guide in a 5mm gap of the median nerve in NHP. In the same study, the CMAP latency was used to quantify the fastest motor NCV across the injury site and the results did not show significant differences in NCV at 300 days in a 5mm gap treated with
either a saline-filled conduit or sural nerve autograft (Archibald et al., 1995). In contrast, the present study showed that the keratin treated group maintained higher NCV values compared to the saline treatment group. Another NHP study by Krarup et al. showed no significant differences in APB CMAP amplitude between direct suture of the median nerve, a 5mm nerve guide and a 5 and 20mm sural nerve autograft after a period of 882 days. However, longer nerve guides (e.g. 20 and 50mm) and autografts (50 mm) had significantly lower APB CMAP amplitude compared to direct suture (Krarup et al., 2002). This would suggest an upper limit of the effectiveness of nerve guides in comparison to autograft over the same gap length. The present study showed no significant differences in CMAP amplitude between uninjured, keratin, and saline conduit-grafted nerves after 365 days although there was a trend toward higher CMAP amplitude in the keratin group that may have been more pronounced with a larger number of animals. Taken together, these data strongly suggest that return to near-normal NCV can be achieved by the addition of keratin hydrogel filler for nerve conduit repairs.

Histological examination revealed that nerves repaired with keratin-filled conduits were larger than the saline treated nerves in both tissue segments analyzed. However, a small area with extremely high axon density observed in native nerves was not observed in the keratin or saline groups. This trend was previously seen in both mouse (Apel et al., 2008; Sierpinski et al., 2008) and rabbit (Hill et al., 2011) tibial nerve studies utilizing the keratin hydrogel as luminal filler. Both of these studies included a sural nerve autograft group and showed the keratin group had significantly larger nerves compared to autograft in the mouse, and larger nerves compared to autograft in the rabbit. In the present study, the native median axon density was significantly higher than both keratin and saline nerve
groups. While the axon density in the keratin treated nerves was greater than the saline treated nerves at both middle and distal nerve segments, this difference was not statistically significant. Interestingly, the keratin nerve group had significantly more axons per nerve than both the saline group and native controls which showed no significant differences. The median nerve repairs in this study were performed to evaluate motor functional recovery and only the recurrent branch of the median nerve was included in the distal conduit. During regeneration, neurons can project axon collaterals which are later pruned once target specificity is achieved (Redett et al., 2005). The use of keratin hydrogel may induce more collateral sprouting or more sensory axons may have grown into motor Schwann cell tubes in the distal nerve tissue.

The frequency distribution of axon diameter showed 2 distinct peaks in the native tissue compared to 1 peak in both the keratin and saline treated nerve groups. These distributions appeared almost identical between the treatment groups with the keratin group having slightly more small diameter axons than the saline group in both the middle and distal sections. There was also a similar shift to slightly larger diameter axons in the distal nerve tissue for both groups, although the distributions remain unimodal. Archibald et al. found similar axon diameter distribution patterns for sural nerve autograft and saline conduit repairs of a 5 mm median nerve gap approximately 45 months after surgery (Archibald et al., 1995). The myelin thickness frequency distribution was also bimodal for the native median nerve compared to unimodal for the keratin and saline groups in both middle and distal segments. Interestingly, the saline group’s distribution peaks were shifted a few tenths of a micron toward a larger size. The slightly larger myelin thickness for the saline group compared to the keratin group may reflect compensation for smaller nerve areas and
lower axon densities, although there were no significant differences in average g-ratio between the saline, keratin and native groups. Larger nerves with higher axon densities will yield a higher NCV as was seen in the motor conduction studies and may help to explain the electrophysiological enhancement seen with the keratin hydrogel compared to the saline conduits, even though the groups had similar axon diameter and myelin thickness.

The earlier return of CMAP latency in the keratin group would be expected to correspond to faster reinnervation of motor targets in the APB muscle. This difference in time to target innervation appeared to manifest in a difference in atrophy between the keratin and saline groups. The effect of muscle atrophy on functional recovery has been well-established with agreement that irreversible atrophy of a denervated muscle occurs sometime between 12 and 18 months following injury (Lee and Wolfe, 2000; Campbell, 2008). Some studies suggest that chronic denervation of distal Schwann cells and not chronic denervation of muscle targets is the main factor contributing to poor functional recovery following nerve injury in a rat model (Kawabuchi et al., 2001; Sulaiman and Gordon, 2009; Gordon et al., 2011). It has been shown that motor nerve regeneration results in enlarged motor unit size with a correlation between the axotomy duration and the ability of regenerating axons to reinnervate muscle fibers (Fu and Gordon, 1995). The quantification of innervated NMJ showed a higher percentage in the keratin group compared to the saline group, although the difference was not significant ($P = 0.14$). Because the APB muscles were used to calculate both myofiber CSA and to label NMJ, measurement of motor unit size was not conducted in this study because AChR morphology was not easily visible.

The keratin biomaterial used in this study is derived from human hair. Extensive toxicity testing of this material has been performed in vitro and in vivo in order to obtain Food and
Drug Administration approval of this material for clinical use. Moreover, experiments have shown that many different cell types remain viable with large concentrations (4.16mg/mL) of the material dissolved in basal media. Biocompatibility studies of subcutaneous implants were also performed in mice showing no adverse effects of the material and degradation within 8 weeks (de Guzman et al., 2011). Despite these promising observations, the potential immunogenicity of keratin biomaterials has not been directly tested and therefore remains an open issue for clinical translation. To determine keratin’s potential immunogenicity in NHP, a keratin bolus injection was performed 12 months following the implantation of a keratin-hydrogel filled conduit in 4 of the study animals. The results of the ELISA performed on the serum of these animals one week after challenge suggests that there is no measurable, adaptive immune response to these processed human keratin proteins.

The working mechanism by which nerve regeneration is promoted by the keratin hydrogel used in this study is still unknown. Beyond the obvious role of keratin as a physical support that resists a proteolytic environment better than a native fibrin matrix, a hypothesis has been postulated that the interaction between the keratin proteins and Schwann cells drives the enhanced nerve regeneration seen in this study and the 3 previously mentioned animal studies (Apel et al., 2008; Sierpinski et al., 2008; Hill et al., 2011). Schwann cells serve many roles in peripheral nerve regeneration such as secretion of signaling molecules that initiates the immune response immediately after traumatic nerve injury, secretion of trophic factors to attract regenerating axons, and actin as physical scaffolds to support axon regrowth (Ide, 1996; Mirsky and Jessen, 1999; Rotshenker, 2011). Previous studies have suggested that keratin proteins are compatible with cells in culture and may induce cell
proliferation, cell adhesion, and alter gene expression (Sierpinski et al., 2008). However, these studies were performed using an immortalized Schwann cell line and need to be repeated with primary Schwann cell explants before definitive conclusions regarding a potential mechanism can be proffered. Any cell-level changes, if present in primary cells, need to be assessed and compared to the relative contribution of structural support to the overall regenerative process.

Peripheral nerve regeneration is a complex set of temporal cellular and molecular events and, if successful, can result in varying levels of functional recovery. There are very few follow-up studies of patients that have undergone conduit graft surgery in large mixed nerves such as the ulnar and median nerve. Lundborg et al. published a 5 year follow-up of 30 patients that underwent either direct suture repair of the median or ulnar nerve or graft with a silicone tube. There were no significant differences in clinical and neurophysiological outcomes between the 2 groups and all patients showed continued improvement throughout the study (Lundborg et al., 2004). Silicone is a bio-inert material that is no longer commonly used in microsurgical repair of peripheral nerves. There are currently several types of bioabsorbable nerve conduits available for clinical use, yet these are only considered effective for repair of small sensory nerves such as the digital nerve (Lohmeyer et al., 2009; Taras et al., 2011; Deal et al., 2012). Preclinical studies have often shown the comparability between autograft repair and conduit repair, albeit for small gaps. There is a need to further test luminal fillers or modified conduits in larger nerve gaps (≥ 5cm) to determine if the two techniques have similar functional outcomes.
Conclusions

This study showed the effectiveness of a keratin biomaterial hydrogel nerve conduit filler for median nerve repair in NHP. The goal of this work was to confirm our earlier findings in studies using rodents and rabbits by employing a more clinically relevant model. The NHP model used in this study is considered relevant because it represents a comparison between the native matrix, fibrin, which quickly replaces the saline used to fill the conduit at the time of surgery, and a biocompatible protein matrix derived from a readily available source, keratin. While the 1cm defect is not a critical size gap for a nerve conduit product (i.e. one that will not heal under normal conditions), it represents an equally challenging test system for both experimental groups, and one in which the fibrin matrix would be expected to do well despite its susceptibility to the proteolytic milieu of injured tissue. Given that the keratin appeared to perform better, this technology may in the future provide surgeons with an off-the-shelf, less technically-challenging alternative to autograft surgery and encourage the practice of conduit repair for gaps up to 3cm, the current limit of commercially available devices.

Acknowledgments

The authors would like to gratefully acknowledge Cindy Andrews, Tammy Cockerham, and Renae Hall in the surgery core at the Wake Forest Institute for Regenerative Medicine, Ken Grant and Paula Moore in the Pathology Department, and Michael Cartwright, M.D. in the Department of Neurology at Wake Forest School of Medicine.

Funding was provided by KeraNetics LLC and the Armed Forces Institute for Regenerative Medicine (DoD Contract # W81XWH-08-2-0032)
Conflict of Interest

Authors Mark Van Dyke and L. Andrew Koman hold stock and are officers in the company, KeraNetics LLC, who provided partial funding for this research. Wake Forest School of Medicine has a potential financial interest in KeraNetics through licensing agreements.

References


Figure 1. Keratin hydrogel enhances recovery of compound motor action potential (CMAP) latency, nerve conduction velocity (NCV), and CMAP amplitude. A) Percent change of CMAP latency is significantly greater for the keratin group over the saline group at 20 weeks. The values converge at 36 weeks and remain similar until the end of study. B) The recovery of NCV is occurs more quickly in the keratin group, remains higher throughout the study and becomes significant at 52 weeks. C) The abductor pollicis brevis CMAP amplitude is greater for the keratin group over the saline group at 52 weeks ($P = 0.051$). The results are expressed as mean ± s.d. (n=8 keratin, n=6 saline, n=4 native controls). **$P < 0.01$. 
Figure 2. Keratin hydrogel results in increased regenerated nerve area, higher axon density and total axons. Regenerated keratin nerves are significantly larger than uninjured native nerves (A & B) for the middle nerve segments and significantly larger than both saline and native nerves in the distal segments (B). Both keratin and saline axon density is significantly lower than uninjured values (C & D). Keratin nerves contain significantly more total axons per nerve than saline and native nerves in both middle and distal tissue (E & F). Results are displayed as mean ± s.d. (n=8 keratin, n=6 saline, n=4 native controls). *** P < 0.001, ** P < 0.01, * P < 0.05.
Figure 3. Toluidine blue staining of 0.5 μm semi-thin sections shows differences in axon density, axon size, and myelin between native and regenerated median nerves. 

A) A photomicrograph of a native median nerve shows both small and large diameter axons and high axon density. Regenerated keratin (B) and saline (C) nerve sections taken from the tissue immediately distal to the conduit have smaller axon diameter and lower axon density. Scale bar = 20 μm.
Figure 4. Keratin and saline regenerated nerves have unimodal distributions of axon diameter. Keratin and saline groups show nearly identical unimodal distributions of axon diameter in both middle (A & B) and distal (D & E) nerve segments compared to the bimodal distribution of the native median nerve (C). F & G) An overlay of the distributions from A-E. Histograms were derived from no less than n=1500 axons per animal per group (n=8 keratin, n=6 saline, n=4 native).
Figure 5. Regenerated median nerves have a unimodal distribution of myelin thickness although average fiber g-ratio is equivalent to native nerve. The keratin group shows a unimodal distribution of myelin thickness at both middle and distal segments (A & D) while the saline group shows its values approaching a bimodal distribution at these segments (B & E). C) Uninjured median nerve has 2 distinct peaks corresponding to small and large diameter axons. F) G-ratio between the axon diameter and myelinated fiber diameter show no significant differences between groups (P = 0.477). G & H) Graphs overlaying the distributions for the middle (A-C) and distal (C-E) median nerve segments with uninjured median nerve. Histograms represent values from at least 200 randomly selected axons per animal (n=8 keratin, n=6 saline, n=4 native nerve).
Figure 6. Keratin enhances abductor pollicis brevis (APB) muscle regeneration and is non-immunogenic. A) Regenerated keratin APB muscles show a trend toward larger average myofiber cross-sectional area than saline muscles ($P = 0.08$). B) APB myofiber density is significantly higher for the keratin muscles compared to saline muscles. C) Cross-sectional photomicrograph of a regenerated APB muscle stained with Masson’s trichrome. Scale bar = 50 μm. D) The percentage of neuromuscular junctions (NMJ) with both pre- and post-synaptic elements is not significantly different between the groups. E) A confocal image of a single APB NMJ showing positive staining for alpha-bungarotoxin (red) and neurofilament H and vesicular acetylcholine transporter (green). Scale bar = 10 μm. F) An ELISA of NHP serum shows no difference in antigen-binding activity between animals that received a keratin bolus and animals naïve to keratin. Human serum albumin as positive control and shows high levels of non-specific binding ($P < 0.01$ for 0.5 μg and 0.001 for 1.0 μg concentration). Results are expressed as mean ± s.d. (n=8 keratin, n=6 saline, n=4 controls, for the ELISA n=3 replicates per sample).
The use of a human hair keratin (HHK) biomaterial hydrogel conduit filler showed significantly better electrophysiological and histological outcome measures compared to saline (SAL) in the non-human primate (NHP) median nerve study. The goal of the NHP study was to evaluate HHK in a long-term study of functional recovery in a clinically relevant animal. The ultimate goal of the HHK nerve project is to promote the clinical translation of an alternative to sural nerve autograft, the current “gold standard” for peripheral nerve injury treatment. The next step in the project was to investigate the HHK biomaterial’s biological activity and mechanism of action. The hypothesis for the following work is that HHK is acting on both inflammatory and regenerative cells in the injured peripheral nervous system to bring about enhanced peripheral nerve regeneration. The following study examines the early cellular response to sciatic nerve injury in rats and compares HHK hydrogel conduit filler to SAL and a well-characterized hydrogel consisting of extracellular matrix components, Matrigel. Following the histological examination of the injured sciatic nerve tissue in the rat study, the cellular interactions were characterized in a cell culture system.
CHAPTER 2

Title: A Human Hair Keratin Hydrogel Promotes Wallerian Degeneration in a Rat Sciatic Nerve Injury Model and Alters Cytokine Signaling in Cultured Schwann Cells†

Abbreviated Title: Keratin Hydrogel Promotes Wallerian Degeneration

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First Author Contribution: Lauren A. Pace provided assistance with the animal surgeries and performed all tissue harvest, histology and histomorphometry, and cell studies

Note: This manuscript is in final preparation for submission to the Journal of Neuroscience

†This study is taken in part from a dissertation submitted to the Neuroscience Program, Wake Forest University Health Sciences, in partial fulfillment of the requirements for the degree of Doctor of Philosophy
Abstract

Peripheral nerve injuries requiring surgery can be repaired by autograft, the clinical “gold standard”, allograft, or nerve conduits. Nerve conduits are often associated with a simplified surgical procedure, have been shown to provide satisfactory outcomes at short gap distances, and accordingly have gained acceptance. Most published clinical studies show the effectiveness of nerve conduits in small size defects in sensory nerves. Many preclinical studies suggest that peripheral nerve regeneration through conduits can be enhanced and defect lengths increased with the use of a biomaterial filler in the conduit lumen. We have previously shown that a luminal hydrogel filler derived from human hair keratin (KOS) can improve electrophysiological and histological outcomes in mouse, rabbit, and non-human primate nerve injury models, but insight into potential mechanisms has been lacking. Based on the premise that KOS provides an instantaneous structural matrix within the lumen, the current study compares the cellular behavior elicited by KOS hydrogel to Matrigel (MAT) and saline (SAL) conduit fillers in a rat sciatic nerve injury model, and investigates the early stages of inflammation in a simplified cell culture model. Conduit repairs of a 1cm defect were performed and the repair site including the conduit and proximal and distal stump tissue were harvested and analyzed at early stages of regeneration. In the proximal conduit, the KOS group showed earlier migration of dedifferentiated Schwann cells (SC) from the proximal nerve end compared to the other groups. The KOS group also showed faster SC de-differentiation, myelin debris clearance, and macrophage infiltration in the distal nerve tissue. In the cell studies, KOS proteins altered SC cytokine release profiles in response to necrotic nerve (NN) tissue and this effect was inhibitory for the migration of THP1 monocytes. Together these data suggest that KOS hydrogel may promote more rapid Wallerian degeneration in
the distal nerve tissue following nerve transection and this effect may be due to altered SC cytokine signaling.

Introduction

Unlike the central nervous system, the peripheral nervous system (PNS) has the ability to self-repair. Peripheral nerve injuries range in severity from compression injuries, which are capable of spontaneous regeneration, to loss of continuity of the nerve trunk which requires surgical intervention for functional restoration (Sunderland, 1978; Campbell, 2008). If a tensionless primary nerve repair cannot be performed, the current recommended treatment is engraftment of the patient’s own nerve tissue, most commonly the sural nerve, to bridge the defect (Dahlin, 2008). Sural nerve autograft is considered the clinical “gold standard” for repair of peripheral nerve defects although this treatment has several limitations: tissue availability, donor site morbidity, and the need for an additional surgery (Lee and Wolfe, 2000; Schlosshauer et al., 2006).

In recent years, several alternatives to autograft have been approved by the US Food and Drug Administration (FDA) for repair of peripheral nerve defects, including acellular allografts (Brooks et al., 2011) and hollow tubes termed nerve conduits which are constructed of type I collagen, polyglycolic acid (PGA), or poly-DL-lactide-caprolactone (Schlosshauer et al., 2006). Preclinical studies in a rat sciatic nerve model have shown that type I collagen conduits are superior to PGA conduits and equivalent to autograft in functional recovery and histological outcomes (Waitayawinyu et al., 2007; Alluin et al., 2009). The majority of clinical data for these conduits (sold as NeuraGen®, Integra LifeSciences) is for repairs of the digital nerve, a small diameter sensory nerve
(Lohmeyer et al., 2009; Taras et al., 2011) and the continuing recommendation for use of nerve conduits is for small gaps (≤ 3 cm) in sensory nerves only (Meek and Coert, 2008; Mackinnon, 2011; Deal et al., 2012).

There are many preclinical studies showing that peripheral nerve regeneration following conduit repair can be improved (e.g. increased gap length, better functional outcomes) with the use of a filler material in the conduit lumen (Chen et al., 2006). We have shown that a novel nerve conduit filler based on a KOS biomaterial hydrogel is capable of electrophysiological and histological enhancement following tibial nerve repair in mice and rabbits (Apel et al., 2008; Sierpinski et al., 2008; Hill et al., 2011) and median nerve repair in non-human primates. Subcutaneous implant studies of the KOS hydrogel in mice revealed that the biomaterial provides a temporary matrix which is degraded by 8 weeks (de Guzman et al., 2011).

Based on our previous study data, we hypothesized that KOS would provide a permissive matrix that was well tolerated by the PNS cells and tissue, and that it would be instantly available for cellular infiltration without the need to build up the normal physiologic fibrin matrix derived from the proximal and distal stump exudate following nerve transection. To investigate these aspects, two models were used: 1) a rat sciatic model to examine early cell infiltration into the conduit lumen, as well as the behavior of SC in the distal stump, and 2) the potential role of inflammatory cells was studied in vitro using primary SC and THP-1 monocytes. We postulated that the presence of a hydrogel matrix immediately after injury would result in a greater influx of resident cells, and that the KOS hydrogel in particular would support a larger regenerative cell population. Moreover, we speculated that the presence of KOS would alter SC behavior as these are
the cells most responsible for regenerative processes at early stages after injury when the
KOS hydrogel would be expected to be intact.

Materials and Methods

Preparation of Keratin Powder and Hydrogel

KOS hydrogels were prepared as described previously (Sierpinski et al., 2008; de
Guzman et al., 2011). Briefly, human hair was oxidized with a 2% peracetic acid solution
and rinsed with water to remove any residual oxidant. Soluble keratins were extracted
into tris(hydroxymethyl)aminomethane (Tris) base and deionized water. The extracted
solution was then dialyzed, concentrated, neutralized, lyophilized, and ground into a fine
powder. The lyophilized keratin was sterilized via \(\gamma\)-irradiation at a dose of 25 kGy and
aseptically reconstituted in phosphate buffered saline (PBS) to form a 15% (w/v)
hydrogel.

Rat Sciatic Nerve Injury Model

Bilateral sciatic nerve transections and repairs were performed in 24 male
Sprague Dawley rats weighing approximately 250g (Harlan Laboratories). The study was
approved by the Wake Forest University Animal Care and Use Committee and conducted
according to the NIH Guide for the Care and Use of Laboratory Animals. The animals
were anesthetized with isoflurane (1.5 to 2.0 volume %) and all procedures were perfomed
under aseptic conditions. The sciatic nerve was transected at the level of the obturator
tendon and a 5 to 6mm portion of the nerve was removed. The proximal stump was placed
approximately 1mm inside one end of a 1cm X 2mm ID bovine collagen I nerve conduit
(NeuraGen, Integra Life Sciences), secured using a single 9-0 nylon microsuture (Ethicon)
and the lumen filled with either a 15% weight/volume (w/v) KOS hydrogel, sterile SAL, or MAT (BD Biosciences) (n=8 per group). The distal stump was similarly placed into the opposite end of the conduit so that contact was made between the tissue and filler material and sutured. Fascia was closed using 5-0 Vicryl suture (Ethicon) and skin was closed with Michel wound clips (Kent Scientific). Buprenorphine (0.01 mg/kg) was administered after closing for analgesia.

*Nerve Histology*

At 3 and 7 days following surgery, the animals were anesthetized with isoflurane (1.5 to 2.0 volume %), euthanized, and the conduit grafts were harvested including 2 to 3mm of proximal and distal nerve tissue. The nerve grafts were measured and transected at the graft midpoint with a scalpel blade. The distal and proximal sections were placed into O.C.T. compound (Tissue-Tek, Sakura Finetek), flash frozen in liquid nitrogen, and stored at -80°C. Prior to sectioning, the blocks were equilibrated to -20°C and 10µm transverse serial cryosections were collected with a cryostat (CM1950, Leica Microsystems) and post-fixed in acetone for 15 minutes at -20°C. The slides were then stored at -20°C until staining. Histology from within the conduit lumen was taken from the proximal 2.5mm of the conduit and is referred to as being from the conduit; histology performed on the stump tissue refers to tissue within the residual nerve stump, immediately adjacent to the gap but within the conduit opening.

*Immunohistochemistry and Immunofluorescence*

All stains were performed at room temperature (RT) using either a Dako Universal Slide Staining System or by hand using an optimized staining technique utilizing tris-
buffered saline/tween 20 (TBS/T) solutions with or without 0.3% Triton X-100 (Sigma Aldrich) depending on the cellular location of the antigen. The slides were rinsed with TBS/T, blocked in 5% serum corresponding to the species of the secondary antibody for 30 minutes, incubated in primary antibody for 2 hours, rinsed with TBS/T and incubated in secondary antibody for 1 hour. Primary antibodies to p75NTR (1:1600, Cell Signaling), NF-L (1:100, Cell Signaling), NF-H (1:200, Cell Signaling), GAP43 (1:100, Cell Signaling), and S100 (1:200, Sigma Aldrich) were used to label the tissue. Secondary antibodies including FITC-anti-mouse (rat adsorbed, 1:100, Vector Labs), Alexa Fluor 594 and 488 goat anti-rabbit (1:1000, Life Technologies) were used to visualize the antigens. A FITC-conjugated Lectin from *Lycopersicon esculentum* (tomato) solution (1:150, 1 hour, Sigma Aldrich) was used to label macrophages. Myelin was labeled with both Sudan Black B solution (5mg/ml in 70% ethanol, 2 hours, Sigma Aldrich) and with FluoroMyelin Red (1:300, 5 minutes, Invitrogen). All stains included a nuclear counterstain with DAPI (1:300, 5 minutes, Sigma Aldrich) and all slides were mounted with ProLong® Gold Antifade reagent (Life Technologies).

**Histomorphometry**

Monochromatic 200x and 630x fluorescent images were obtained for each stain using a Leica DM4000 upright microscope (Leica Microsystems) or a Zeiss LSM 510 confocal microscope (Carl Zeiss) with identical capture settings for each group. Three images per animal per timepoint were obtained and analyzed using ImageJ software (NIH). The images were converted to a binary format and either stain density (total pixels/total area) or cell number measures were determined and averaged for each animal. The tissue section area was traced and measured in µm² and stain density was determined by dividing
the area of the stain by the total area for each section and expressing the value as a percentage. Macrophage infiltration was quantified in Image J by superimposing a grid over the images, manually counting cells that crossed the gridlines in the same pattern across each image, and dividing the cell count by the total area analyzed.

**Growth Cone Advancement and Migrating SC**

The conduits were fastened to the nerves ends via 9-0 epineurial nylon microsuture (Ethicon). The suturing technique placed a 1-2mm portion of the proximal and distal nerve tissue inside the conduit lumen and the sutures were visible using light microscopy. The maximum distance of growth cone advancement from the proximal nerve end was quantified by identifying the growth cones with GAP43/NF-L double immunofluorescence and counting the number of sections between the growth cone and the proximal suture. The total cell number and p75 stain density in the proximal conduit was quantified at the level of the growth cones. The approximate number of p75+ cells migrating from the proximal nerve was quantified by multiplying the total cell number by the p75 stain density percentage for N=3 images for each animal.

**Primary Schwann Cell Culture**

To isolate primary Schwann cells, post-natal day 4 Sprague Dawley rat pups were euthanized, sterilized with 70% ethanol, and bilateral sciatic nerve harvest was performed. The nerves were collected in sterile collection/plating media containing high glucose DMEM (Thermo Scientific), 1% antibiotic/antimicotic (A/A, Thermo Scientific), 1% GlutaMAX (Life Technologies) and 10% fetal bovine serum (FBS, Thermo Scientific). The nerves from 10-12 pups were split into 2 wells of a 6 well tissue culture plate (BD
Biosciences) and dissociation media containing 0.12% collagenase type I (Worthington Biochemical), 0.25% trypsin (Sigma Aldrich), and 1% A/A was added for 5 hours at 37°C. The dissociation reaction was quenched with the addition of DMEM with 10% FBS and the tissue was gently triturated up to 10 times through a 22g needle until a cell suspension was obtained. The cells were centrifuged at RT for 5 minutes at 1500 rpm, the media was aspirated, and the cell pellet was resuspended in plating media containing 10% FBS, the cells were counted with a hemocytometer and plated on human placenta laminin-coated (10µg/ml, Sigma Aldrich) 10cm tissue culture plates (BD Biosciences) at a density between 50,000 and 75,000 cells/cm². After 24 hours, the fibroblasts were purified from the sciatic nerve cultures with the addition of serum free DMEM containing cytosine β-D-arabinofuranoside (5µg/ml, Sigma Aldrich) for 72 hours. The cells were then washed 3 times with PBS and SC proliferation media containing basic fibroblast growth factor (bFGF, 50ng/ml, Sigma Aldrich) and forskolin (4µM, Sigma Aldrich) was added to the cells. The cells were allowed to proliferate for 7 days with media changes every 2-3 days before passaging. All experiments were performed after the second passage.

**Immunocytochemistry**

The SC were characterized with antibodies for S100 (1:200, Sigma) and p75 (1:1600, Cell Signal) to confirm the purity of the cultures. The cells were passaged and plated at a density of 40,000 to 50,000 cells/cm² and allowed to attach overnight in laminin-coated (10µg/ml, Sigma) wells of a 48 well tissue culture plate (BD Biosciences). The cells were washed with TBS/T solution and fixed for 20 minutes at RT with 4% paraformaldehyde (PFA) in TBS/T and briefly washed in TBS/T. The cells were blocked with 5% goat serum (Thermo Scientific) for 30 minutes before S100 and p75 antibody
solutions were placed on the cells for 2 hours followed by 2 TBS/T washes and then an Alexa Fluor 488 or 594-conjugated secondary antibody solution (Goat anti-rabbit, Life Technologies) was placed on the cells for 1 hour. Following 2 TBS/T washes, nuclear labeling was performed with DAPI (1:300, Life Technologies) and the cells were imaged using a Zeiss Axiovert 100 inverted microscope (Carl Zeiss).

**Primary Schwann Cell cytokine media**

To induce cytokine release in the SC, necrotic sciatic nerve tissue harvested from adult female Sprague Dawley rats was added to the cell media for 24 hours. Harvested sciatic nerves were subjected to 6 cycles of freezing in liquid nitrogen and thawing at 37°C creating necrotic nerves (NN). The NN were measured and a 5mm segment was added to a single well of a 6 well plate. After 24 hours, the NN were removed from the wells, the media was aspirated and centrifuged at 12,000 rpm for 10 minutes to remove any cell debris, and the media was aliquoted and either assayed immediately or stored for short periods at -20°C.

**Schwann Cell Supernatant ELISA**

To analyze rat inflammatory cytokines in SC media, multianalyte ELISArrays (SABiosciences) were utilized. The SC were passaged and plated 200,000 cells per well on laminin-coated 6 well plates. NN were created using the previously mentioned method. Experimental media was derived by dissolving KOS powder into the media at a concentration of 10µg/ml. The treatment groups were SC no treatment, SC + NN, SC + NN + KOS, and SC + KOS. The relative concentration of 12 inflammatory cytokines was determined by colorimetric detection using a SpectraMax M2 microplate reader (Molecular
Devices). The optical density (OD) values were derived by subtracting a negative control and the concentration was expressed as a percentage of the standard for each cytokine. The OD values were also converted to percent change from no treatment for each cytokine by subtracting the no treatment values from the experimental values, dividing by the no treatment value and multiplying by 100. The values are an average of n= 3 independent experiments.

*BrdU Proliferation Assay*

A Bromodeoxyuridine (BrdU) proliferation assay was performed by coating a 96 well plate with laminin (10 µg/ml, Sigma Aldrich), passaging the SC and plating at a density of 20,000 cells/well in SC plating media containing 10% FBS. The cells were allowed to attach overnight before experimental media was added for 24 hours. BrdU solution was added to the cells for 20 hours and the cell proliferation was evaluated by colorimetric detection using a SpectraMax M2 microplate reader. Experimental media included 1 mg/ml, 100 µg/ml, and 10 µg/ml KOS and 1 mg/ml, 100 µg/ml, and 10 µg/ml MAT dissolved in complete SC media. Complete SC media containing 10% FBS was used as a positive control. The values are expressed as the average absorbance values for n=2 independent assays.

*THP-1 Migration Assays*

THP-1 monocytes were obtained from the American Type Culture Collection and expanded in RPMI 1640 cell media with l-glutamine (Thermo Scientific) supplemented with 1% penicillin-streptomycin (pen-strep, Thermo Scientific), 10% FBS (Thermo Scientific), and 0.05mM 2-mercaptoethanol (Sigma Aldrich) in 25cm² culture flasks (BD
Biosciences). QCM Chemotaxis Cell Migration Assays (Millipore) were performed by passaging and resuspending the cells in RPMI assay media containing 1% pen-strep and 0.5% FBS and pipetting the cells at a density of 750,000 cells/ml into a 96 well modified Boyden chamber apparatus for 6 hours. The bottom feeder tray was loaded with experimental media including the NN SC media mentioned previously. RPMI with lipopolysaccharide (LPS, 1µg/ml, Sigma Aldrich) and RPMI with 10% FBS were used as positive controls and serum free DMEM was used as a negative control. The cells that migrated towards the experimental media were detached from the porous membrane, lysed and labeled with CyQuant dye, and fluorometric detection was performed using a SpectraMax M2 microplate reader. The values are expressed as average relative fluorescent units (RFU) for n=3 independent assays.

Statistics

For the nerve tissue histomorphometry, growth cone advancement, BrdU proliferation assay, and THP-1 migration assays, one-way or two-way analysis of variance (ANOVA) with Bonferroni post-hoc analysis were performed using Prism 4.0 software (GraphPad). For the multi-analyte ELISArray, a two-way ANOVA with Bonferroni post-hoc analysis was performed using GraphPad Prism 4.0. Histomorphometry data is expressed as mean ± standard deviation (s.d.) and the cell assay data is expressed as mean ± standard error of the mean (s.e.m.); p < 0.05 is considered statistically significant.
Results

Rat Sciatic Nerve Proximal Conduit

The proximal conduit lumen was examined for the presence and advancement of axonal growth cones and migrating cells. An antibody for NF-L (1:100, Cell Signaling) was used to label axons and a GAP43 antibody (1:100, Cell Signaling) was used to label regenerating axons (Figure 1A). The uninjured portion of the proximal nerve tissue was identified with the NF-L label and consecutively distal slides were used to identify the distance of axonal sprouting from the proximal nerve end into the conduit lumen. By 3 days, the MAT group showed significant advancement of the growth cone with a mean value of 35.0±19.1 µm compared to 15.0±10.0 µm and 12.5±9.6 µm for the KOS and SAL groups (p < 0.01). At the 7 day time point, the MAT had increased growth cone advancement of 80.0±11.5 µm, which was significant compared to the KOS and SAL groups (p < 0.001). The KOS group had growth cone advancement of 37.5±5.0 µm, which was significant compared to the SAL group with advancement of 13.3±4.7 µm (p < 0.01, Figure 1B). The total cell number was evaluated at the level of the growth cones with a DAPI label (Figure 1D) and the KOS group (2077.3±309.3 cells/mm²) had significantly higher values compared to the SAL group (1221.0±121.3 cells/mm², p < 0.05), and slightly higher values than the MAT group (1601.2±466.9 cells/mm²) at 3 days. There were no significant differences between the 3 treatment groups at 7 days (KOS 2330.6±733.1 cells/mm², MAT 3213.6±1273.7 cells/mm², SAL 2044.4±1065.1 cells/mm²). A p75 antibody was used to label dedifferentiated SC at the level of the growth cones (Figure 1C). The KOS group (26.4±3.2%, p < 0.01) and MAT group (23.3±1.8%, p < 0.05) had significantly higher expression of p75 compared to the SAL
group (16.0±3.2%) at 3 days. By the 7 day time point, the values were equivalent (KOS 26.5±4.8%, MAT 22.9±4.7, SAL 22.1±3.2, Figure 1E). The p75 expression values and total cell numbers were used to calculate the number of p75+ cells migrating from the proximal nerve tissue (Figure 1F). At 3 days, the KOS group (550.6±121.2 cells) had significantly higher numbers of p75+ cells compared to the SAL group (196.7±46.9 cells, p < 0.05) but not significantly different from the MAT group (366.6±81.8 cells). By 7 days, both the KOS (593.0±68.2 cells, p < 0.05) and MAT (758.5±375.3 cells, p < 0.01) groups had significantly higher numbers of p75+ cells migrating from the proximal nerve tissue compared to the SAL group (347.1±94.3 cells).

Rat Sciatic Nerve Distal Stump Tissue

To examine the possible effects of hydrogel conduit fillers on Wallerian degeneration, the distal nerve stump was harvested, sectioned and stained with antibodies for p75NTR to label dedifferentiated SC (Figure 2A&B), NF-H to label axons (Figure 2A&B), and S100 to label total SC. DAPI was used to quantify total cells and FluoroMyelin Red (Figure 3A&B) was used to examine myelin phagocytosis. A FITC-conjugated tomato lectin reagent was used to visualize macrophage infiltration (Figure 3D). At 3 days, the KOS and MAT groups had significant upregulation of the p75NTR compared to the SAL group with stain density values of 34.9±4.2%, 26.8±4.1%, and 15.8±1.6% respectively (p < 0.01 and 0.05). By the 7 day time point, the values were equivalent between the KOS (20.8±4.1%), MAT (23.8±6.8%), and SAL (23.1±1.8%) groups (Figure 2C). The S100 labeling revealed no significant differences between the groups at 3 days although the KOS nerves had slightly higher expression (72.2±6.0%) compared to the MAT (59.9±12.3%) and SAL (47.7±17.9) groups. At 7 days, all groups
had similar S100 expression values (43.8±8.3% KOS, 42.1±12.7% MAT, and 41.6±8.4%, Figure 2E). There were no significant differences in axon density (KOS 4149±818, MAT 3212±1227, SAL 5642±1890 axons/mm\(^2\)) between the 3 groups at 3 days. At 7 days, the axon density was vastly reduced and similar for all 3 groups (KOS 606±156, MAT 707±331, SAL 916±169 axons/mm\(^2\), Figure 2D). At 3 days, the myelin density was similar between the KOS (35.2±13.4%), MAT (44.5±7.1%), and SAL (42.2±12.0%) groups but by 7 days, the myelin density was significantly lower in the KOS group (13.9±1.3%) compared to the MAT (22.0±3.4%) and SAL groups (23.3±2.8%, p < 0.05, Figure 2C). Because myelin phagocytosis has been shown to be performed independently by both macrophages and SC in vitro (Rotshenker, 2011), the extent of macrophage infiltration was evaluated. At 3 days, the SAL group (79.0±15.2 cells/mm\(^2\)) had significantly higher tomato lectin positive cells compared to the KOS group (45.8±2.5 cells/mm\(^2\)) but no significant difference compared to the MAT group (61.0±11.2 cells/mm\(^2\), Figure 3E). By 7 days, the KOS group had significantly lower tomato lectin positive cells (139±14.5 cells/mm\(^2\)) compared to both the MAT and SAL groups with values of 188.6±14.1 cells/mm\(^2\) and 210.8±3.8 cells/mm\(^2\), respectively (p < 0.01 and 0.001, Figure 3F). There were no significant differences between total cell number in the distal tissue between the 3 groups at 3 days (KOS 1260±55 nuclei/mm\(^2\), MAT 999±27 nuclei/mm\(^2\), SAL 1446±399 nuclei/mm\(^2\)) or 7 days (KOS 1755±122 nuclei/mm\(^2\), MAT 1633±201 nuclei/mm\(^2\), SAL 1860±187 nuclei/mm\(^2\), Figure 2F).

**Immunocytochemistry**

Primary SC cultures were imaged using light microscopy during purification and the difference in morphology between the SC and fibroblasts is easily detectable (Figure
4A). The p75 immunostaining allowed visualization of highly purified primary SC cultures following cytosine arabinoside treatment and passaging (Figure 4B).

**Cytokine media and SC Supernatant ELISArrays**

NN placement in the SC cultures was successful in altering cytokine release in the cell supernatant. There were 2 distinct patterns of cytokine expression: the first appeared to be a result of the NN treatment and the second appeared to be a result of the KOS in the media. The presence of NN + KOS significantly increased expression of IL-6 (8.09±1.82% of standard) compared to no treatment (2.92±1.84% of standard, p < 0.001), and KOS only (3.23±1.56% of standard, p < 0.01) and IFN-γ (5.16±1.78% of standard) compared to no treatment (1.00±0.59% of standard, p < 0.05) and NN only (0.32±0.32% of standard, p < 0.01, Figure 5A). The cytokine expression was converted to percentage change from the no treatment control values and a clear pattern of increase, decrease, or no change was visible (Figure 5B).

**BrdU Proliferation**

The media containing 10% FBS showed significantly greater proliferation in primary SC after 24 hours (p < 0.001) compared to all other groups with a mean absorbance value of 0.423 OD. None of the KOS and MAT concentrations investigated were significantly different from SC growth media or DMEM without mitogens with absorbance values of 0.124 and 0.095 OD, respectively. Of the experimental media preparations, the 1mg/ml MAT had a slightly higher absorbance value of 0.157 OD compared to the other treatments. The KOS treatments did not show a dose response with similar values of 0.128, 0.107, and 0.109 OD for the 1 mg/ml, 100 µg/ml and 10 µg/ml
KOS concentrations. The MAT concentrations did show a slight increase in proliferation with the highest concentration of 1 mg/ml (0.157 OD) although the 100 µg/ml and 10 µg/ml MAT treatments were equivalent with values of 0.129 and 0.131 OD (Figure 6A).

**THP-1 Migration Assay**

The migration assays revealed that THP-1 cells readily migrate into media containing a chemoattractant such as FBS or LPS. The FBS and LPS treatments showed the highest RFU levels of 46.6±3.3 and 46.5±4.2 at 6 hours. There was cell migration into each cell media treatment although the SC-treated media containing 10 µg/ml KOS + NN and the SC no treatment group had significantly lower migration than the FBS and LPS controls with average values of 23.4±0.9 and 27.8±1.9 RFU, respectively (p < 0.01). The remaining SC-treated media groups had higher RFU values of 33.8±0.9 for the KOS only and 37.1±2.6 for NN only and SC media containing forskolin and bFGF had an average value of 35.9±5.6 RFU. None of the other treatment groups were significantly different from the positive controls (Figure 6B).

**Discussion**

Nerve conduits are approved for clinical use to bridge small defects in peripheral nerves and are most commonly used to repair small diameter sensory nerves such as digital nerves. For nerve conduits to completely replace autograft as the gold standard, they must be capable of providing clinically relevant functional recovery at short and long gap distances, in both motor and mixed motor nerves. In order to accomplish this, many types of filler materials have been used in numerous preclinical studies, primarily using rodent models. These fillers are based on the concept that the transected nerve ends
exude a fluid that polymerizes into a fibrin matrix, allowing cells to attach and migrate as axon regeneration occurs from the proximal end (Chen et al., 2006). Conduits are typically filled with saline at the time of surgery, but this liquid quickly leaches out of the repair site and is replaced by the native fibrin matrix. Fillers must be capable of acting as a suitable replacement for fibrin, must not prematurely degrade so that their scaffolding function is lost, but also must not persist so long as to impede axonal sprouting. Keratin is a suitable choice based on these structural criteria because there are no natural keratinases produced by mammals, yet KOS biomaterials are not completely impervious to proteolytic degradation (Kaluzewska et al., 1991). Consequently, they are biodegradable, but at a slower rate than other extracellular matrix (ECM) proteins. The result, when engineered properly, is a KOS hydrogel scaffold that provides initial support for regenerative cells without premature structural failure, but also a low initial solids content and gradual clearance that accommodates regenerating tissue.

Aside from a structural role, the purpose of this study was to investigate other possible mechanisms of KOS biomaterial hydrogel, in particular and starting with an ability to support a greater regenerative cell influx into the conduit lumen, presumably by providing sites for cell migration and adhesion as has been shown by other in vitro studies (Reichl, 2009). Because MAT has been successfully used as a nerve conduit filler and been shown to induce neurite sprouting of dorsal root ganglia in vitro, it was chosen as a positive hydrogel control for this study (de Guzman et al., 2010). MAT is heterogeneous consisting of laminin, entactin, collagen, and small concentrations of growth factors such as IGF-1 and TGF-beta which makes it an excellent material for cell applications (Benton et al., 2011)
KOS hydrogel has been shown to significantly enhance nerve regeneration in both mouse and rabbit models (Apel et al., 2008; Sierpinski et al., 2008; Hill et al., 2011). These studies were of long-term functional recovery following tibial nerve transection and repair and compared KOS hydrogel conduit filler to SAL and sural nerve autograft, considered the clinical “gold standard” (Schlosshauer et al., 2006). The mouse study outcomes showed electrophysiological and histological enhancement in the KOS group compared to the other treatment groups at 6 weeks and 6 months (Apel et al., 2008; Sierpinski et al., 2008). In the rabbit study, the KOS hydrogel group showed significant electrophysiological improvement compared to the SAL and autograft groups and histological evaluation showed KOS and autograft to be similar with an improvement over SAL-filled conduits (Hill et al., 2011). These findings were significant because the autograft control used in both models was a multi-strand sensory nerve autograft, which is used clinically and represents the repair technique with the most potential for beneficial outcomes. Other forms of autograft used in preclinical animal models (e.g. the so-called “snip and flip” where a nerve segment is removed, flipped 180° and re-attached, or contralateral nerve segments are exchanged) are inferior repair techniques because they introduce tension into the nerve. However, neither study addressed the question of why the KOS hydrogel filler worked as well or better than autograft. Because the degradation of KOS hydrogel has been examined in mouse subcutaneous implants and the material was found to be relatively fast-degrading (de Guzman et al., 2011), this study hypothesized that the KOS hydrogel acts early in the regenerative process, perhaps initiating a cascade of events that at least contribute to the electrophysiological and histological outcomes seen in the long-term functional studies. Silicone chamber models
of nerve regeneration have shown that the direction of cellular infiltration into the conduit lumen occurs from the proximal nerve end and begins within one week after injury (Heath and Rutkowski, 1998; Chen et al., 2006). The cell population has been initially characterized as fibroblasts and SC migrating ahead of regenerating axons from the proximal nerve tissue in a rat sciatic nerve crush injury model (Parrinello et al., 2010). The current study revealed that using KOS hydrogel as a conduit filler material modestly increases cellular migration from the proximal nerve tissue at 3 days yet by 7 days there were no significant differences between the groups (Figure 1D). Interestingly, the proportion of migrating cells expressing the p75 receptor was significantly increased for the KOS group compared to the SAL group at both 3 and 7 days while the MAT group did not show a significant increase in p75+ cells compared to the SAL group until 7 days. These results suggest that KOS hydrogel may induce an earlier infiltration of SC into the conduit lumen compared to SAL and MAT conduit fillers. However, examination of the nerve growth cones showed that regenerating axons had advanced farther in the MAT group at 3 days, although the KOS hydrogel did allow modest growth cone advancement by 7 days, which was significant compared to the SAL controls. Together, these data suggest that the KOS hydrogel conduit filler may allow earlier cellular infiltration into the conduit lumen but MAT may provide a better structural material to support axonal regeneration. The potential interaction between the KOS hydrogel and SC was further investigated in the distal nerve tissue as SC also play an early role in the inflammatory response to nerve injury (Ide, 1996; Gaudet et al., 2009; Rotshenker, 2011).

Prior to the current study, a pilot study of rat sciatic nerve injury was performed comparing KOS and SAL conduit fillers in a 1cm defect. Examination of macrophage
infiltration in the distal nerve tissue at 3 and 7 days showed significantly fewer macrophages in the KOS group compared to SAL (Pace et al., unpublished). This result led to the hypothesis that the presence of KOS in the conduit may be altering the inflammatory response of PNS resident cells to nerve injury.

Following nerve transection, the distal tissue undergoes Wallerian degeneration which is a complex series of molecular and cellular events resulting in the clearance of axonal and myelin debris from the distal site of injury to the distal targets (Shim and Ming, 2009). The process is initiated by sodium ion-dependent (Na⁺) calcium (Ca²⁺) influx into the axoplasm resulting in activation of calpain, a protease necessary for cytoskeletal and axonal degeneration (Hirata and Kawabuchi, 2002; Gaudet et al., 2011). Immediate and temporal changes occur in the SC following nerve transection. Guertin et al. showed increases in expression and activation of the SC neuregulin receptor erb2 in the distal nerve at 10 minutes following sciatic nerve axotomy, attenuation by 3 hours and another period of upregulation from 3-14 days after injury (Guertin et al., 2005). Two days after nerve transection in young rodents, axon fragmentation and SC dedifferentiation begins. It is thought that SC sense tissue damage through toll-like receptor (TLR) activation by TLR ligands on damaged axons such as degraded ECM proteins. SC dedifferentiation is followed by proliferation which peaks around 4 days (Gaudet et al., 2011). Resident macrophages, which reside within the endoneurium, make up only 2-4% of the cell population in rodent peripheral nerves (Hirata and Kawabuchi, 2002). Mueller et al. investigated the early role of these cells during Wallerian degeneration following sciatic nerve crush injury in rats. Two days after injury, the cells undergo morphological changes corresponding with their activation and begin myelin
phagocytosis which was confirmed with co-labeling for myelin basic protein (MBP) and macrophage markers ED1 and Iba1. Only the Iba1 positive cells were found to undergo a period of proliferation between 2 and 7 days with a peak at 3 days (Mueller et al., 2001). Nerve transection injury allows infiltration of bone-marrow derived macrophages from the periphery; a process that begins within hours due to disruption of the blood nerve barrier and peaks at 7 days following local production of cytokines and chemokines (Gaudet et al., 2011; Rotshenker, 2011). Wallerian degeneration of the distal nerve tissue is essential for successful peripheral nerve regeneration because myelin debris is inhibitory for axonal regeneration (Gaudet et al., 2011; Rotshenker, 2011). If Wallerian degeneration rates could be accelerated in the clinic, functional recovery in patients may also be improved.

Based on the foregoing, it seemed appropriate to investigate the effect, if any, that filling a conduit with KOS would have on the cellular activity in the distal stump, specifically the role of SC in Wallerian degeneration, particularly since cell infiltration into the conduit lumens did not appear to show any significant differences by 7 days. The 3 treatment groups in this study showed no differences in axon density in the distal stump and thus axon fragmentation, but significant differences in macrophage infiltration and myelin phagocytosis. The KOS treated nerves had the highest p75 expression in the distal tissue at 3 days suggesting that SC dedifferentiation and proliferation may be occurring more rapidly. The amount of myelin debris and the number of macrophages was reduced in the distal tissue with the use of KOS in the conduit. This data suggests that the SC and resident macrophages may be phagocytosing the myelin debris earlier in the Wallerian degeneration process. Because the total cell number was similar between the groups
while the macrophage number was significantly lower in the KOS group by 7 days, KOS may also promote SC proliferation. The total S100 stain showed slightly higher levels of the protein in the KOS treated tissue only at 3 days and therefore if SC proliferation is increased by KOS, it may be occurring before that time point. A possible theory for the significantly lower macrophage counts at 7 days in the KOS treated nerves could be altered cytokine/chemokine expression of the resident cells, resulting in reduced macrophage recruitment which peaks at this time point (Gaudet et al., 2011).

During Wallerian degeneration of the distal nerve tissue, a complex network of cytokine, chemokine, and growth factor signaling between many different cell types is initiated and the precise patterns of cytokine release have been extensively characterized. Shamash et al. showed that SC upregulate TNF-α mRNA expression, protein synthesis, and secretion during Wallerian degeneration along with IL-1α and IL-1β protein synthesis and secretion (Shamash et al., 2002). This increase in cytokine production by SC is concomitant with the activation of the phospholipase A_2 (PLA_2) enzyme which causes the initial degradation of the myelin sheath and may be directly controlled by SC cytokines (Gaudet et al., 2011). The SC-derived cytokines are also partially responsible for the recruitment of monocytic cells from the periphery that differentiate into macrophages following infiltration into injured tissue (Gaudet et al., 2011). SC and resident macrophages can secrete pro-inflammatory cytokines IL-1, IL-6, and IL-12 which can serve to recruit macrophages as well as anti-inflammatory cytokines IL-10 and TGF-β which may inhibit inflammation (Ruohonen et al., 2005). IL-4, IL-10, and IL-13 are thought to play an anti-inflammatory or neuroprotective role by reducing production of inflammatory cytokines IL-1 and TNF-α. IL-6, although considered an inflammatory
cytokine, may have anti-inflammatory functions which are linked to the presence of other cytokines that can alter its function. INF-\(\gamma\) upregulates major histocompatibility complex (MHC) class II antigen expression and controls expression of IL-1, TNF-\(\alpha\), IL-10 and other chemokines (Hanisch, 2002). SC express MHC II and may act as antigen-presenting cells in order to phagocytose myelin debris during early Wallerian degeneration (Gaudet et al., 2011). Herrero et al. found that IL-10 anti-inflammatory activity in cultured blood monocytes is directly controlled by IFN-\(\gamma\) (Herrero et al., 2003).

Lee et al. found that cultured SC inflammatory activation by necrotic neuronal cells (NNC) occurs via TLR2 and TLR3. NNC treatment increased expression of inflammatory genes TNF-\(\alpha\), iNOS, LIF and MCP-1 after 3 hours. When SC from TLR2 and TLR3-knockout animals were subjected to NNC treatment, the upregulation of the TNF-\(\alpha\) and iNOS genes was suppressed suggesting that TLR ligands from injured nerve tissue could be the activating factors of SC in early Wallerian degeneration (Lee et al., 2006). Inflammatory activation of cultured SC was performed in the current study to evaluate the differences in cytokine release between cells treated with KOS proteins, NN, and a combination of these components. The presence of KOS + NN in the cell supernatant resulted in significant increase of IL-6 and IFN-\(\gamma\) after 24 hours. Both of these cytokines has a well-characterized early role in Wallerian degeneration and changes in their expression could alter the cellular response to injury. The NN-treated SC media was also used to investigate the migration of THP1 monocytes and results showed that the combination of NN + KOS proteins significantly reduced the number of migrating THP1 cells. The altered SC cytokine expression profiles in the ELISArrays which served
to inhibit migration of THP1 cells may suggest a possible mechanism of action for the KOS hydrogel.

The likely role of a KOS biomaterial hydrogel conduit filler is primarily structural, but based on the in vivo and in vitro data in this study, a contribution to the regenerative process appears to derive at least in part from altered SC activity. A potential postulate that fits these data is that in the distal nerve tissue following transection, cellular interaction with KOS proteins at the gel-stump interface causes dedifferentiation of SC and greater release of IFN-γ and IL-6 proteins, which results in resident macrophage activation, more effective clearance of myelin debris, and ECM remodeling by the resident cells. Increased IL-6 signaling, which can serve to downregulate cytokine production (Rotshenker, 2011), may also reduce the extent of macrophage recruitment.

**Conclusion**

This study presents the first evidence of interactions between KOS proteins and PNS cells at early time points both in vitro and in vivo. The KOS hydrogel conduit filler has been shown to be equivalent to the clinical “gold standard” for nerve repair in a 2cm tibial nerve defect in rabbits (Hill et al., 2011) and a 4 mm tibial nerve defect in mice (Apel et al., 2008; Sierpinski et al., 2008). The KOS hydrogel’s ability to promote peripheral nerve regeneration and functional recovery must be compared to sural nerve autograft utilizing a larger (≥ 3cm) nerve defect model in order for a true determination of its efficacy as an autograft alternative. Biocompatibility studies of the KOS biomaterial have been performed in mice and show no adverse reactions (de Guzman et
al., 2011) although more extensive immunogenicity testing must be completed before the material can be made clinically available. KOS hydrogel is a simple and effective nerve conduit filler that seems to promote a more rapid response of the PNS to traumatic injury via interactions with resident cells. Nerve injury patients may benefit from the translation of this technology as a clinically available alternative to autograft surgeries.

Acknowledgments

The authors would like to gratefully acknowledge the Wake Forest Department of Orthopedic Surgery for their assistance with this study. Funding was provided by KeraNetics LLC, the Errett Fisher Foundation, and the Armed Forces Institute for Regenerative Medicine (DoD Contract # W81XWH-08-2-0032)

Conflict of Interest

Author Mark Van Dyke holds stock and is an officer in the company, KeraNetics LLC, who provided partial funding for this research. Wake Forest School of Medicine has a potential financial interest in KeraNetics through licensing agreements.

References


Figure 1. Matrigel (MAT) enhances axon sprouting and Keratin (KOS) increases P75 expression in the injured proximal nerve. A. Immunofluorescent staining for GAP43 (red) and NF-L (green) with a DAPI counterstain shows high levels of co-localization at the regenerating nerve growth cone in a MAT nerve at 7 days. 400x magnification. Scale bar = 50 µm. B. Growth cone advancement shows significantly increased growth at 3 days in the MAT group compared to KOS and SAL. By 7 days, both KOS and MAT treatment have significantly greater advancement of the nerve growth cone into the conduit compared to SAL. C. Immunofluorescent staining for P75 (red) and NF-L (green) shows upregulation of P75 in Schwann cells migrating along with sprouting axons in a SAL nerve at 3 days. 200x magnification. Scale bar = 50 µm. D. Total cell number is significantly higher in the KOS over the SAL group at 3 days. E. P75 expression shows significant upregulation in KOS and MAT groups compared to SAL at 3 days. F. Number of P75+ cells is significantly higher in the KOS group over SAL at 3 days and MAT and KOS are significantly higher than SAL at 7 days. Values expressed as mean ± s.d. *** p < 0.001, ** p < 0.01, * p < 0.05.
Figure 2. Keratin (KOS) hydrogel-treated distal nerves show increased P75 expression while axon density, S100 expression, and total cell number are similar between groups. A & B. Immunofluorescent labeling with antibodies for P75 (red) and NF-H (green) shows high expression of P75 at 3 days (A) and lowered expression of NF-H by 7 days (B) in a KOS injured sciatic nerve. 100x magnification. Scale bars = 50 µm. C. Quantification of percent stain density shows significantly higher expression of P75 in KOS and Matrigel (MAT)-treated sciatic nerves over Saline (SAL) nerves at 3 days. By 7 days, the values are similar between groups. Axon density (D), and S100 expression (E) and total cell number (F) is similar between the groups at 3 and 7 days. Values expressed as mean ± s.d. ** p < 0.01, * p < 0.05.
Figure 3. Keratin (KOS) hydrogel-treated distal nerves show more rapid phagocytosis of myelin debris and less macrophage infiltration than Matrigel (MAT) and Saline (SAL) groups. A & B. 630x confocal images showing decrease in myelin staining density using FluoroMyelin Red labeling between 3 (A) and 7 (B) days following sciatic nerve repair with a SAL conduit. Scale bars = 20 µm. C. Quantification of myelin staining density shows significantly less myelin in the distal nerve tissue at 7 days in the KOS nerves compared to MAT and SAL. D. 200x image of FITC-tomato lectin labeling shows macrophage infiltration in MAT-treated distal nerve tissue at 7 days. Scale bar = 50 µm. E. Quantification of macrophage infiltration into the distal nerve tissue at 3 days shows significantly less macrophages in the KOS group compare to SAL. F. By 7 days, macrophage infiltration is significantly lower in the KOS group compared to both MAT and SAL. Values are expressed as mean ± s.d. *** p < 0.001, ** p < 0.01, * p < 0.05.
Figure 4. Sciatic nerve explants yield highly purified primary Schwann cell cultures. 
A. A brightfield image of passage 1 sciatic nerve explants shows fibroblast (green arrowheads) contamination prior to purification. Fibroblasts retain a flat triangular morphology. 200x magnification. 
B. Purified passage 2 Schwann cell cultures 7 days after cytosine arabinoside treatment. Immunolabeling with S100 (green) and DAPI (blue). 200x magnification.
Figure 5A. Keratin (KOS) + necrotic nerves (NN) alters cytokine expression in Schwann cell (SC) supernatant. A. Average expression of 12 cytokines and chemokines shows altered expression with the addition of NN, KOS, and KOS + NN compared to no treatment after 24 hours in primary SC cultures. Values are expressed as mean ± s.e.m. percentage of standard for each cytokine. ** p < 0.01, * p < 0.05.
Figure 5B. Data in Figure 5A expressed as percent change from no treatment group allows visualization of altered expression for each cytokine. Values expressed as mean ± s.e.m. for N=3 independent experiments.
Figure 6. Keratin (KOS) and Matrigel (MAT) do not induce proliferation in cultured Schwann cells (SC) and SC-treated media alters THP1 cell migration. A. A BrdU proliferation assay shows that MAT and KOS added to cell media do not affect SC proliferation compared to 10% fetal bovine serum (FBS) after 24 hours. *** p < 0.001. B. SC-treated media with KOS + necrotic nerves (NN) significantly reduces THP1 cell migration compared to 10% FBS and 1µg/ml lipopolysaccharide as positive controls after 6 hours. ** P < 0.01. Values are expressed as mean ± s.e.m.
The study of human hair keratin (HHK) biomaterial hydrogel as a nerve conduit filler originated in 2004 during a first year graduate student’s rotation project examining peripheral nerve regeneration of a 4mm mouse tibial nerve defect. This study sought to compare a conduit filled with HHK hydrogel to the best possible positive control: a sural nerve cable autograft. Most animal studies of nerve regeneration utilize an autograft control created by removing a portion of the nerve to be grafted, rotating the tissue 180°, and suturing it back into place (Archibald et al., 1991; Alluin et al., 2009; McGrath et al., 2010). This does not reflect the technique for autologous nerve grafting used in the clinic and instead generates an inferior repair with a severed vascular supply, limited diffusional exchange, and most likely tension along much of the length of the nerve. Therefore, comparison to a multi-strand cable autograft represented the most rigorous assessment possible. The HHK hydrogel conduits in the mouse study showed significant improvement in electrophysiological outcome measures compared to both sural nerve autografts and saline-filled (SAL) conduits, which represent the current technique used for conduit implantation (Apel et al., 2008; Sierpinski et al., 2008). Following the mouse study, the HHK conduit filler was evaluated in a larger nerve defect in a larger animal: a 2cm rabbit tibial nerve model. The rabbit study revealed similar electrophysiological outcome measures to the mouse study for the same 3 treatment groups HHK conduit, SAL conduit, and sural nerve cable graft (Hill et al., 2011). The project then progressed to determine the efficacy of the HHK conduit filler in a clinically relevant animal: the non-human primate (NHP).
The overall hypothesis for this work is that HHK conduit filler enhances peripheral nerve regeneration via early cellular interactions in the injured PNS. A long-term study of functional recovery in a NHP median nerve study was employed to confirm the effectiveness of the HHK hydrogel. A rat sciatic nerve injury model was then utilized to identify the early cellular events that occur with the use of HHK hydrogel in the nerve conduit. The cellular interactions during these early events were then characterized in a simplified cell culture system. These studies were performed with the goal of promoting the clinical translation of an effective alternative to the current “gold standard” for nerve repair through the assessment of its mechanism of action.

In the NHP median nerve study, concerns about animal welfare and increased study costs led to the decision to exclude a sural nerve cable graft control and only HHK and SAL filled conduit groups were included. One cohort of animals underwent unilateral median nerve repair allowing the contralateral uninjured median nerve to serve as one type of positive control. Like the mouse and rabbit studies, the HHK group showed significant electrophysiological enhancement over the SAL group including a faster return of nerve conduction latency, higher nerve conduction velocity, and increased compound muscle action potential of the abductor pollicis brevis muscle. The enhanced electrophysiology outcome measures corresponded to larger nerve area, higher axon density, less muscle atrophy, and higher myofiber density in the HHK regenerated tissues over SAL.

One of the limitations of the NHP study was that only motor functional recovery could be examined. The recurrent branch of the median nerve innervates the muscles of the thenar compartment and first and second lumbricals and the palmar branch provides
sensory innervation to the palmar and dorsal aspects of the hand (Lundborg et al., 2004). The NHP nerve study focused solely on motor recovery due to an anatomical variation noted in the animals. The bifurcation of the median nerve, which normally occurs after exiting the carpal tunnel in humans, occurred in the forearm and therefore only the recurrent branch of the median nerve was included in the distal conduit to provide the most favorable environment for nerve regeneration and reinnervation of the distal targets.

The NHP study outcome measures provided evidence that HHK could enhance nerve regeneration and return of motor function in a clinically relevant injury model. In addition, initial immunogenicity testing showed no adverse events following a challenge bolus injection of the HHK biomaterial 12 months after initial exposure to HHK hydrogel at the time of nerve repair with an HHK-filled conduit. The NHP nerve study illustrated the safety and efficacy of the HHK biomaterial with the goal of gaining FDA approval for clinical trials. Since the NHP study began, a Request for Designation has been submitted to the FDA, various Good Laboratory Practice safety tests have been conducted, and continued testing of the HHK biomaterial is currently underway. An important concern in the clinical translation of a biomaterial is its biological activity and mechanism of action. These questions were initially addressed using the rat sciatic nerve studies.

There are published studies illustrating the HHK biomaterial’s biocompatibility with various cell types and initial studies of the interactions between HHK proteins and cells of the peripheral nervous system (PNS), although these studies employ a Schwann cell (SC) line (Apel et al., 2008; de Guzman et al., 2011). Based upon the hypothesis that HHK supports a robust cellular infiltration into the conduit following nerve repair, a rat
sciatic nerve pilot study was performed to compare HHK and SAL conduit fillers. Evaluation of axonal regeneration at 14, 21, and 28 days and cellular infiltration at 3, 7, and 14 days after injury did not reveal a meaningful difference between the treatment groups. Examination of macrophage infiltration in the distal nerve stump at 3 and 7 days after injury did reveal a trend toward fewer cells in the HHK tissue compared to SAL. Based upon these initial results, another rat sciatic nerve study was designed to compare the inflammatory and regenerative cellular response to nerve injury between conduit repairs with HHK hydrogel, SAL, and a hydrogel positive control, Matrigel (MAT). MAT was selected because it is secreted basement membrane containing various extracellular (ECM) components which incorporate well-characterized cell binding domains into their structure. MAT also contains trace amounts of growth factors shown to enhance cell viability. The exact structure of HHK proteins in the hydrogel form is still under investigation but it has been hypothesized that the amino acid sequence of these proteins may contain similar cell binding motifs to endogenous ECM (Sierpinski et al., 2008).

The rat sciatic study revealed that MAT was capable of inducing significant advancement of the nerve growth cones from the proximal nerve end compared to both HHK and SAL at 3 and 7 days although, at the 7 day time point, the HHK group had significantly greater advancement than SAL. The distal nerve data showed that HHK hydrogel in the conduit induces significantly higher expression of the SC de-differentiation marker p75NTR compared to SAL and increased expression compared to MAT at 3 days. Another enhancement seen with the HHK conduits was significantly faster degradation of the myelin debris in the distal nerve tissue by 7 days compared to
both MAT and SAL. Interestingly, the HHK-treated nerves had significantly less macrophage infiltration than the SAL group at 3 days and by 7 days, the difference was significant compared to both MAT and SAL. Together, these data suggest that the HHK conduit filler promotes an altered inflammatory response to distal nerve injury. This altered response may partially contribute to the enhanced nerve regeneration seen in the mouse, rabbit, and NHP studies as the HHK conduit filler seems to accelerate the clearance of degenerated myelin which is inhibitory for axonal regeneration (Gaudet et al., 2011; Rotshenker, 2011). However, the relative contribution of this effect to overall nerve regeneration is not known.

Many studies have shown that inhibition of macrophage infiltration is detrimental for peripheral nerve regeneration (Ramaglia et al., 2007; Gaudet et al., 2009) and therefore, results showing that it may lead to enhanced nerve regeneration may seem counterintuitive. During Wallerian degeneration, macrophages function to facilitate an orchestrated immune response to injury by other macrophages, SC, and fibroblasts. They release pro-inflammatory cytokines to recruit blood monocytes and later anti-inflammatory cytokines to signal the beginning of tissue repair (Ruohonen et al., 2005). The process of macrophage polarization in which macrophages respond to injury and differentiate into the pro-inflammatory M1 phenotype or the anti-inflammatory M2 phenotype is well-characterized in the central nervous system (CNS) but has not been as closely studied in the PNS. It has been hypothesized that the recruited macrophages that infiltrate injured nerve tissue and peak in numbers by 7 days following injury are the anti-inflammatory M2 phenotype that function to signal the end of the inflammatory response.
(Rotshenker, 2011). Preliminary in vitro studies of THP1 monocytes have shown that HHK may be capable of differentiating these cells into an M2 phenotype.

The SC of both the distal and proximal nerve tissue had an increased expression of p75NTR for HHK conduit filler compared to the other groups. This suggests a rapid de-differentiation of these cells. Because the release of cytokines by SC during Wallerian degeneration has been well-characterized (Shamash et al., 2002; Guertin et al., 2005), a method to examine “Wallerian degeneration in a dish” was developed. All myelinating primary SC in culture without neuronal cells are the de-differentiated phenotype expressing p75NTR and studies have shown that they can be induced to release cytokines into the cell supernatant by the addition of necrotic neuronal cells (Lee et al., 2006). Necrotic adult sciatic nerves (NN) were created by freeze/thaw and the addition of this tissue to SC cultures resulted in increased expression of several cytokines as shown by ELISArrays. Addition of HHK proteins to the NN media changed the SC cytokine release patterns and these altered cytokine levels showed a reduction in the chemoattractant ability of NN media to induce migration of THP1 cells that were used as a model of undifferentiated macrophages.

Despite the progress made in this project, there were limitations in several experiments. Although the HHK conduit filler in the rat sciatic study did not work as well as MAT to induce growth cone advancement, the presence of the HHK in the conduit did show significant improvement over SAL by 7 days. The distal tissue data showed a snapshot of cellular events at 2 time points and therefore utilizing more numerous and earlier time points in future studies may allow a better understanding of the current work. Also, the difficulties in obtaining and expanding primary SC in culture placed limitations...
on the cell assays. The cytokine expression network in Wallerian degeneration begins within hours after injury and continues for up to 3 weeks (Ruohonen et al., 2005). Repeating the ELISArrays with more time points would allow an analysis of temporal expression instead single data points at 24 hours. The RNA collected from the SC in the NN experiments may be used to investigate signaling pathways with a PCR array which would also give a better understanding of how the altered cytokine expression in the presence of HHK may be occurring. ELISAs on the cytokines with the most increased or decreased expression levels could also be performed to allow the expression to be quantified as concentration instead of the percentage of a standard.

If additional experiments can be performed in the in vitro studies, future directions for this work could include the identification of a signaling pathway involved in the SC NN cytokine experiments and investigation of the temporal expression of cytokines in response to NN. SC toll-like receptors (TLR) are likely involved as previous in vivo and in vitro studies have implicated them in the SC injury response. Reduced cytokine expression in response to necrotic neuronal cells has been shown with the use of SC from TLR knock-out animals (Lee et al., 2006). Following the correct identification of a specific TLR or signaling pathway, the sciatic nerve study could be repeated in a knockout mouse and should reveal no differences between HHK and SAL conduits for all outcome measures. Another important future direction is to continue the study of the HHK biomaterial’s ability to induce macrophage polarization of THP1 monocytes by performing fluorescent-activated cell sorting analysis of the M1 and M2 phenotypes following HHK treatment and this work is currently underway.
Future work may also be performed to better characterize the HHK biomaterial. The extracted keratins in the HHK hydrogel contain multiple subtypes with different chemical properties (de Guzman et al., 2011). The ability to isolate and test these different subtypes in vitro and in vivo may yield a more effective HHK biomaterial for nerve regeneration.

If the HHK hydrogel is FDA approved, it may prove to be effective in not only the currently available nerve conduits but in the next generation conduits. Much of the current peripheral nerve regeneration tissue engineering research is focused on providing structural fibers or filaments in the conduit lumen to allow cell migration and electrical stimulation through the conduit has also been shown to increase axonal regeneration in rat sciatic nerve injury models (Nectow et al., 2012). Incorporation of growth factors into the materials used to engineer the conduits is also common. HHK hydrogel conduit filler was used in combination with conduits engineered with glial derived growth factor microspheres in a rat sciatic nerve injury model and showed significant histological improvements over SAL conduits after 6 weeks (Lin et al., 2012). Other studies have shown that HHK hydrogel is capable of sustained release of antibiotics (Saul et al., 2011) and continued drug delivery studies are underway. Because HHK has been shown to interact with SC, combining it with the next generation conduits may achieve much greater levels of functional recovery than previously reached in the mouse and rabbit studies and the current work.

The mechanisms of peripheral nerve regeneration are exceedingly complex and involve a wide array of cell types with differential expression of many cell adhesion molecules, growth factors, and cytokines (Ide, 1996; Fu and Gordon, 1997). The
experiments in this work provide evidence to support a mechanism that may contribute, at least in part, to enhanced peripheral nerve regeneration, yet they also raise additional questions about the HHK conduit filler’s role in influencing cell behavior.

Preclinical studies of conduit fillers are conducted with the goal of developing an equivalent alternative to autograft surgery that can allow patients moderate to excellent levels of functional recovery under optimal conditions (Lundborg et al., 2004; Ray and Mackinnon, 2010). The current clinical recommendation for conduits is in small diameter sensory nerves in gaps of ≤ 3 cm (Taras et al., 2005; Schlosshauer et al., 2006; Taras et al., 2011). While improvement of outcomes using conduits is a laudable goal for filler materials and a clear benefit to patients, a more challenging objective would be for a filler material to allow nerve regeneration and functional recovery in large nerve defects (≥ 5 cm) so that complete obsolescence of autograft can be achieved. Current studies of median nerve regeneration in a NHP 5 cm defect are being conducted by another group to ascertain whether the HHK hydrogel is effective in a nerve gap of this size.

The data in this document provides a better understanding of how a novel nerve conduit filler derived from HHK can be used to enhance peripheral nerve regeneration and it also raises many additional questions to be addressed in future studies.

References


APPENDIX 1

Title: A Human Hair Keratin Biomaterial Hydrogel Enhances Sciatic Nerve Regeneration in a Rat Model

Introduction

Peripheral nerve injury requiring surgery occurs in approximately 2-3% of trauma patients (Rodrigues et al., 2012). The current recommended surgical intervention for nerve transection injuries in which a tensionless primary end-to-end repair cannot be performed is to bridge the nerve defect with a portion of the patient’s own nerve tissue, referred to as autograft. Autografts may result in donor site complications and are limited by tissue availability (Matsuyama et al., 2000). Nerve conduits provide a clinical alternative to autograft and many preclinical studies have suggested that functional recovery may be improved with the use of biomaterial hydrogels or cell fillers in the conduit lumen (Chen et al., 2006). A human hair keratin (HHK) hydrogel was evaluated as a conduit luminal filler in mouse (Apel et al., 2008; Sierpinski et al., 2008) and rabbit (Hill et al., 2011) tibial nerve injury models and showed enhanced histological and electrophysiological outcomes compared to saline (SAL) and autograft groups. Only late time points were examined in these studies (e.g. 6 weeks) so no information about the formative stages of regeneration was collected. The current study examines the early events of nerve regeneration in a 1cm rat sciatic nerve defect and compares HHK to SAL, which is currently used as standard conduit luminal filler (Lohmeyer et al., 2009).
Materials and Methods

Rat sciatic nerve injury model

Thirty 250g male Sprague Dawley rats (Harlan Laboratories) were randomized into SAL and HHK groups and underwent unilateral sciatic nerve transection and repair of a 1cm defect with a NeuraGen® conduit (Integra Life Sciences). All animal procedures were approved by the Wake Forest University Animal Care and Use Committee and conducted according to the NIH Guide for the Care and Use of Laboratory Animals. Anesthesia was maintained with isoflurane (1.5 to 2.0 volume %) and all procedures were performed under sterile conditions. The sciatic nerve was transected at the level of the obturator tendon, the nerve ends were entubulated with a 1cm X 2mm ID conduit and secured via epineurial suture with 9-0 nylon (Ethicon), and either a 15% weight/volume (w/v) HHK hydrogel or sterile SAL was introduced into the conduit lumen. The subcuticular layers were approximated using 5-0 Vicryl suture (Ethicon) and the skin was closed with Michel wound clips (Kent Scientific). Immediately following the procedure, buprenorphine (0.01 mg/kg) was administered for analgesia.

Preparation of Keratin Hydrogel

HHK hydrogel was prepared as described previously (Sierpinski et al., 2008; de Guzman et al., 2011). Briefly, human hair was oxidized with a 2% peracetic acid solution and rinsed with water to remove any residual oxidant. Soluble keratins were extracted into successive treatments of tris(hydroxymethyl)aminomethane (Tris) base buffer and deionized water. The extracted solution was then dialyzed, concentrated, neutralized, lyophilized, and ground into a fine powder. The lyophilized keratin was sterilized via $\gamma$-
irradiation at a dose of 10 kGy and aseptically reconstituted in phosphate buffered saline (PBS) to form a 15% (w/v) hydrogel.

**Nerve Histology**

At 3, 7, 14, 21, and 28 days following surgery, the animals were anesthetized with isoflurane (1.5 to 2.0 volume %), euthanized, and the nerve graft tissue was harvested including 1 to 2mm of proximal and distal nerve tissue, placed into O.C.T. compound (Tissue-Tek, Sakura Finetek), flash frozen in liquid nitrogen, and stored at -80°C. The tissue blocks were equilibrated to -25°C and 10 µm longitudinal serial cryosections were collected with a cryostat (CM1950, Leica Microsystems) using positive charged slides (Xtra®, Leica), post-fixed in acetone for 15 minutes at -20°C and stored at 4°C.

**Immunohistochemistry and Immunofluorescence**

All immunofluorescent stains were performed at room temperature (RT) using either a Dako Universal Slide Staining System or by hand using an optimized staining technique utilizing tris-buffered saline/tween 20 (TBS/T) solutions with 0.3% Triton X-100 (Sigma Aldrich). The slides were rinsed with TBS/T, blocked in 5% serum corresponding to the species of the secondary antibody for 30 minutes, incubated in primary antibody for 2 hours, rinsed with TBS/T and incubated in secondary antibody for 1 hour. Primary antibodies to neurofilament heavy chain (NF-H,1:200, Cell Signaling) and integrin alpha M (OX42,1:50, Santa Cruz Biotechnology) and a FITC-anti-mouse (rat adsorbed, 1:100, Vector Labs) secondary antibody was used to label the tissue. All slides were mounted with ProLong® Gold Antifade reagent with DAPI (Life Technologies) to visualize nuclei.
Histomorphometry

For the NF-H and DAPI labels, 50x magnification monochromatic images were obtained of the entire 1cm conduit at 14, 21, and 28 days for NF-H and 3, 7, and 14 days for DAPI (n=3 animals per group per time point) with a Zeiss upright microscope and photo-merged using Photoshop CS software (Adobe). The merged images were converted to binary format and the stain density and total cell numbers were quantified using ImageJ software (National Institutes of Health). For the NF-H label, a grid of 10 1mm sections was overlaid on the conduit image, and the stain density was quantified for each section by dividing the stain area in pixels$^2$ by the total area in pixels$^2$. For the DAPI label, the entire conduit lumen was traced and the area calculated in mm$^2$. Nuclei/mm$^2$ was quantified using the analyze particles count function. For the OX42 label, macrophage infiltration was quantified by obtaining three 200x magnification images per animal with a Leica upright microscope, superimposing a grid over the images, and manually counting cells that crossed the gridlines in the same pattern across each image.

Statistics

A two-way analysis of variance (ANOVA) with Bonferroni post hoc analysis was performed for the NF-H stain density, total cell counts, and OX42 cell counts using GraphPad Prism 4.0 software (GraphPad). A P value of 0.05 was considered statistically significant and all data is expressed as mean ± standard error of the mean (s.e.m).
Results

Nerve Histomorphometry

NF-H immunostaining for the 14, 21 and 28 day time points allowed visualization of regenerating axons from the proximal nerve end to the distal side of the conduit. The process began after 14 days post-injury for both groups but occurred more robustly in the HHK group (Figure 1A). NF-H staining density was modestly increased for the HHK nerves compared to SAL for the distal conduit sections at 28 days (Figure 1B). The data is summarized in Table 1.

DAPI quantification showing cellular infiltration into the conduit lumen (counts include the proximal and distal nerve tissue inside the conduit) showed a greater number of cells in the HHK versus SAL group at the 3 and 14 day time points (Figure 2). At 3 days, the HHK nerves had 555.6±186.6 cells/mm² compared to 301.5±100.8 cells/mm² for the SAL group. At the 7 day time point, the HHK group had 357.7±72.6 cells/mm² in the conduit while the SAL group had 364.6±131.3 cells/mm². By 14 days, the HHK group showed an increase in cellular infiltration with 500.6±91.1 cells/mm² and the SAL group showed a decrease in cellular infiltration with 146.2±14.4 cells/mm². There was a significant difference between the groups for the treatment variable (P < 0.05, two-way ANOVA) but Bonferroni post hoc analysis did not detect any significant differences between the groups at each time point.

Counts of OX42 positive cells showed less macrophage infiltration in the distal nerve tissue at 3 and 7 days for the HHK (329.3±26.0 and 346.7±43.7 cells, respectively) group compared to SAL (452.0±30.6 and 448.0±62.1 cells, respectively). In the proximal
nerve tissue, the HHK group also showed fewer OX42 positive cells at 3 days (346.7±43.7 cells) compared to SAL (627.0±13.9 cells) and 7 days (448.0±62.1 cells HHK, 661.7±27.4 cells SAL, Figure 3). The distal tissue data showed a significant difference between the groups for the treatment variable (P < 0.05, two-way ANOVA) but Bonferroni post-hoc analysis indicated no significant difference between the groups at either time point. The proximal tissue showed no significant difference between the groups (P = 0.14, two-way ANOVA).

Discussion

The NF-H stain showed the occurrence of axonal regeneration which began after 14 days and the progression of the nerve growth cones from the proximal conduit to the distal nerve end by 28 days in both HHK and SAL groups. There was a slight increase in NF-H staining density in the HHK group compared to the SAL group in the more distal sections of the conduit and although this difference was not significant, it suggests the possibility that axonal elongation may be occurring more rapidly when HHK conduit filler is used. However, axon sprouting from the proximal stump has been shown to occur as quickly as 5 hours after injury (Ide, 1996). Because axon sprouting was not visible with the NF-H stain until after 14 days for both the HHK and SAL groups, examining the proximal tissue using transverse sections may allow a more accurate comparison.

The sequence of events occurring in the nerve conduit lumen following implantation has been well-characterized using silicone chambers. Within the first day, the luminal space is initially filled with a fluid containing neurotrophic factors and structural proteins exuded by the injured nerve ends. Within a week, a fibrin bridge forms
between the proximal and distal tissue and provides sites of attachment for migrating cells (Heath and Rutkowski, 1998). The migrating cells are primarily fibroblasts and Schwann cells (SC) and they migrate ahead of regenerating axons. SC also migrate along the extending axons in order to synthesize a new myelin sheath (Parrinello et al., 2010). The increased conduit cellular infiltration in the HHK group at all time points may be due to the fact that the HHK hydrogel also provides sites of attachment for migrating cells, which reduces the need for the fibrin bridge. Nerve regeneration is rate-limited by the anterograde transport of membrane components from the cell body and it is unlikely that this rate can be altered. Therefore, the presence of a structural material, like HHK, in the conduit would most likely begin the process of cell migration and axonal elongation at an earlier time point after injury (Ide, 1996).

Following nerve transection, Wallerian degeneration of the distal stump begins to occur within hours (Ramaglia et al., 2007). The SC de-differentiate, proliferate, and begin to phagocytize the axonal and myelin debris (Ide, 1996; Mirsky and Jessen, 1999; Hirata and Kawabuchi, 2002). Resident and later recruited macrophages bolster the phagocytotic process during Wallerian degeneration and the peak of phagocytic cell infiltration occurs at 7 days (Hirata and Kawabuchi, 2002; Ramaglia et al., 2007; Ramaglia et al., 2009). The OX42 label, also known as CD11b or Integlin alpha M, is used to identify macrophages in the injured nerve tissue and is generally thought to be a marker for activated, recruited macrophages in the injured PNS (Ramaglia et al., 2007). More specifically, the OX42 antibody labels cells expressing complement receptor CR3, which has been implicated in opsonin-dependent myelin phagocytosis during Wallerian degeneration (Hirata and Kawabuchi, 2002).
The HHK group showed fewer OX42-positive macrophages in both the distal and proximal nerve tissue 3 and 7 days post-injury. Previous long-term studies of the HHK hydrogel have shown enhanced histological and electrophysiological outcomes compared to SAL in mouse (Apel et al., 2008; Sierpinski et al., 2008) and rabbit (Hill et al., 2011) tibial nerve injuries. The trend toward less OX42 macrophage infiltration of the distal tissue in the current study may be partially due to the increased involvement of resident macrophages and SC in myelin phagocytosis. *In vivo* studies have shown that both SC and macrophages are capable of myelin phagocytosis independently of one another (Rotshenker, 2011). An altered biological response to nerve injury has been shown in studies that inhibit the complement system in rats leading to less macrophage accumulation and axonal damage following nerve crush injury. (Ramaglia et al., 2007; Ramaglia et al., 2009)
Figure 1. A. Representative photo-merged images of the conduits stained for NF-H (green) and DAPI (blue) at 50x magnification shows the advancement of regenerating axons and cells from the proximal nerve tissue between the 14 and 21 day time points. Regenerated axons reach the distal side of the conduit by 28 days in both HHK and SAL groups. B. Quantification of NF-H stain density for 10 1mm sections through the conduit shows the increase in NF-H stain volume throughout the conduit by 28 days which corresponds with axonal regeneration. N=3 animals, values expressed as mean ± s.e.m.
Figure 2. Quantification of cellular infiltration using a DAPI label shows temporal differences in cell number within the groups and differences between the HHK and SAL groups at 3 and 14 days. N=3 animals, values expressed as mean ± s.e.m.

Figure 3. A. Quantification of OX42-positive cells in the distal nerve tissue shows an increased number of this macrophage phenotype at 3 and 7 days in the SAL group compared to the HHK group. B. In the proximal tissue, there were no differences between the groups. N=9 images, values expressed as mean ± s.e.m.
<table>
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<td>Time Point 1mm 2mm 3mm 4mm 5mm 6mm 7mm 8mm 9mm 10mm</td>
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<td>14 days</td>
<td>58.6±6.1 39.7±15.3 4.7±4.3 1.1±1.1 0.22±0.2 0.06±0.02 0.09±0.08 0.23±0.12 0.76±0.64 4.93±2.5</td>
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<tr>
<td>21 days</td>
<td>72.0±5.2 61.8±16.5 26.6±6.5 14.8±2.1 9.2±3.5 6.4±3.3 7.1±3.0 5.6±3.8 4.3±3.1 3.3±2.7</td>
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<tr>
<td>28 days</td>
<td>43.8±1.7 45.6±12.3 28.8±12.8 20.4±6.9 15.4±4.2 12.8±6.1 13.5±5.5 12.3±4.2 8.2±3.6 5.8±2.8</td>
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<tr>
<td>Saline</td>
<td>Neurofilament Stain Density (mm from Proximal Nerve)</td>
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<tr>
<td></td>
<td>Time Point 1mm 2mm 3mm 4mm 5mm 6mm 7mm 8mm 9mm 10mm</td>
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<tr>
<td>14 days</td>
<td>41.6±10.0 34.9±7.9 9.0±6.6 2.8±2.4 0.42±0.37 0.34±0.22 0.13±0.05 0.31±0.21 6.7±3.0 4.7±2.3</td>
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<tr>
<td>21 days</td>
<td>48.6±17.7 78.2±2.2 47.1±5.3 26.8±1.9 12.7±2.6 5.8±2.6 3.6±1.7 3.2±1.8 1.7±1.1 2.0±0.51</td>
</tr>
<tr>
<td>28 days</td>
<td>63.2±2.7 47.5±0.80 16.6±6.4 13.1±2.5 8.7±1.6 8.8±2.9 4.7±1.1 9.1±0.94 6.9±2.1 6.1±4.9</td>
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**Table I.** Summary of stain density values for the data in Figure 1B. Values expressed as mean ± s.e.m.
Title: Investigation of Dorsal Root Ganglia Neurite Outgrowth on Human Hair Keratin and Matrigel Substrates

Introduction

Many animal studies have illustrated the benefit of using nerve conduit luminal filler materials to increase gap length and improve functional recovery following peripheral nerve transection. These materials may consist of extracellular matrix (ECM) components, growth factors, and cells (Schlosshauer et al., 2006; Dahlin, 2008). To determine if a human hair keratin (HHK) biomaterial hydrogel may be capable of inducing axon sprouting following nerve injury in vivo, the HHK biomaterial was compared to Matrigel (MAT), a substrate material that has been shown to induce neurite outgrowth of explanted dorsal root ganglia (DRGs) (de Guzman et al., 2010).

Materials and Methods

DRG isolation and culture

All animal procedures were approved by the Wake Forest University Animal Care and Use Committee and conducted according to the NIH Guide for the Care and Use of Laboratory Animals. Post-natal day 6 Sprague Dawley rat pups were euthanized, sterilized with 70% ethanol, and DRGs were dissected under a microscope and collected in high glucose DMEM (Thermo Scientific) containing N-2 (1:100, Life Technologies), B-27 (1:50, Life Technologies), GlutaMAX supplements (1:100, Life Technologies), nerve growth factor (NGF, 50ng/ml, PeproTech), and 1% antibiotic/antimicotic (Thermo
The DRGs were plated and allowed to attach overnight to HHK-coated (90µg/ml) or MAT-coated (30 µg/ml, growth factor reduced, BD Biosciences) sterile 22x22mm square glass coverslips in wells of a 6 well tissue culture plate before 300µl of media was added. All coverslip coatings were left on for 3 hours at room temperature (RT), excess solution removed, and rinsed with PBS prior to seeding DRGs. One half of the media volume was changed every 2 days. One DRG was placed on each corner of the coverslip for n=4 DRGs per well. At 24, 48, and 72 hours the DRGs were fixed with 4% paraformaldehyde (Sigma Aldrich) for 20 minutes, stained with an antibody for neurofilament H (NF-H, 1:200, Cell Signaling), visualized with a FITC-conjugated anti-mouse secondary antibody (rat-adsorbed, 1:100, Vector Labs), and counter-stained with DAPI (1:300, Life Technologies). Either 25x or 50x images were obtained with a Zeiss inverted microscope and neurite extension was examined qualitatively. Sample size was n=3 DRGs per group per time point.

**Preparation of Keratin Powder**

HHK powder was prepared as described previously (Sierpinski et al., 2008; de Guzman et al., 2011). Briefly, human hair was oxidized with a 2% peracetic acid solution and rinsed with water to remove any residual oxidant. Soluble HHK was extracted into successive treatments of tris(hydroxymethyl)aminomethane (Tris) base and deionized water. The extracted solution was then dialyzed, concentrated, neutralized, lyophilized, and ground into a fine powder. The lyophilized HHK was sterilized via γ-irradiation at a dose of 10 kGy and dissolved to a concentration of 90µg/ml in sterile phosphate buffered saline (SAL) for use as a culture substrate coating solution.
Results

DRG isolation and culture

At 24 hours, the DRGs had attached to both the HHK and MAT-coated coverslips but no neurite extension was noted by the NF-H label (Figure 4 A&B). At 48 hours, neurite sprouting was noted in the MAT DRGs but not the HHK (Figure 4 C&D). By 72 hours, the MAT cells had sprouted numerous neurites whereas the HHK group showed modest sprouting of less than 5 neurites per DRG (Figure 5). DAPI labeling showed increased cell migration away from the DRGs as time progressed which suggests that there were Schwann cells migrating ahead of sprouting neurites in both groups (data not shown).

Discussion

HHK has been used as conduit luminal filler in mouse and rabbit tibial nerve injury models (Apel et al., 2008; Sierpinski et al., 2008; Hill et al., 2011). These long-term studies of functional recovery showed electrophysiological and histological enhancement of nerve regeneration with the use of HHK hydrogel compared to SAL and improved or equivalent outcome measures to sural nerve autograft, the clinical “gold standard”. This purpose of this study was to investigate whether or not the HHK biomaterial may be acting as a substrate to induce axonal sprouting in the previous studies. Results showed that HHK induced no identifiable neurite outgrowth in DRG explants compared to MAT as the positive control.
Figure 4. **A & B.** After 24 hours, DRGs on both HHK (A) and MAT (B) substrates show no neurite outgrowth. **C & D.** By 48 hours, DRGs on the MAT (D) but not HHK (C) substrate begin to show neurites (indicated by white arrowheads).
Figure 5. A & B. At 72 hours, DRGs on the HHK substrate (A) begin to show modest neurite outgrowth (white arrowhead) compared to robust neurite outgrowth on the MAT substrate (B).
Title: Protocol for Sciatic Nerve Explants and Schwann Cell Isolation and Culture

This protocol is based on the studies published by Brockes et al. (Brockes et al., 1979; Brockes and Raff, 1979; Brockes et al., 1980) and utilizes post-natal day 4 Sprague Dawley rat pups.

The day before:

Note: All media preparation and plate coating should be performed in sterile conditions using a biosafety cabinet

1) Prepare and autoclave surgical kit: 1 pair large surgical scissors, 1 pair 6” Mayo scissors, 1 pair 4” undermining scissors or dissecting scissors, 1 scalpel handle, 1 square forceps, 1 needle driver, 1 straight tip fine point forceps

2) Prepare Schwann cell (SC) collection/plating media: 10% heat-inactivated fetal bovine serum (FBS), 1% anti-biotic/anti-mitotic (A/A), and 1% GlutaMAX (Invitrogen) in high glucose DMEM (Thermo Scientific) and aliquot ~12ml into 1 15ml conical tube for each litter

3) Prepare laminin coating solution and coat plates: Remove laminin (Sigma Aldrich) from -80°C and thaw on ice, make 10μg/ml coating solution in ice-cold PBS, pipette the coating solution into a 10 cm tissue culture plate (4ml is sufficient) and coat 1 plate per litter. Leave solution on at RT overnight or for 2 hours at 37°C then aspirate the coating solution and allow to dry. Dry coated plates may be stored at 4°C for up to 4 weeks.
4) Prepare dissociation media: High glucose DMEM, 1% A/A, 1% GlutaMAX, 0.25% trypsin from bovine pancreas (Sigma Aldrich) and 0.12% collagenase type I (Worthington Biochemical), and make fresh for every use

Tissue Harvest:

Note: All tissue harvest should be performed using aseptic technique including repeated sterilization of gloves and surgical instruments with 70% EtOH

1) Decapitate rat pup with large surgical scissors and sterilize the dorsal aspect with 70% EtOH

2) Secure hindlimbs to 10cm dissecting dish with 0.5 in. needles with the dorsal aspect facing up (a dissecting dish can be made by pouring liquid paraffin into a 10cm culture plate and allowing to harden)

3) Trim the skin away from both the dorsal hindlimbs beginning at the lower leg and halfway up the back using the square forceps and 6” Mayo scissors

4) Place a 11-0 scalpel blade on the scalpel handle using the needle drivers

5) Under the microscope, carefully separate the overlying muscle tissue and expose the sciatic nerve by trimming a superficial portion of the fascia and muscle using the 4” undermining scissors at the level of the obturator tendon (the landmark appears as a white line running from the knee to the sacrum, the nerve is directly caudal to the femur)

6) Using the 4” undermining scissors and fine point forceps, harvest both sciatic nerves one at a time by first transecting the proximal side while holding a portion of the nerve distal to the transection point and then transect the distal side.
7) Place the all nerves from 1 litter in 1 SC collection/plating media tube

**Tissue Dissociation and Cell Isolation**

**Note:** Cell viability is improved for multiple litters by beginning the tissue dissociation for the first litter before harvesting tissue from the next

1) Carefully aspirate the SC collection media from the harvested nerves that will have formed a small pellet at the bottom of the conical tube

2) Using a sterile 1000µl pipette tip, pipette the nerves from the tube and divide them into 2 wells of a 6 well tissue culture plate (if the nerves get stuck, use a second pipette tip to stretch and pull them away from the first)

3) Remove all residual media from the wells by tilting the plate 45° and aspirating any media with a pipette tip (this is very important as any FBS will inactivate the trypsin)

4) Place 1.5 ml dissociation media in each well and incubate for 5 hours at 37°C

5) Stop the dissociation reaction by adding 1.5 ml of SC collection/plating media to the wells

6) Gently triturate the dissociated tissue using a 3ml luer lock syringe with a 23 to 22g needle, triturate up to 10 times until a cell suspension is obtained (there will be some residual nerve tissue in the suspension) and perform this separately for each well

7) Centrifuge the cells for 5 min. at 22°C and 1500 rpm

8) Aspirate the media from the cell pellet and resuspend the pellet in fresh SC collection media (2-3 ml per well used)
9) Count the cells with a hemocytometer

10) Just prior to plating the cells, equilibrate the laminin-coated plate with SC collection media for ~30 seconds then aspirate

11) Plate the cells at a density of 50,000 to 75,000 cells/cm² and allow to attach for 24 hours

**Fibroblast Purification and Culture Media**

1) Prepare fibroblast purification media: Dissolve cytosine arabinofuranoside (Sigma Aldrich) to a concentration of 5µg/ml in high glucose DMEM, 1% A/A, and 1% GlutaMAX

2) Aspirate the SC collection media from the cells after 24 hours and add fibroblast purification media for 72 hours

3) Prepare SC culture media: high glucose DMEM, 1% A/A, 1% GlutaMAX, 50 ng/ml bFGF (Sigma Aldrich), and 4µM forskolin (Sigma Aldrich)

4) After 72 hours, aspirate the fibroblast purification media and wash the cells 3X in sterile PBS

5) Add SC culture media to the cells and culture for ~7 days with media changes every 2 days before passaging

**Passaging**

1) Prepare laminin-coated plates as previously described

2) Detach cells by adding 0.05% trypsin (Thermo Scientific) and incubating for 5 minutes

3) Add an equal volume of SC collection/attachment media

4) Lightly scrape the cells with a sterile cell scraper to ensure all cells are detached
5) Centrifuge at 1500 rpm for 5 min. and resuspend cells in SC collection/attachment media

6) Plate cells at a density of 20,000 cells/cm²

7) Change media to SC culture media after 24 hours

References


CURRICULUM VITAE

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I. Education

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Ph.D., Neuroscience, Program in Neuroscience, Wake Forest School of Medicine, Winston-Salem, NC

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B.A., Psychology, Department of Psychology and Behavioral Sciences, Louisiana Technical University, Ruston, LA

II. Awards and Honors

2nd place- Integrative Sciences, Graduate School of Arts and Sciences Graduate Student Research Day Poster Competition, Wake Forest University, 2011.

2nd place- Best Presentation by an Advanced Student, Neuroscience Program, Wake Forest School of Medicine, 2010.

Travel Award- The 8th International Stem Cell School in Regenerative Medicine, Karolinska Institute, Stockholm, Sweden, 2010

Magna cum laude Graduate, Lousiana Technical University, 2000

Presidential Scholarship, Louisiana Technical University, 1997-2000

III. Publications


IV. Abstracts/ Scientific Presentations

National Conference Podium Presentations


Regional Conference Podium Presentations


National Conference Posters


"Human Hair Keratins Activate Schwann Cells via Integrin Receptors and Promote Regeneration of Large Peripheral Nerve Defects in a Rabbit Model" Society for Neuroscience: Chicago, IL 10/2009

Regional Conference Posters


"Cellular Interactions with a Human Hair Keratin Hydrogel Enhance Peripheral Nerve Regeneration" Wake Forest Graduate School of Arts and Sciences Graduate Student Research Day: Winston-Salem, NC 3/2010