A Human Hair Keratin Hydrogel Scaffold Enhances Median Nerve Regeneration in Nonhuman Primates: An Electrophysiological and Histological Study

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A human hair keratin biomaterial hydrogel scaffold was evaluated as a nerve conduit luminal filler following median nerve transection injury in 10 *Macaca fascicularis* nonhuman primates (NHP). A 1 cm nerve gap was grafted with a NeuraGen[®] collagen conduit filled with either saline or keratin hydrogel and nerve regeneration was evaluated by electrophysiology for a period of 12 months. The keratin hydrogel-grafted nerves showed significant improvement in return of compound motor action potential (CMAP) latency and recovery of baseline nerve conduction velocity (NCV) compared with the saline-treated nerves. Histological evaluation was performed on retrieved median nerves and abductor pollicis brevis (APB) muscles at 12 months. Nerve histomorphometry showed a significantly larger nerve area in the keratin group compared with the saline group and the keratin APB muscles had a significantly higher myofiber density than the saline group. This is the first published study to show that an acellular biomaterial hydrogel conduit filler can be used to enhance peripheral nerve regeneration and motor recovery in an NHP model.

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.^{1–3} Techniques for surgical management of peripheral nerve transection injuries vary depending on injury severity, although primary end-to-end neurorrhaphy is the preferred treatment. 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 to attain a tension-free repair.⁴ 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 as they are technically challenging, and result in increased surgical time and donor-site morbidity.⁵

Alternatives to autograft are currently under investigation in both preclinical studies and clinical trials.^{6,7} Hollow tubes, referred to as nerve guides or nerve conduits, constructed of collagen type I (NeuraGen, Neuroflex[™], and NeuroMa-

trix[™]), polycaprolactone (Neurolac[®]), and polyglycolic acid (Neurotube[®]) are available for clinical use. 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.^{7–10} However, there is a paucity of clinical studies showing the effectiveness of nerve conduits and they are currently recommended for repair of small gap lengths (≤ 3 cm) in sensory nerves only.^{7,9,11–14} There are few clinical studies examining the effectiveness of bioabsorbable conduits in large mixed motor and sensory nerve gaps and currently, autologous nerve graft or allograft repairs are recommended to achieve functional recovery.^{7,9,12,15} For bioabsorbable conduits to become an alternative treatment method in small and large gaps in mixed motor and sensory nerves, peripheral nerve regeneration through these devices needs to be improved.^{16–18}

Before nerve repair with a conduit, resection of the injured nerve tissue is performed.¹⁹ Inside the conduit, it is recommended to fill the lumen with saline before closure, but the nerve ends exude a fluid that quickly forms a native fibrin matrix that displaces the saline, thereby providing a

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native scaffold for migrating cells such as the Schwann cells (SC).²⁰ Based upon this phenomenon, luminal fillers consisting of extracellular matrix proteins, SC, and growth factors have been used in experimental studies in combination with nerve conduits to enhance nerve regeneration. In pre-clinical experiments, these fillers have shown modest to significant improvement of functional recovery in several animal models.^{20–24} However, relatively few of these studies have progressed down a translational pathway toward clinical trial. 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.^{16,17,25} Keratin biomaterial has the distinct advantage of being resistant to proteolytic degradation.²⁶ Therefore, keratin biomaterial forms a scaffold that persists in the nerve conduit and can be made to have a controlled degradation rate that is longer than other natural protein filler materials. However, the keratin scaffold can be remodeled by infiltrating cells and does not present an impediment to axonal regrowth. Moreover, keratins have been shown to be more biocompatible than synthetic materials.²⁷ 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 a nonhuman primate (NHP) nerve injury model. A relatively small gap (i.e., 10 mm) was chosen to ensure that a native fibrin matrix would form in the saline group and not degrade prematurely, thereby allowing for a direct comparison of these two matrices. 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

Nonhuman 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 5 cm proximal to the middle wrist crease, and a 5 to 6 mm portion of the nerve was removed. A 1 cm $\times$ 2 mm 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 eight cases, the conduit lumen was filled with a 15% weight/volume (w/v) keratin hydrogel and in six cases, the conduit lumen was filled with sterile saline. The conduit was then interposed between the nerve ends creating a 10 mm 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 subdermal 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 before extubation and every 8 h for 24 h for analgesia.

Preparation of keratin hydrogel

Keratin hydrogels were prepared as described previously.^{16,27} 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 the 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 by means of γ -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 before 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) (Fig. 1A). Before 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 6 mm tin surface electrode (Rochester Electro-Medical, Inc.) placed 2 mm distal to the muscle insertion or a 0.40 mm subdermal needle electrode (Cadwell Laboratories) placed within the muscle (Fig. 1A). 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 the 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 two stimulation electrode distances, the proximal site being proximal to the lesion, with a single 1 ms pulse of 20 mA and used to calculate the nerve conduction velocity (NCV) by the following formula: $NCV = \frac{\text{distance}_2 - \text{distance}_1 \text{ from the APB muscle (in mm)}}{\text{latency}_2 - \text{latency}_1 \text{ (in ms)}}$ ²⁸

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 midconduit and 1 mm distal to the distal graft suture were postfixed in 1% osmium tetroxide, dehydrated in increasing concentrations of ethanol, and embedded using epoxy resin. Semithin sections (0.5 μ m) were cut using an LKB III Ultramicrotome (LKB Instruments), mounted on slides, and stained with 1% Toluidine blue.

Nerve morphometry

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

Muscle histology

The APB muscles were harvested 12 months after surgery following terminal electrophysiology measurements, fresh frozen in liquid nitrogen, and stored at -80°C . Before analysis, the APB muscles were placed into the O.C.T. compound (Tissue-Tek, Sakura Finetek) and $10\text{-}\mu\text{m}$ cryosections were collected with a cryostat (CM1950, Leica Microsystems) and used to examine the cross-sectional fiber area and fiber density (Fig. 6C–E). 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 $10\text{-}\mu\text{m}$ sections were obtained at $200\times$ magnification with a Zeiss microscope. The myofiber cross-sectional area (CSA) and fiber density were 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.

Antibody titer

Following the final electrophysiology procedure, 4 of the animals that received keratin hydrogel underwent a bolus injection procedure with a sterile keratin solution (0.4 mg/kg in a $4\text{ w/v\%}$ 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 h after 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 20°C for 30 min before centrifugation to separate the serum. Serum samples from two 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) for normalization during the enzyme-linked immunosorbent assay (ELISA). 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 20°C overnight, using the collected serum as the primary antibody, an HRP-goat anti-human IgG (1:1000; Life Technologies) as the secondary antibody, and the TMB substrate (Thermo Scientific) for detection. The detected signal was compared with a standard curve using an anti-basic hair keratin K81 antibody (Progen Biotechnik). Solutions of the anti-basic hair keratin K81 antibody (i.e., without serum) were used as a positive control to ensure that the assay could detect a known amount of keratin antibody. Samples were analyzed at two concentrations of $0.5\text{ }\mu\text{g/mL}$ and $1.0\text{ }\mu\text{g/mL}$ total protein and all concentrations were assayed in triplicate.

Statistical analysis

All data are presented as mean $\pm$ 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 the Bonferroni post-test using GraphPad 4.0. In all tests, $p < 0.05$ was considered statistically significant.

Results

Motor conduction studies

At 12 weeks following surgery, none of the animals showed a detectable return of APB CMAP latency (Fig. 1A; saline group shown as representative), 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 ($60.2 \pm 25.4\%$) compared with the saline group with values of ($95.3 \pm 11.4\%$, $p < 0.01$; Fig. 1B). The percent difference in CMAP latency remained lower for the keratin group compared with the saline group until 36 weeks after surgery (Fig. 1A). At 44 weeks, the CMAP latency was similar in both groups (keratin $39.1 \pm 23.6\%$ vs. saline $45.4 \pm 9.2\%$ Fig. 1B). The recovery of baseline NCV was significantly higher in the keratin group compared with the saline group at 52 weeks ($78.5 \pm 17.4\%$ and $50.3 \pm 20.3\%$, respectively; Fig. 1C). The CMAP amplitude at 52 weeks was not significantly different between the keratin ($11.7 \pm 5.3\text{ mV}$), saline ($8.7 \pm 3.7\text{ mV}$), and uninjured control ($16.6 \pm 4.0\text{ mV}$) groups (Fig. 1D, $p = 0.051$).

Nerve morphometry

Analysis of the midconduit nerve area showed a significantly larger mean area for the keratin group ($2.3 \pm 0.8\text{ mm}^2$) compared with the native nerve ($1.0 \pm 0.33\text{ mm}^2$, $p < 0.05$, Fig. 2A), but no significant difference from the saline group ($1.6 \pm 0.68\text{ mm}^2$). The distal nerve area was significantly larger for the keratin group ($2.9 \pm 0.9\text{ mm}^2$) compared with the saline group ($1.7 \pm 0.5\text{ mm}^2$, $p < 0.05$) and native nerve ($1.0 \pm 0.33\text{ mm}^2$, $p < 0.01$, Fig. 2B). The axon density of the middle and distal nerve segments showed no significant differences in the average density between the keratin group (10110 ± 1929 and 9054 ± 1821 axons/ mm^2) and the saline group (6926 ± 1991 and 7663 ± 854 axons/ mm^2). Native

FIG. 1. Keratin hydrogel enhances recovery of compound motor action potential (CMAP) latency, nerve conduction velocity (NCV), and CMAP amplitude. **(A)** Representative CMAP waveforms show latency in milliseconds (ms, first arrowhead from left) and amplitude in millivolts (mV, second arrowhead from left) for baseline measurements, loss of function at 12 weeks in a saline nerve, and return of function at 36 weeks in both keratin and saline nerves [the inset axes indicate waveform scale for amplitude (*y* axis) and latency (*x* axis)]. **(B)** 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. **(C)** The recovery of NCV occurs more quickly in the keratin group, remains higher throughout the study, and becomes significant at 52 weeks. **(D)** 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 $\pm$ SD ($n=8$ keratin, $n=6$ saline, $n=4$ native controls). ** $p<0.01$. Color images available online at www.liebertpub.com/tea

median nerve axon density was also analyzed showing significantly higher values (18166 ± 3897 axons/ mm^2) compared with the keratin and saline middle and distal values ($p<0.0001$, Fig. 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, $p<0.01$, Fig. 2E, F). Photomicrographs taken of the nerve sections at $630\times$ magnification allowed visualization of decreased axon density, smaller axon diameter, and differences in myelination between the native (Fig. 3A), keratin (Fig. 3B), and saline nerves (Fig. 3C). The 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 $1\mu\text{m}$ and $9\mu\text{m}$ 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^{29,30} (Fig. 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 $2\mu\text{m}$ (Fig. 4A, B, D–G). The myelin thickness of the native nerve also displayed a bimodal distribution with a small peak at $0.5\mu\text{m}$ and major

peak at $1.7\mu\text{m}$ (Fig. 5C, G, H). Again, the keratin and saline groups had a unimodal distribution for myelin thickness at both the middle and distal tissue segments. In the middle nerve segments, the keratin group had a peak at $0.6\mu\text{m}$, while the saline group had a peak at $0.9\mu\text{m}$ (Fig. 5A, B, G). In the distal tissue, the keratin nerves showed a peak between 0.4 and $0.7\mu\text{m}$ and the saline group showed a peak at $0.9\mu\text{m}$ (Fig. 5D, E, H). Quantification of the g-ratio showed no significant differences between the keratin (0.468) and saline (0.435) treatment groups and native nerve controls (0.426 , $p=0.477$, Fig. 5F).

Muscle morphometry

Analysis of the APB muscle myofiber CSA (Fig. 6C–E) showed a larger average myofiber size for the uninjured ($7676 \pm 2045\mu\text{m}^2$) and keratin ($6776 \pm 1973\mu\text{m}^2$) treatment groups compared with the saline group ($4878 \pm 1049\mu\text{m}^2$, $p=0.081$, Fig. 6A). The keratin muscles showed 12.2% atrophy compared with 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/ mm^2) and keratin group (542.5 ± 175.9 fibers/ mm^2) compared with the saline group (220.2 ± 38.1 fibers/ mm^2 , $p=0.0021$, Fig. 6B).

FIG. 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 are 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 $\pm$ SD ($n=8$ keratin, $n=6$ saline, $n=4$ native controls). *** $p<0.001$, ** $p<0.01$, * $p<0.05$. Color images available online at www.liebertpub.com/tea

Antibody titer

The ELISA revealed that none of the animals receiving a bolus injection of keratin had a detectable difference in the antigen binding activity of the serum compared with the control animals that were keratin naïve (KN) (Fig. 7). The highest detected value of 5019 ± 2702 ng/mL actually corresponded to one of the KN animals. In addition, the albumin control, used to assess the potential background signal from the serum itself, was strongly positive. The anti-basic hair keratin K81 antibody utilized as a positive control gave values for the $0.5 \mu\text{g}$ (721 ± 255 ng/mL) and $1.0 \mu\text{g}$ (1962 ± 1145 ng/mL) concentrations that indicated an appropriate dose response, thus indicating that the assay was functioning appropriately. No adverse reaction to the keratin bolus injection was noted. These data suggest that keratin

does not elicit an adaptive immune response when implanted.

Discussion

It is important to note that the saline-filled conduits were not expected to remain as such. As described by Lundborg,³¹ nerve conduits quickly become filled with exudates from the severed nerve stumps and a fibrin matrix forms to act as a natural scaffold for regeneration. In choosing a 1 cm 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)²⁶

FIG. 3. Toluidine blue staining of 0.5- μ m semithin sections shows differences in the 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 μ m. Color images available online at www.liebertpub.com/tea

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. In addition, a control group using sensory nerve autograft was considered, but this is not a common clinical repair strategy at the 1 cm gap distance and would likely not be considered by a skilled surgeon if better nerve conduit filler technologies were commercially available.

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 reinnervated 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 5 mm 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 5 mm gap treated with either a saline-filled conduit or sural nerve autograft.²⁹ In contrast, the present study showed that the keratin-treated group maintained higher NCV values compared with the saline treatment group. Another NHP study by Krarup *et al.* showed no significant differences in the APB CMAP amplitude between direct suture of the median nerve, a 5 mm nerve guide, and a 5 and 20 mm sural nerve autograft after a period of 882 days. However, longer nerve guides (e.g., 20 and 50 mm) and autografts (50 mm) had a significantly lower APB CMAP amplitude compared with direct suture.³² 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 the 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^{16,17} and rabbit²⁵ tibial nerve studies utilizing the keratin hydrogel as luminal filler. Both of these studies included a sural nerve autograft group and showed that the keratin group had significantly larger nerves compared with autograft in the mouse, and larger nerves compared with autograft in the rabbit. In the present study, the native median axon density was significantly higher than both keratin and saline nerve groups. Whereas 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

FIG. 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 with 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). Color images available online at www.liebertpub.com/tea

evaluate motor recovery and only the recurrent branch of the median nerve was included in the distal conduit. During regeneration, neurons can project axon collaterals that are later pruned once target specificity is achieved and future studies may be used to determine if increased collateral sprouting in the keratin group led to a significantly greater number of axons per nerve compared with the saline group at 12 months.³³

The frequency distribution of the axon diameter showed 2 distinct peaks in the native tissue compared with 1 peak in both the keratin- and saline-treated nerve groups. These distributions appeared almost identical between the treatment groups in both the middle and distal nerve sections. Conversely, Archibald *et al.* found that the axon diameter distribution patterns for sural nerve autograft and saline conduit repairs of a 5 mm median nerve gap were similar, although this was approximately 45 months after surgery.²⁹ The myelin thickness frequency distribution was also bi-

modal for the native median nerve compared with unimodal for the keratin and saline groups in both the middle and distal segments. This may simply reflect the correlation of myelin thickness and axon size that has been demonstrated in another study,³⁴ which was also found to be more pronounced in mature nerve compared with regenerating nerve. 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.^{4,35} 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.³⁶

FIG. 5. Regenerated median nerves have a unimodal distribution of myelin thickness although the 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) The uninjured median nerve has two distinct peaks corresponding to the small and large diameter axons. (F) The G-ratio between the axon diameter and myelinated fiber diameter shows no significant differences between groups ($p=0.477$). (G, H) Graphs overlaying the distributions for the middle (A–C) and distal (D–F) 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). Color images available online at www.liebertpub.com/tea

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* 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 (4.16 mg/mL) of the material dissolved in basal media.²⁷ Biocompatibility studies of subcutaneous implants were also performed in mice showing no adverse effects of the material and degradation within 8 weeks.²⁷ Despite these promising observations, the potential immunogenicity of keratin biomaterials has not been directly tested and therefore remains an open issue for clinical translation.

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 biomaterial scaffold that resists a proteolytic environment better than a native fibrin matrix, a hypothesis has been postulated that the interaction between the keratin proteins and SC drives the enhanced nerve regeneration seen in this study and the 3 previously mentioned animal studies.^{16,17,25} Previous studies have suggested that keratin proteins may induce cell proliferation, cell adhesion, and alter gene expression.¹⁶ However, these studies were performed using an immortalized SC line and need to be repeated with primary SC explants before definitive conclusions regarding a potential mechanism can be

FIG. 6. Keratin enhances abductor pollicis brevis (APB) muscle regeneration. **(A)** Regenerated keratin APB muscles have a larger average myofiber cross-sectional area than saline muscles ($p = 0.08$). **(B)** APB myofiber density is significantly higher for the keratin muscles compared with saline muscles. $**p < 0.01$. **(C–E)** Cross-sectional photomicrographs of uninjured APB muscle **(C)**, regenerated keratin APB muscle **(D)** and regenerated saline APB muscle **(E)** stained with Masson's trichrome. Scale bars = 50 μm. Color images available online at www.liebertpub.com/tea

proffered. 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 who have undergone conduit graft surgery in large mixed motor nerves such as the ulnar and median nerve. Lundborg *et al.* published a 5-year follow-up of 30 patients who 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 two groups and all patients showed continued improvement throughout the study.³⁷ Silicone is a bioinert

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.^{7,11,14} Preclinical studies have often shown the comparability between autograft repair and conduit repair, although for small gaps. There is a need to further test luminal fillers or modified conduits in larger nerve gaps (≥ 5 cm) 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. Whereas the 1 cm 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 3 cm, the current limit of commercially available devices.

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FIG. 7. Keratin appears to be nonimmunogenic. An ELISA of NHP serum shows no difference in the antigen-binding activity between animals that received a keratin bolus injection 12 months after initial exposure to implanted keratin and animals naïve to keratin (KN). The positive control (anti-K81 antibody) shows the expected dose response. However, the human serum albumin control shows high levels of nonspecific binding ($**p < 0.01$ for 0.5 μg and $***p < 0.001$ for 1.0 μg concentration). Results are expressed as mean $\pm$ SD ($n = 8$ keratin, $n = 6$ saline, $n = 4$ controls; for the ELISA $n = 3$ replicates per sample). Color images available online at www.liebertpub.com/tea

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Disclosure Statement

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

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