Award Number: W81XWH-12-1-0511

TITLE: Large Extremity Peripheral Nerve Repair

PRINCIPAL INVESTIGATOR: Robert W. Redmond Ph.D.

CONTRACTING ORGANIZATION: Massachusetts General Hospital
Boston, MA 02114

REPORT DATE: October 2013

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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14. ABSTRACT

High velocity projectiles and fragments from improvised explosive devices (IED) cause traumatic tissue damage with approximately 20-30% of all extremity injuries and >80% of penetrating injuries being associated with peripheral nerve damage, typically involving large segmental nerve deficits. Standard repair of such injuries uses autologous nerve graft, secured by suture. Outcomes are often unsatisfactory and poor recovery of function adversely affects quality of life and return to active duty. We are investigating a sutureless, light-activated technology for sealing nerve grafts to produce an immediate water-tight seal that protects and optimizes the regenerating nerve environment. Our studies have shown that biocompatible chemical crosslinking of thin amnion and SIS sections considerably strengthens the materials and protects them from rapid biodegradation in vivo that would compromise their function as nerve wrap sealants during the regeneration process. Outcomes of rodent studies of segmental nerve deficit repair using isograft show the best performing wrap/fixation method to be sutureless photochemical tissue bonding with the crosslinked amnion wrap. This approach will now be taken into final rodent testing with allograft prior to ultimate deployment in a large animal model to transition to human trials.

15. SUBJECT TERMS- none provided

16. SECURITY CLASSIFICATION OF:

| a. REPORT | U |
| b. ABSTRACT | U |
| c. THIS PAGE | U |

17. LIMITATION OF ABSTRACT

UU

18. NUMBER OF PAGES

31

19. TELEPHONE NUMBER (include area code)

USAMRMC
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Introduction.

The goal of the research performed in this project is to develop a new technology for repair of peripheral nerve injuries involving significant neural deficit with improved functional outcomes for the wounded warrior. The research addresses drawbacks of current methods of suture attachment of nerve grafts and involves development of both a sutureless fixation method to place the nerve graft and an optimal wrap material to seal the endoneurial environment for regeneration. Reduction in needle trauma, reduced inflammation and scarring and sealing the endoneurial environment should all contribute to improved clinical outcomes.

Body

Task 1– Determine mechanical properties, seal strength and resistance to biodegradation of candidate photochemical nerve wrap biomaterials. (Months 1-10)

Task 1a. Regulatory approval of use of human tissue by Partners (MGH) IRB and review and approval by USAMRMC Office of Research Protections (human amniotic membrane, HAM). (Months 1-4, MGH: Winograd/Redmond)

Regulatory approval for the use of discarded human tissue (Amniotic membrane) was obtained from both the MGH Institutional Review Board and the USAMRMC Office of Research Protections in August 2012.

Task 1b. Regulatory approval of rodent sciatic nerve for nerve wrap bond measurements by MGH IACUC and review and approval by USAMRMC Office of Research Protections (ACURO). (Months 1-4, MGH: Redmond)

Approvals for the rodent protocols to be used in Task 2 were obtained from the MGH IACUC (protocol #2012N000117) and ACURO approval on 11/19/2012.

Task 1c. Mechanical testing of AxoGuard® nerve protector (Months 2-4, MGH: Redmond)

The AxoGuard nerve protector proved to be too thick for facile use in photochemical tissue bonding experiments in the rodent model. It was not possible to wrap this material around the small caliber rat sciatic nerve without undue mechanical tension on the wrap that tended to disrupt the contact between nerve and wrap. This required a search for a different source of commercial nerve wrap material, described below in Task 1i.

Task 1d. Processing of HAM and crosslinking with EDC to make xHAM. (Months 4-6, MGH: Redmond)

Task 1e. Mechanical testing (ultimate stress and Young’s Modulus) of HAM and xHAM. (Months 4-6, MGH: Redmond)

We have completed processing of human amniotic membrane (HAM) and chemical crosslinking with EDC/NHS to make the crosslinked HAM that should resist biodegradation in vivo. A chemical crosslinking system (EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide), a water soluble agent used with N-hydroxysuccinimide (NHS) for coupling carboxyl groups with primary amines to form amide bonds in proteins) was used at different concentrations under conditions of one hour incubation at room temperature and the resultant mechanical properties measured using a microtensiometer. Figures 1A and 1B show the effect of chemical crosslinker on the maximum stress and Young’s modulus...
(stiffness) of HAM, measured using a microtensiometer. As expected, crosslinking imparts a greater
strength and stiffness to the HAM, especially at the higher concentrations used.

**Task 1f. Determine resistance of nerve wraps to collagenase digestion. (Months 4-6, MGH: Redmond)**

Biodegradation of HAM as a function of EDC/NHS treatment was determined in the presence of
0.1% collagenase, a high concentration used in our laboratory for extraction of chondrocytes from
cartilage. Two assays were used (a) time to complete dissolution and (b) rate of release of amine
containing amino acids using the fluorescamine assay.

As can be seen in Figure 2, crosslinking of HAM with EDC/NHS has a large effect on the ability of
HAM to resist biodegradation. Figure 2A shows that the uncrosslinked HAM is dissolved in the first
hour of treatment whereas all treated samples remain intact even up to 24 h. A more detailed
approach using the fluorescamine assay (Figure 2B) to detect amino-acid residues released on
degradation shows that increasing EDC/NHS reduces rate and extent of degradation measured in this
fashion. This is a highly positive result as a major limiting factor for use in nerve repair would be
rapid degradation of the HAM wrap in vivo and this treatment affords considerable protection.

**Task 1g. Rat sciatic nerve harvest from 20 Lewis rats. (Months 6-8, MGH: Randolph/Winograd)**

**Task 1h. Measure bonding strengths of wraps to ex vivo sciatic nerve (months 6-8, MGH: Redmond)**

One of the strategies for sutureless graft fixation in this project involves photochemical bonding of a
nerve wrap at the graft/nerve stump junction. Studies above show that chemical crosslinking with
EDC/NHS strengthens the wrap material and increases its resistance to biodegradation. It is however,
important to evaluate whether this chemical crosslinking could interfere with the ability to
photochemically bond the wrap material around the epineurium. Thus, rat sciatic nerves were
harvested from donor rats immediately post-euthanasia (Task 1g) and bonding of the wrap around the
nerve ends performed following application of 0.1% Rose Bengal dye in saline to wrap and
epineurium with illumination at 532 nm. The HAM wrap/nerve sample was then mounted in a
microtensiometer, as shown in Figure 3 and the tensile load increased until bond failure.

The bond strength of the EDC/NHS treated HAM remains unchanged until the highest tested
concentration of 8mM/2mM (EDC/NHS), when a statistically significant decrease is observed with
respect to control (p<0.05). At this higher concentration the xHAM becomes brittle and more difficult
to handle. Figure 4 shows the data for bond strength as a function of treatment parameters.

In Task 1h we focused on determining the failure strength of the bond formed between ex vivo nerve
segments as a function of the fixation procedure, in preparation for the corresponding rat experiments
in Task 2. Figure 5 shows the results obtained with the three fixation methods under study (a)
epineurial suture, (b) fibrin glue and (c) photochemical tissue bonding (PTB) with a wrap material.
All methods induced bonding between the nerve segments with bond strength in the order of
suture>PTB>fibrin glue. Conventional epineurial suturing using six 10.0 nylon sutures resulted in the
strongest bond. This bond was significantly greater than any of the bonds created by PTB (p<0.05).
The strength of the bonds created by PTB were not significantly different from those created
following 4-suture epineurial repair. The caliber of the rat sciatic nerve is comparable to a human
digital nerve. The use of four epineurial sutures in this situation is clinically realistic and is therefore
supportive of the bond strength imparted by PTB. Little difference was seen between uncrosslinked
HAM or crosslinked xHAM, except at the highest crosslinker concentration used. In the rat repair
model in Aim 2 we have chosen to use the crosslinked HAM due to its increased resistance to enzymatic degradation. Although bond strength is not really an issue in nerve repair, which should ideally be tension-free, these results show that the PTB method can provide fixation strengths approaching that of conventional microsurgery and that the PTB repair is unlikely to be disturbed in vivo.

The results in Figure 5 were obtained with PTB parameters of 0.1% Rose Bengal with 532 nm light delivered at an irradiance of 0.5 W/cm² and a fluence of 60 J/cm². To further explore the optimal dosimetry conditions for the rat experiments in Aim 2, we performed a fluence dependence study using light delivered at an irradiance of 0.5 W/cm² for various durations. Figure 6 shows the fluence dependence of the bond strength formed using PTB/xHAM (4mM/1mM (EDC/NHS)) to reattach the nerve segments ex vivo. A fluence of 60 J/cm² resulted in superior bond strength in comparison to 30, 120 and 240 J/cm² (p<0.05). Interestingly, bond strength was significantly weaker with the highest fluence, presumably due to increasing friability of the amnion wrap. Those nerve/wrap preparations treated with no illumination predictably had negligible bond strength. An irradiance of 0.5W/cm² and a fluence of 60J/cm² equates to an illumination duration of 120 seconds (60 seconds per nerve/wrap side) and this was felt to be clinically acceptable for use in the animal studies in Aim 2.

The anatomy of HAM is shown pictorially in Figure 7. In vivo, the epithelial layer is in contact with the amniotic fluid while the spongy layer is in contact with the chorion. During preparation the amnion is de-epithelialized but there remains the question as to “which way up” the HAM should be bonded to the nerve. To that end we performed experiments where we were careful to note the surface in contact with the nerve and the resultant bond strength obtained after bonding with 60 J/cm² of 532 nm light delivered at an irradiance of 0.5 W/cm². Figure 8 shows that the bond strength between nerve and amnion was not significantly different to the epithelial and chorionic surfaces. This finding has helped simplify processing and storage of the amnion and also intra-operative handling.

The outcome of these experiments has helped confirm the following optimum conditions to apply to the in-vivo rodent survival operations in aim 2:

- Amnion cross-linked with 4mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)/1mM H-hydroxysuccinimide (NHS)
- Laser irradiance of 0.5 W/cm² and fluence of 60 J/cm²
- Amniotic epithelial and chorionic surfaces bond equally well

Task 1i. Data analysis, conclusions and consideration of alternative wrap materials, if required (Months 6-10, MGH: Redmond)

In this task we focused on determining the best candidate commercial wrap to evaluate in PTB studies for nerve repair. As outlined in the original project proposal, our plan was to use a tubular swine intestinal submucosa (SIS) product called AxoGuard manufactured by AxoGen. Following initial trials this product was found to be unsuitable as it was too thick and possessed too much inherent shape memory to permit easy wrapping around the small diameter rat sciatic nerve. Intimate contact between tissue surfaces is an essential pre-requisite for photochemical tissue bonding. Following this discovery, we conducted a thorough search of alternative commercially available biomaterials that could satisfy our needs.

The following products were sampled and assessed for their conformability and bonding ability:
1. AxoGuard (multi-layer SIS – AxoGen)
2. NeuraGen (Collagen – Integra)
3. NeuraWrap (Collagen – Integra)
4. Tenoglide (Collagen – Integra)
5. NeuraMend (Collagen – Stryker)
6. NeuraMatrix (Collagen – Stryker)
7. Colafilm (Collagen – Innacol)
8. Amniofix (Collagen – MiMedx)
9. SurgiMend (Collagen – TEI Biosciences)
10. Oasis (Single layer SIS – HealthPoint)

Options 1-9 were also found to be unsuitable due to similar problems regarding excessive material thickness, stiffness, shape memory and inability of the material to conform satisfactorily around the rat sciatic nerve.

Option 10 is a single layer SIS product that met our requirements. Oasis is a product marketed and distributed as a wound dressing by HealthPoint. Although the material is approximately double the thickness of human amnion, it was sufficiently thin to allow circumferential nerve wrapping and close contact between wrap and epineurium. In fact, we discovered that both AxoGuard and Oasis SIS products are manufactured by Cook Medical. AxoGuard is simply a multi-layered SIS product. Given that the large animal studies with large caliber nerves in Aim 3 will use AxoGuard we feel that the single layer SIS material is totally appropriate for these small animal studies.

Biomechanical testing of the single layer SIS material showed a Young’s modulus (Fig. 9) and a maximum load to failure (Fig. 10) that were considerably greater than that of human amnion (See Figs. 1 and 2). Similar to earlier HAM studies the chemical crosslinking of SIS with increasing concentrations of EDC/NHS, gave an increase in Young’s modulus and maximum load to failure. In fact, we discovered that both AxoGuard and Oasis SIS products are manufactured by Cook Medical. AxoGuard is simply a multi-layered SIS product. Given that the large animal studies with large caliber nerves in Aim 3 will use AxoGuard we feel that the single layer SIS material is totally appropriate for these small animal studies.

As with the human amnion nerve wraps, it was important for us to confirm that, in addition to increasing the resistance to enzymatic degradation, EDC/NHS crosslinking of SIS did not interfere with photochemical tissue bonding. Figure 12 shows that there was no significant drop in bond strength measured by ex-vivo tensiometer testing. Interestingly, bond strength between SIS nerve wrap and sciatic epineurium was significantly greater than that found with untreated and crosslinked human amnion.

**Milestones** for Task 1 include the following, with decisions taken at the joint meetings of Partnering PI’s, held at MGH, WRNMMC and mutual conferences.

- Obtain MGH and DOD approvals for all protocols
- Determine mechanical properties of wrap materials and establish suitability for use in PTB. Modify processing of wraps and utilize alternate wraps if necessary
- Determine bond strength of wraps to ex-vivo nerve and synthetic graft. Modify processing of wraps and utilize alternate wraps if necessary.
• Collate results and determine best nerve wraps to use in Task 2

All the above milestones have been met so far, as described above.

• Prepare publications and presentations based on Task 1 research results

A joint presentation entitled “Large Extremity Peripheral Nerve Repair” by N. Fairbairn, J. Ng-Glazier, A. Meppelink, MA Randolph, JM Winograd, IL Valerio, ME Fleming, IE Kochevar and RW Redmond was given at the Military Health System Research Symposium (MHSRS) symposium in Fort Lauderdale, August 18-21, 2013.

Task 2 – Determine efficacy of nerve regeneration in a rodent model of segmental nerve deficit injury as a function of wrap and fixation procedure. (Months 6-22).

Task 2a. Regulatory approval for rodent study of segmental deficit repair by MGH IACUC and review and approval by USAMRMC Office of Research Protections (ACURO). (Months 1-4, MGH: Redmond/Winograd/Randolph)

This aim involves a large study of peripheral nerve repair in a rat sciatic nerve model using isogeneic Lewis rats. The animal protocol for these experiments has been reviewed and received IACUC approval at the Massachusetts General Hospital (protocol #2012N000117) and was also granted ACURO approval on 11/19/2012.

Task 2b. Rodent surgeries for segmental deficit and repair using isograft with 110 Lewis rats. (Months 6-8, MGH: Winograd/Randolph)

A total of 110 rodents have now undergone surgery, as shown in Table 1. Rodent survival surgeries commenced on 2/26/2013 and were completed on 5/10/2013.

Task 2c. Biweekly functional recovery testing by gait analysis in isograft study. (Months 6-14, MGH: Redmond)

Functional recovery in each rodent during the 5-month follow-up period has been measured by monthly walking track analysis using the well-established Sciatic Function Index (SFI) as calculated from the paw-prints of the rodents as a function of time after surgery. As predicted, those animals in the negative control group (no repair following nerve deficit injury) experienced no functional recovery as illustrated by a complete lack of correction of the sciatic function index (-96.2+/−3.7). A value of -100 indicates zero functional recovery. Despite performing well in ex vivo experiments, those isografts wrapped with the commercially sourced SIS material performed worst out of all biological nerve wraps. In SIS+suture and SIS+PTB groups, this was statistically significant in comparison to standard repair (positive control). Isografts wrapped with cross-linked human amnion and secured with PTB (xHAM+PTB) exhibited the greatest functional recovery value although this did not reach statistical significance in comparison to the positive control group (SFI = -67.93+/−5.11 vs -71.69+/−4.80). There was no statistically significant difference between any of the remaining experimental groups compared to the positive control group. Table 2 provides a summary of the functional data.

It should be noted that although walking track analysis is a well-accepted outcome measure for functional assessment following rodent peripheral nerve surgery, the method has several recognised limitations that have been encountered. The inked footprints are rarely perfect and are open to
considerable inter-observer variability. Walking track analysis is also limited by experimental hind-paw clawing. Although this was not a major problem, a small number of rodents at later time points in the majority of experimental groups were excluded from the calculation of mean SFIs due to unmeasurable footprints. Clawing results due to injury and incomplete recovery of the nerve supply to intrinsic musculature in the hind foot and may be exacerbated by the lack of physical rehabilitative measures that would ordinarily be introduced in human subjects. Although not thoroughly assessed, the presence of clawing did not seem to affect the reinnervation and retention of gastrocnemius muscle mass and therefore this problem may be limited to walking track analysis. Thus, SFI results are useful but a decision regarding which wrap and fixation method to proceed with in the next step cannot be taken solely on this metric.

Task 2d. Gastrocnemius muscle harvest and muscle mass retentions in isograft study (Months 12-14, MGH: Redmond)

Following sacrifice, both experimental left sided gastrocnemius muscle and contralateral right sided control muscle were harvested from each rodent. Wet muscle mass was recorded immediately following harvest and percentage muscle mass retention calculated. Mean muscle mass retention in the negative control group was only 9.2% +/- 0.92. As expected, this was significantly less that achieved in the standard repair group. Greatest muscle mass retention occurred in the xHAM+PTB group and the increase over standard repair was statistically significant (67.3% +/- 4.44 vs 60.0% +/-5.16; P=0.02). There was no statistically significant difference between the positive control group and any of the remaining experimental groups. Wraps secured with fibrin glue performed consistently better than those secured with suture although these effects did not reach statistical significance. Table 3 provides a summary of muscle mass retention data.

Task 2e. Histomorphometric analysis of proximal and distal fibers in isograft study (Months 12-16, MGH: Redmond/Winograd)

Following sacrifice, left sciatic nerves were harvested and sent for histology. Nerves were harvested from a distance 5mm proximal to the proximal isograft neurorrhaphy site to 5mm distal to the distal neurorrhaphy. Following 24-hours of fixation, each nerve was cut into proximal, mid-graft and distal sections. Following dehydration and epoxy resin embedding, 1-micron slices of each specimen were cut and mounted for histomorphometric analysis. Axon counts and G-ratio (marker of myelination) will be measured from scanned images. All histologic specimens have been collected and prepared for sectioning. Around 40% of the samples have been sectioned at this point and the remainder should be finished in the next few weeks. At that point we will commence image analysis which should be completed by the end of the year.

Task 2f. Determination of axonal migration, endoneurial scarring in isograft study. (Months 12-16, MGH: Redmond/Randolph/Winograd)

Previous studies from our lab have suggested that, in addition to creating a water tight seal at the neurorrhaphy site, photochemical tissue bonding also reduces the formation of fibrinous adhesions around the nerve. Although this is difficult to quantify, observations following rodent sacrifice in this study have supported this (Figure 13).

Task 2g. Decision on wrap/fixation method for Avance® nerve graft studies in rodent model. (Month 16,
As detailed in the original project proposal, our intention was to purchase the Avance processed human allograft from AxoGen for use in the final phase of the rodent studies. After discussion with AxoGen and a review of the most recent literature on the use of human versus rat allograft in rodent models, we were advised that the human sourced nerve may not be optimal for rodent studies (due to cross-species immunoreactivity being greater than originally expected) and that for the purpose of these studies it would be better use processed rodent allograft. We have since harvested, frozen and stored donor rat sciatic nerve before shipping to AxoGen for processing. A Material Transfer Agreement has been agreed between MGH and AxoGen and delivery of the processed nerve is expected in November. This should coincide with full analysis of the histomorphometric data and will hopefully prevent any delay between establishing the optimum wrap/fixation method and the commencement of the remaining 2 groups of n=10 rodent allograft surgeries.

Although the vital histomorphometry data is not yet complete, early indications from both muscle mass retention and walking track data support the use of PTB/xHAM as the lead fixation/wrap method for the next stage in rodent studies using processed allograft. The SIS material did not perform as well as expected from ex vivo studies. In two animals in the SIS+PTB group, nerve repairs were not successful. At sacrifice the repaired sciatic nerves were not in continuity. On analysis, these animals were indistinguishable to animals in the no repair (negative control) group. These two animals were excluded from the statistical analysis of gastrocnemius muscle mass retention. Although SIS was the most suitable commercially available product we could find, it is twice as thick as human amniotic membrane (100 microns vs 50 microns). During survival surgeries, it was noted that photochemical bonding between SIS and epineurium was less successful when compared to amnion. It is likely that this compromised the formation of a water tight seal at the repair site and reduced the overall strength of repair, leading to dehiscence.

**Milestones** for Task 2 include the following, with decisions taken at the quarterly meetings of Partnering PI's, held alternately at MGH and WRNMMC.

- Obtain MGH and DoD approvals for all protocols.
- Complete all rodent surgeries and repair groups for isograft study.
- Determine lead wrap/fixation method for best functional recovery in isograft model and proceed with this method for Avance® nerve graft study
- Complete all rodent surgeries and repair groups for Avance® nerve graft study
- Analyze recovery of function and histomorphometry data and determine whether to proceed with Avance® nerve graft or autograft for large animal study in Aim 3
- Prepare publications and presentations based on Task 2 research results.

The first two milestones are complete and milestone 3 awaits only the histomorphometric analysis to make the decision regarding which wrap/fixation method to use in the processed isograft study.
Key Research Accomplishments:

- Demonstrated that human amniotic membrane (HAM) can be strengthened by a biocompatible crosslinking process.
- Demonstrated that crosslinking dramatically increases resistance of HAM to biodegradation, thus, increasing its longevity as a wrap for nerve graft sealing in vivo.
- Demonstrated that chemical, crosslinking of HAM does not affect its ability as a wrap for photochemical sealing over nerve graft coaptation sites.
- Demonstrated that photochemical sealing of crosslinked HAM (xHAM) over nerve graft coaptation sites can be performed in a facile manner in a rat sciatic nerve model.
- Demonstrated that commercial single layer SIS (swine intestinal submucosa, Oasis) is a stronger material than HAM and can be further strengthened by chemical crosslinking with increased resistance to biodegradation.
- Demonstrated that both SIS and HAM and their crosslinked derivatives can be used as photochemical wraps in vivo over nerve graft coaptation sites in a facile manner in a rat sciatic nerve model.
- Demonstrated improved nerve regeneration in a functional recovery model (SFI, sciatic function index) using PTB/xHAM wrap compared to standard (suture) of care microsurgery.
- Demonstrated improved nerve regeneration in a muscle mass retention model (contralateral control (unoperated) vs treated sciatic nerve graft) using PTB/xHAM wrap compared to standard (suture) of care microsurgery.
Reportable Outcomes:

Conference Presentations
Conclusion:

At the end of the first year of this project there have already been some notable discoveries that may impact military health care in the near future. We have demonstrated that biocompatible chemical crosslinking can be used to strengthen thin nerve wraps and increase resistance to biodegradation such that the wrap retains its sealing ability throughout the time taken for the regenerating axons to traverse the nerve graft and pass the distal coaptation site. Thus, we have been able to replicate prior successful outcomes on nerve transection injuries with uncrosslinked wraps where longevity of the wrap in vivo is not as important. The light-activated sealing of the nerve wrap around the coaptation sites obviates the need for suture attachment of the graft and a host of advantages result from the lack of needle injury, inflammation and scarring, possible infection and axonal scape that can reduce functional recovery and cause neuroma formation. To date, processed crosslinked human amnion, a thin biological membrane (< 50 micron) has demonstrated the best potential as a nerve wrap for photochemical sealing in rodent models in vivo, with other commercial nerve wraps having proven less suitable due to greater thickness and inability to conform to the dimensions of the rat sciatic nerve. However, ex vivo testing of swine intestinal submucosa (SIS) has been promising and as one scales the nerve up in size in large animal studies in Task 3 of the project this material is worth revisiting.

The next important step in the overall project is the use of the optimum wrap/fixation method in a rodent model using processed allograft nerves instead of autograft, the latter being the standard of care. In many seriously wounded combatants the injuries are so severe, including multiple amputation, that there is no availability of donor autograft and alternative nerve bridges must be sought. Processed allograft is currently under investigation as it can be readily available and stored ready for use. The next step in our project will determine if the advantages inherent in the sutureless wrap/fixation method using processed allograft can reproduce outcomes similar to, or better than, autograft reconstruction. If so, this will represent major progress in the treatment of peripheral nerve injury associated with military trauma. This approach is capable of rapid commercialization and translation into military medicine. The IP has been filed and the materials involved can be easily stored in a prolonged manner for rapid deployment.
Table 1: Rodent survival operations to date (✓ = operation completed)

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<thead>
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<th>Rodent</th>
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Table 2: Summary of sciatic function index (SFI) outcomes at 150 days post-repair as a function of treatment group.

<table>
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<tr>
<th>Experimental group</th>
<th>Mean 5-month SFI</th>
<th>SD</th>
<th>P value *</th>
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<tr>
<td>Negative control</td>
<td>-96.23</td>
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<td>Positive control</td>
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<td>HAM+suture</td>
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<td>xHAM+fibrin</td>
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<td>SIS+suture</td>
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<td>SIS+PTB</td>
<td>-84.97</td>
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*comparison between treatment group and positive control group
**Table 3:** Summary of gastrocnemius muscle mass retention data at 150 days post-repair as a function of treatment group.

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Mean left gastrocnemius muscle mass retention (%)</th>
<th>SD</th>
<th>P value*</th>
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</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>9.2</td>
<td>0.92</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Positive control</td>
<td>60.0</td>
<td>5.16</td>
<td>1</td>
</tr>
<tr>
<td>HAM+suture</td>
<td>56.0</td>
<td>5.60</td>
<td>1</td>
</tr>
<tr>
<td>HAM+fibrin</td>
<td>59.8</td>
<td>5.43</td>
<td>1</td>
</tr>
<tr>
<td>HAM+PTB</td>
<td>62.5</td>
<td>4.01</td>
<td>1</td>
</tr>
<tr>
<td>xHAM+suture</td>
<td>57.7</td>
<td>5.12</td>
<td>1</td>
</tr>
<tr>
<td>xHAM+fibrin</td>
<td>62.7</td>
<td>4.30</td>
<td>1</td>
</tr>
<tr>
<td>xHAM+PTB</td>
<td>67.3</td>
<td>4.44</td>
<td>0.02</td>
</tr>
<tr>
<td>SIS+suture</td>
<td>54.9</td>
<td>4.46</td>
<td>0.68</td>
</tr>
<tr>
<td>SIS+fibrin</td>
<td>58.5</td>
<td>5.44</td>
<td>1</td>
</tr>
<tr>
<td>SIS+PTB</td>
<td>54.1</td>
<td>3.18</td>
<td>0.37</td>
</tr>
</tbody>
</table>

*comparison between treatment group and positive control group
**Figure 1A:** Effect of EDC/NHS crosslinker concentration on maximum stress of HAM (n=5, * p<0.1, ** p<0.05).

![Graph showing mean maximum stress of HAM vs. crosslinker concentration](image-url)

Mean max stress (MPa) vs. crosslinker concentration (mM EDC/mM NHS)
Figure 1B: Effect of EDC/NHS crosslinker concentration on Young’s Modulus of HAM (n=5, * p<0.1, ** p<0.05).
Figure 2A: Effect of EDC/NHS crosslinking on gross degradation time on incubation of control and crosslinked HAM samples with 0.1% collagenase in PBS at 37°C.

** A **

** test stopped at 24 hours due to persistence of amnion

Crosslinked concentration (mM EDC/mM NHS)

<table>
<thead>
<tr>
<th>Crosslinked concentration (mM EDC/mM NHS)</th>
<th>Time (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.75</td>
</tr>
<tr>
<td>1/0.25</td>
<td>24**</td>
</tr>
<tr>
<td>2/0.5</td>
<td>24**</td>
</tr>
<tr>
<td>4/1</td>
<td>24**</td>
</tr>
<tr>
<td>8/2</td>
<td>24**</td>
</tr>
</tbody>
</table>
**Figure 2B:** Effect of EDC/NHS crosslinker concentration on amine containing amino-acid release detected by fluorescamine assay on incubation of control and crosslinked HAM samples with 0.1% collagenase in PBS at 37°C.

Fluorescamine degradation test for crosslinked human amnion

![Graph showing the effect of EDC/NHS crosslinker concentration on amine containing amino-acid release detected by fluorescamine assay.](image-url)
Figure 3: Schematic cartoon of wrap/nerve complex secured in the grips of the mechanical testing device.
Figure 4: Bond strength between rat sciatic nerve ex vivo and HAM wrap as a function of crosslinking with photochemical bonding using 532 nm light delivered at 0.5 W/cm² and a total fluence of 60 J/cm² (n=5, ** p<0.5).
Figure 5: nerve-amnion bond strength as a function of fixation method using 532 nm light delivered at 0.5 W/cm$^2$ and a total fluence of 60 J/cm$^2$ (n=5).
Figure 6: Nerve-amnion bond strength using PTB as a function of fluence (J/cm²) using 532 nm light delivered at 0.5 W/cm² (n=5, ** p<0.5).
Figure 7: Schematic representation of the anatomy of human amniotic membrane (HAM)
Figure 8: Amnion-nerve bond strength via PTB as a function of which surface was used as interface with nerve using 532 nm light delivered at 0.5 W/cm$^2$ and a total fluence of 60 J/cm$^2$. 
Figure 9: Effect of EDC/NHS crosslinker concentration on Young’s Modulus of SIS (n=5, *p<0.5)

Young’s Modulus of untreated and crosslinked SIS nerve wraps

![Bar chart showing Young's Modulus of untreated and crosslinked SIS nerve wraps.](chart.png)
Figure 10: Effect of EDC/NHS crosslinker concentration on Young’s Modulus of SIS (n=5, * p<0.5).

Maximum load to failure of untreated and crosslinked SIS nerve wraps

n=5

Untreated SIS  xSIS (1mM)  xSIS (2mM)  xSIS (4mM)  xSIS (8mM)

SIS wrap preparation

Mean load (N)
Figure 11: Digestion rates of crosslinked SIS as a function of crosslinker concentration in 0.1% collagenase solution

Fluorescamine degradation test for crosslinked swine intestinal submucosa (SIS)
Bond strength between sciatic nerve and candidate nerve wraps with varying crosslinker concentration

n=5

Mean load to failure (N)

Nerve wrap preparation

Untreated HAM  xHAM (1mM)  xHAM (2mM)  xHAM (4mM)  xHAM (8mM)  Untreated SIS  xSIS (1mM)  xSIS (4mM)
Figure 13: A. Findings following sacrifice in standard repair (positive control) group. Note the extent of fibrinous adhesions around proximal and distal neurorrhaphy sites (arrows). B. Findings following sacrifice in xHAM+PTB group. Note the relative absence of fibrinous adhesions around proximal and distal neurorrhaphy sites.