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TITLE: Acceleration of Regeneration of Large-Gap Peripheral Nerve Injuries Using Acellular Nerve Allografts plus amniotic Fluid Derived Stem Cells (AFS).

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CONTRACTING ORGANIZATION: Wake Forest University Health Sciences, Winston-Salem, NC 27157

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14. ABSTRACT
Major accomplishments this year include successful seeding of AFS into ANA. This accomplishment also documented that these cells remained viable up to 72 hours after seeding. The seeded constructs were used to repair critical-sized, large gap nerve injury in rats and their functional recovery was monitored longitudinally using digital video gait analysis as well as electrophysiologic and histologic outcomes. The data analysis is not complete, but preliminary data indicate that this AFS seeded ANA used for nerve repair results in an improved functional outcome for the rats compared to a nerve autograft, the current gold standard for tension-free repair of transected peripheral nerves.
The coming year will utilize these techniques for repairing large-gap (6 cm) nerve injuries in non-human primates.
These represent a more translational model of peripheral nerve injury and repair.

15. SUBJECT TERMS

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INTRODUCTION:

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INTRODUCTION:

The current research addresses repair of large gap peripheral nerve injuries. Clinically, nerve injuries greater than 3-5 cm have poor outcomes, regardless of repair techniques. One of factors limiting the re-growth of the axon across a large nerve gap may be the lack of trophic factors in the extracellular matrix of the interposed nerve graft. It is hypothesized that amniotic derived tissues possess trophic factors that support axonal re-growth and that incorporation of these tissues into an acellular nerve allograft will result in a nerve allograft with an enhanced potential to re-grow across a large nerve gap. This research will optimize cellular seeding of nerve allografts and functional assessment of that optimal construct in a rat sciatic nerve defect. Acellular nerve allografts with and without Amniotic Fluid Derived Stem Cells (AFS) will be used to repair large nerve gaps in rats (15 mm). The outcomes of these surgeries will be compared to those obtained with autograft nerve repairs that currently have the best outcomes for large-gap peripheral nerve repair. These techniques then will be employed in anon-human primate model (macaca fasciculata) of large-gap (6 cm) peripheral nerve injury and repair. Functional outcomes also will be assessed in this model. Finally, an intervention to prevent the degenerative changes that occur in neuromuscular junctions following delayed nerve injury/repair will be studied. If successful, the potential for the denervated muscle to regain function after nerve repair would be increased.

KEYWORDS:

Peripheral nerve injury, nerve allograft, amniotic derived stem cells, rats, macaca fasiculata, cell seeding of scaffolds

OVERALL PROJECT SUMMARY:

HYPOTHESES/OBJECTIVES

We hypothesize that acellular nerve allografts (ANA) can be seeded with amniotic fluid-derived stem cells (AFS) to promote and accelerate nerve regeneration. The presence of the AFS will provide support for the regenerating axons without the requirement of becoming Schwann cells. The specific aims to address this hypothesis are noted below:

SPECIFIC AIMS

Specific Aim 1: To demonstrate the ability to seed ANA with AFS using sub-atmospheric pressure (SAP) in vitro. Cell culture will be utilized to establish that the AFS cells remain on the allograft scaffold and that they do not differentiate into another cell type. Control cultures will employ ANA's with topically applied AFS but without SAP.

a. Follow-up experiments will examine Schwann cell migration in the presence of seeded allografts
b. Decellularization of species-specific mixed motor nerve tissue will be performed using decellularization and oxidation to improve the porosity of the allograft construct and enhance AFS cell seeding potential

Specific Aim 2: To establish the feasibility of using AFS seeded ANA's in large gap nerve repairs in vivo.

a. Rodent studies using ANA with/without AFS to repair large gap nerve defects
b. Enhancement of regenerative rate will be investigated
c. Motor end plate preservation studies to maintain muscle potential for re-innervation
d. Non-human primate studies in pre-clinical testing.

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Investigators: Initiating Principal Investigator – Thomas L. Smith, PhD
Partnering Principal Investigator – Zhongyu John Li, MD, PhD
Animal Use at this site: Animals will be used at this site

Progress over the past 24 months:

SOW Task 1 Specific Aim 1 (months 1-12):
In vitro studies to demonstrate the ability to seed Acellular nerve allografts (ANA) with Amniotic fluid derived stem cells and tissue (AFS) using subatmospheric pressure (SAP).

Task 1.1 (months 1-6) Cell seeding using SAP. Tests first will employ fibroblasts (NIH/T3T cells) and will examine the ability of the subatmospheric pressure seeding device (SAPSD) to improve penetration of the fibroblasts into the ANA. Secondarily, the magnitude and duration of exposure to SAP resulting in the greatest cell seeding density within the center of the ANA will be identified. Cell culture will be utilized to establish that the AFS cells remain on the allograft scaffold and that they do not differentiate into another cell type. Control cultures will employ ANA's with topically applied AFS but without SAP.

a. Decellularization of species specific mixed motor nerve tissue will be performed using decellularization and oxidation to improve the porosity of the allograft construct and enhance AFS cell seeding potential

Progress Task 1.1:

- Cell culture for Schwann cells has been established in the investigator's laboratory using explanted Schwann cells from donor rats.
    - Yields from explants are low, but that is expected. Improvements on the techniques are being employed to increase the yield of these cells.
    - This is a critical step because we will need to provide a cell culture environment that supports the cellularized nerve constructs.
    - A Schwannoma cell line also has been established so that pilot studies of cell seeding experiments can utilize adequate numbers of cells.

- Green Fluorescent Protein expressing fibroblasts (NIH/T3T cells) have been obtained and stocks of these cells are preserved in liquid nitrogen. These cells allow clear visualization of cell distributions within the experimental scaffolds.

- Material transfer agreements are in place and acellular nerve allografts for both humans and rats have been obtained from AxoGen.

- Material transfer agreements are in place and amniotic tissues have been obtained from NuTech (26-11-2013)

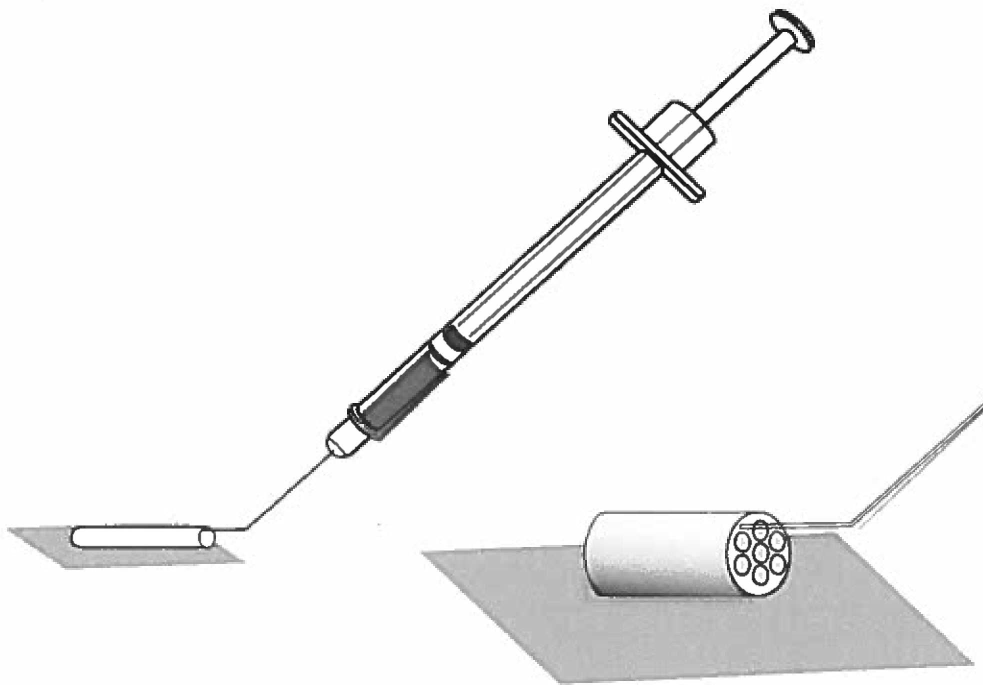
- Cell seeding experiments began in January 2014
    - Four series of cell seeding experiments have been performed using subatmospheric pressure (SAP) as well as static seeding. One million cells have been applied to scaffolds under SAP's of
        - -40 cm H_2O
        - -30 cm H_2O
        - -20 cm H_2O
        - -15 cm H_2O
    - Cell seeding of the ANA using SAP has not been adequate. The chambers providing SAP have been modified to maximize application of SAP to the acellular nerve scaffold.
- Sciatic nerves from 45 Lewis rats were harvested bilaterally, frozen in saline, and shipped to AxoGen for decellularization and processing. AxoGen could not obtain an adequate number of ANA from these donor nerves because the nerves from Lewis rats differ from those normally processed by AxoGen (from Sprague Dawley rats). AxoGen has provided us with ANA obtained

from Sprague Dawley rats and has documentation that these ANA can be implanted in Lewis rats.

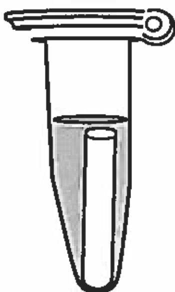
Cell seeing of 1.5 cm long ANA was successful using an injection technique of AFS cells into the ends of the graft and beneath the epinurium of the graft near the mid-point followed by perforation of the epinurium using a microneedle array. The AFS-seeded ANA then was cultured for 72 hours. The perforation of the epinurium allows diffusion of nutrients to maintain AFS viability following injection into the midsubstance of the ANA. Cell viability of AFS was documented in the ANA following 72 hours of incubation. This construct then was chosen for the repair of 1.5 cm nerve defects in the rat sciatic nerve during *in-vivo* studies.

Cell Seeding on allografts

$1X10^6$ AFS cells were injected underneath the epineurium of the decellularized sciatic nerve allografts using a 26 G syringe. Seeded graft were placed vertically at the bottom of a small centrifuge tube covered with DMEM containing 20% FBS for overnight then transferred to a 48 well plate for additional 48 hours.



Sciaticnerve graft



Sciatic nerve graft standing vertically in media overnight

48 hours

Task 1.1 complete

Task 1.2 (months 6-12) Using the pressures established in 1.1, AFS will be seeded onto the ANA. Flow cytometry and cell markers then will be utilized to document that the AFS do not differentiate after being seeded onto the ANA. If the AFS undergo a phenotypic change after seeding on the ANA, the new phenotype will be identified and measures will be employed to prevent this differentiation.

- We are resolving the cell seeding issues noted above. (months 1-12)
- Cell seeding issues resolved (months 12-18)
- Cell viability documented

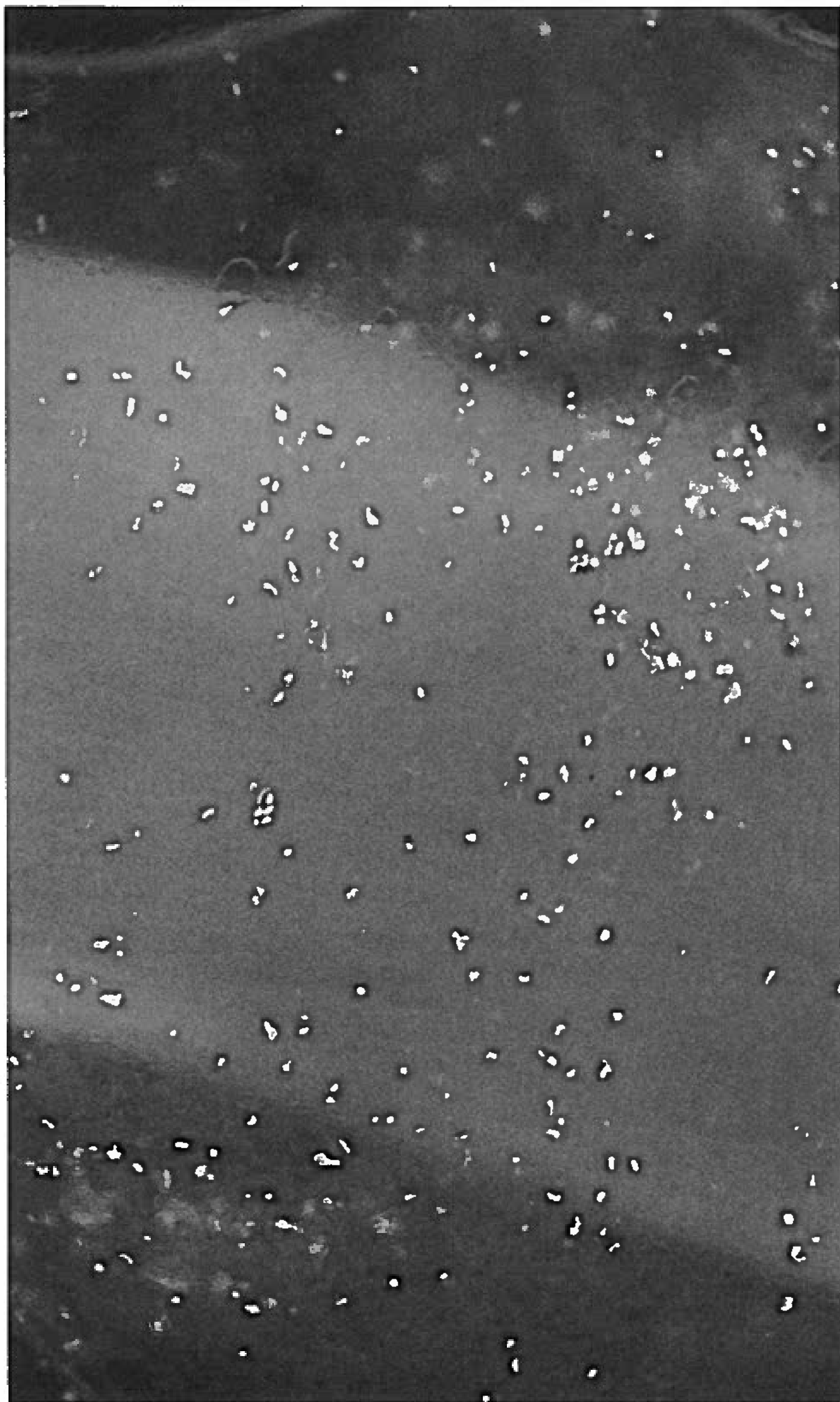
Progress on Task 1.2:

DAPI staining on longitudinal and cross sections of grafts showed cells spread evenly through the nerve fibers.

Longitudinal section of a sciatic nerve allograft -DAPI staining showed AFS cells nuclei appeared bright blue. Magnification X100

Table 1 Number of AFS cell-seeded allografts (as of 6/9/15)

Implanted AFS- Seeded Allograft	7
Control AFS-Seeded Allograft for testing cell infiltration	9



In vitro AFS cells seeded graft. $1x10^6$ AFS cells were injected under epineurium into the allograft. DAPI staining showed cells were viable 72 hours post injection.

Task 1.3 (months 6-18) Cell culture will be employed to study the migration of Schwann cells onto the AFS seeded scaffold. Commercially available Schwann cells (from Schwannoma cell lines) will be co-cultured with the AFS seeded ANA's. Parallel studies of Schwann cell infiltration of non-AFS seeded ANA's also will be performed. The density of Schwann cells in the middle of the ANA's will be assessed histologically at three different time points after initiating co-culture of the Schwann cells. These time points will be at 12 hours, 24 hours, and 48 hours.

Progress on Task 1.3:

- Co-culture systems are being established
- Accellular nerve allografts for rats (Sprague Dawley) have been received from AxoGen
- Migration studies of labeled cells within grafts currently are underway using labled AFS cells and 7T MRI imaging. (months 18-24)

Task 1.4 (months 12-18, if necessary) If the cell seeding results of 1.3 are unacceptable (poor seeding of the ANA), nerves will be decellularized and oxidized according to the techniques of Whitlock et al. (2007). This technique results in a more porous allograft structure. If the oxidation of the nerve allograft tissue is too aggressive, the techniques can be modified by decreasing the concentration of and duration of exposure to peracetic acid during the oxidation phase of the tissue treatment.

Task 2 Specific Aim 2 (months 6-36): In vivo studies to establish the feasibility of using this construct in large gap nerve repairs.

Task 2.1 (months 6-18) – ANA with AFS for long gap nerve repairs will be studied using Lewis Rats as experimental subjects. A large gap nerve injury (1.5 cm) will be performed and the gap will be repaired immediately with an ANA construct alone (Group 1), an ANA construct with AFS cells (Group 2), or with an autograft (nerve segment is cut out, reversed, and sewn back in place)(Group 3). All surgeries will be performed using aseptic microsurgical technique. Outcomes of nerve injury/repair will be assessed at 1 month, 2 months, and 4 months post injury.

a. Outcomes – Outcomes assessed will include: Walking track analysis as an indicator of return of motor control. Walking track analysis will be performed at 1 month, 2 months, and 4 months post injury. Each animal will be compared to their preinjury walking track values. Use of this technique will permit use of the highly sensitive repeated measures analysis of variance for these animals. This technique will reveal even slight differences between groups. The number of animals required per group to achieve statistical power will be reduced using this experimental design.

Histologic analysis of nerve recovery at the end of 4 months. Axon counts on the post injury nerve segments will be performed according to the methods of Ma (2002, 2007). In addition, axon morphology will be assessed and compared between treatment groups.

Analysis of neuromuscular junction (NMJ) density. The number of neuromuscular junctions per mm2 of muscle tissue within the normal distribution of motor end plates will be determined and compared between groups. (Ma 2007, 2002)

Fate of AFS in ANA's following regeneration. Two approaches will be used: first, immuno-histochemistry will be employed to identify the AFS cells. In parallel, studies using green fluorescent protein labeled AFS cells will be initiated. These will allow us to monitor the fate of the AFS cells after several weeks of implantation.

Muscle force generation will be assessed following the last walking track analysis to assess the degree of motor recovery. These studies will utilize techniques developed in this laboratory. (Stone 2007, 2011)

Progress Task 2.1:

Progress Q1

- A DigiGate video analysis system for quantifying gait in rats and performing walking track analysis has been purchased and delivered to our laboratories. The company CEO has provided on-site instruction in its use and we have begun training and assessing rat gait. The DigiGate computer is also connected to our institutional web server. This has allowed us to utilize and test the on-line assistance provided by the DigiGate company. (20-11-2013)
- Lewis rats, the strain identified for these studies have been obtained and we are learning techniques for training these animals to walk on the DigiGate. (05-12-2013)

Progress Q2

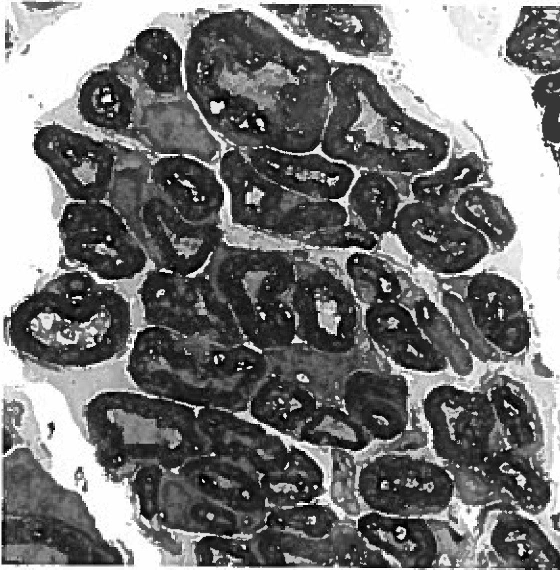
- Nerve autograft repairs of sciatic nerve injuries have been performed on the first six treadmill trained Lewis rats. These surgeries were uneventful and all animals have had their staples removed. The first animals to undergo nerve autograft repairs will be tested on the DigiGate device at 1 month post-surgery (first animals tested on 01-04-2014). Additional testing of these animals will be performed at two and four months post-surgery.
- Surgeries to create and repair sciatic nerve injuries will be performed in the next cohort of treadmill trained rats beginning 01-04-2014

Progress Q3

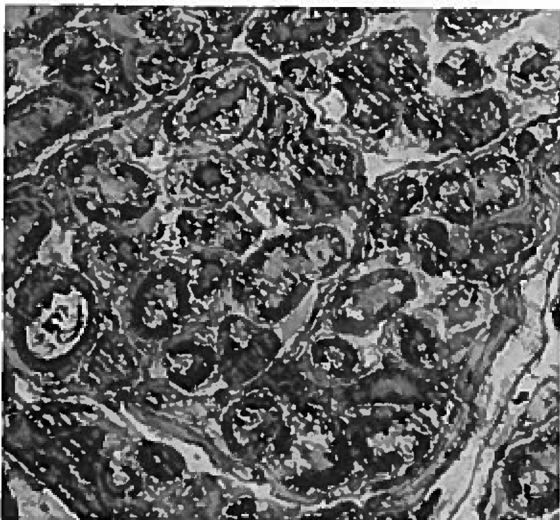
- Two groups of rats underwent surgical transection of the sciatic nerve on the left side with repair of the injured nerve using either a nerve autograft (Group 3; nerve segment obtained from the same rat) or a nerve allograft (Group 1; AxoGen supplied acellular human nerve of appropriate size).
- Rats were tested on the gait analysis device (DigiGate) before injury, and at 1 month, 2 months, and 4 months. In summary, several components of the rats' gait are significantly altered by sciatic nerve injury. Their gait parameters did not return to pre-injury values after 4 months. There were no remarkable differences between allograft and autograft nerve repair outcomes, which is in itself notable.
- Muscle function data also were collected and these results are still being analyzed.
- Gross muscle weights on the nerve injury side were significantly lower than on the intact contralateral side, suggesting muscle atrophy occurred following nerve injury. This atrophy was not reversed four months after nerve repair.

Progress Q4

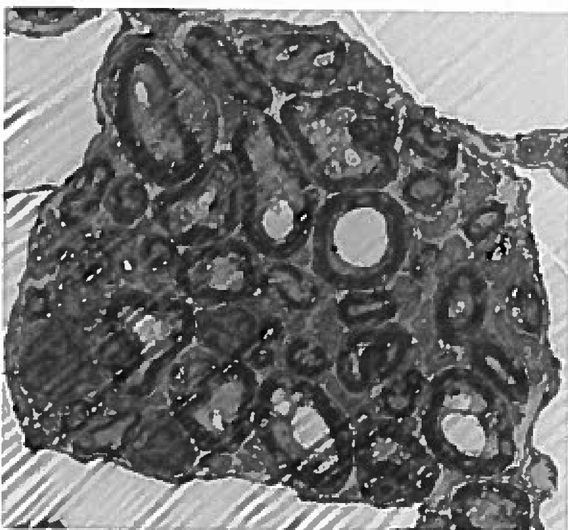
- Histology is continuing to assess axon counts as well as neuromuscular junction density



Electron micrograph of nerve autograph



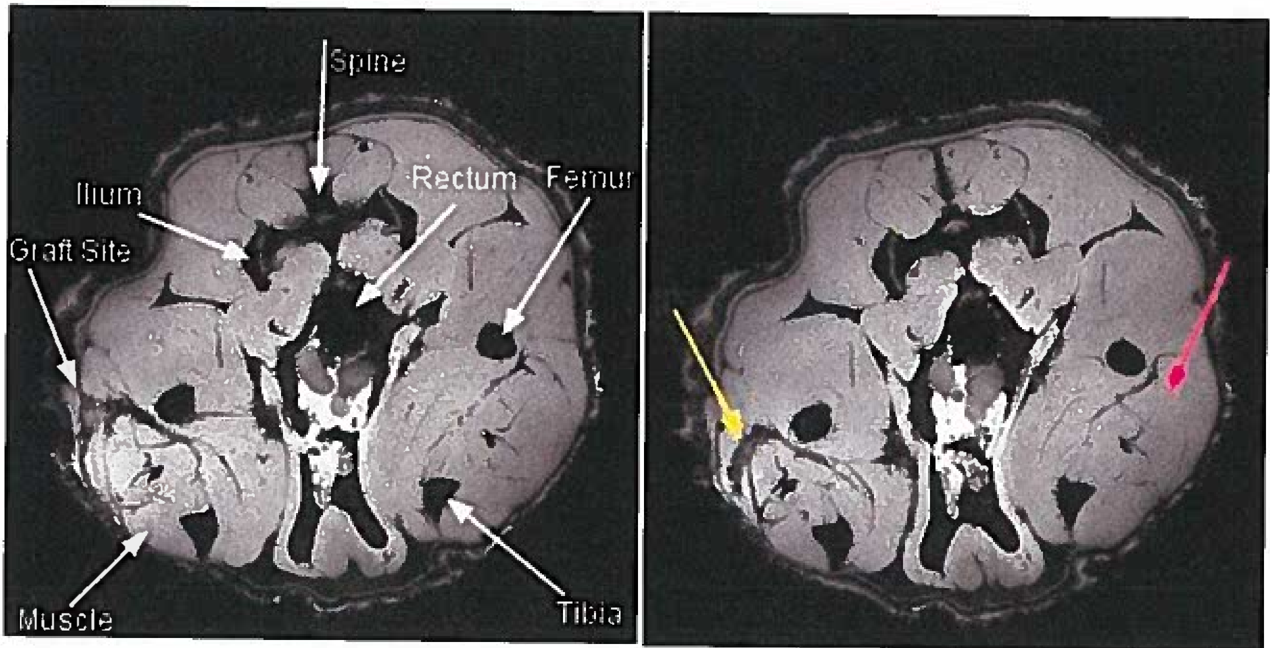
Electron micrograph of nerve allograft



Electron micrograph of nerve allograft + AFS

Representative electron micrographs of myelinated axons in the distal nerve stump of the rat, 1 mm distal to the suture line (Magnification: 3700X)

- Tracking of AFS cells in-vivo is being pursued through nano-particle labeling of cells and use of a 9T MRI to image these cells



T2 images of AFS cells labled with micron-sized iron oxide particles (yellow arrow) 1 week following graft implantation into sciatic nerve defect.

Progress Months 12-24

- All experimental groups of rats have been placed on study. Groups I-II have been studied through the 4 month time period following surgery. Group III (ANA + AFS) is finishing their 4 month post-surgery evaluation in Q1 of year 3 of this grant. Preliminary functional data (at 2-moths post-surgery) from gait analysis has been assessed for all three groups. The results have been discussed in an abstract submitted to the Orthopaedic Research Society Annual meeting for 2016 (attached as Appendix 1).
    - Briefly, at two months it was determined that ANA + AFS (Group III) demonstrated improvements in gait parameters compared to autograft repairs (Group I), particularly in the Sciatic function index.
    - Four month data are summarized in Table 2.

Functional and Histological Outcomes			
	Autograft	ANA	ANA+AFS
Stance/Swing Ratio	0.66 ± 0.22	0.64 ± 0.23	0.66 ± 0.22
Ataxia Coefficient	1.06 ± 0.29	1.27 ± 0.3	1.35 ± 0.23
Overlap Distance	0.79 ± 0.34	0.42 ± 0.19	0.71 ± 0.33 *
Step Angle Degree	0.9 ± 0.33	0.98 ± 0.37	0.97± 0.36
Paw Angle Degree	2.01 ± 0.25	2.88 ± 0.36	2.09 ± 0.22 **
Stride Length	1.1 ± 0.19	1.18 ± 0.28	1.16 ± 0.14
Paw Drag	1.38 ± 0.3	1.23 ± 0.38	1.08 ± 0.31 *
Stance Width	1.41 ± 0.28	1.04 ± 0.33	1.2 ± 0.21 *
Axis Distance	1.58 ± 0.25	1.13 ± 0.36	1.35 ± 0.23 *
Midline Distance	1 ± 0.22	1.25 ± 0.27	0.92 ± 0.17
SFI	9.02 ± 0.63	5.41 ± 0.63	7.29 ± 0.55 *
Wet Muscle Mass Ratio (GM)	0.52 ±0.02	0.50 ±0.01	0.51 ±0.05
Gastrocnemius CAMP Ratio	0.29 ± 0.05	0.27 ± 0.04	0.39 ± 0.05 *
Myelin Thickness (μm)	1.14 ± 0.22	0.69 ± 0.09	0.88 ± 0.13 **
Axon Diameter (μm)	2.29 ± 0.28	1.96 ± 0.24	2.36 ± 0.36 **
Fiber Diameter (μm)	3.93 ± 0.28	2.86 ± 0.25	3.84 ± 0.3 **
G Ratio (AD/FD)	0.58 ± 0.02	0.68 ± 0.02	0.61 ± 0.01 **

*p<0.05, **p<0.01

Table 2. Results of functional and histological analysis at the end of 4 months post nerve injury. ANA plus AFS cells group showed significantl improvement in gait function, compound evoked muscle action potentials (CMAP), myelin thickness and axon diameter compared to ANA group alone (*p<0.05, **p<0.01), closely resembling the best outcomes obtained from autograft group.

Task 2.2 (months 12-24) – Motor end plate preservation to increase functional recovery following denervation/reinnervation of the affected muscle will be studied in a separate cohort of rats. This group (n=10) will be subjected to nerve injury and repair using a 15 mm nerve defect and autologous nerve repair as in 2.1. A beta 2 agonist (fenoterol) will be administered via an osmotic minipump to the denervated gastrocnemius complex at a dose rate of 1.4 mg/kg/day in a total volume of 24 microliters. This drug and dosing regimen has been demonstrated to reduce and reverse muscle wasting in rats (Ryall 2003). It is hypothesized that it may reverse the loss of NMJ surface area and number following denervation. This may allow greater recovery following reinnervation.

A control group of injured rats (n=10) treated with vehicle for the beta2 agonist only will also be studied. Muscle force generation and histology to examine neuromuscular junction density will be performed at 120 days.

- An amendment requesting additional rats to pursue this study was approved by the Wake Forest IACUC. Accordingly, this amendment is being prepared for submission to the USAMRMC ACURO so that these studies can be initiated.

Task 2.3 (months 18-36) – Large gap nerve repairs will be studied in nonhuman primates. The nerve reconstruction constructs utilized in study 2.1 [ANA construct alone (Group 1), an ANA construct with AFS cells (Group 2)] will be employed bilaterally in a randomized fashion (right arm v. left arm) to repair a large gap nerve defects (6 cm) in macaca fasciculata monkeys. Electrophysiologic testing as well as functional assessments (grasp and pinch ability) will be assessed longitudinally on a bimonthly basis (beginning 3 months post surgery) for 12 months following large nerve gap repair of the median nerve. At the end of 1 year, the animals will be euthanized. The median nerve from the elbow to the wrist crease will be removed bilaterally for histologic study and the muscle tissue of the thenar complex will be recovered bilaterally.

- The results from Task 2.1 are encouraging and procedures are underway to procure test subjects through the Wake Forest School of Medicine Non-Human Primate Program and the Wake Forest University Animal Resources Program. Vervet monkies will be used instead of m. fasciculate because they are less expensive, they are available immediately and will not require quarantine, and they are of comparable size.
- An extension of the original contract will be required to complete these studies because they require at least a 12 month follow-up period to appropriately assess functional recovery.

KEY RESEARCH ACCOMPLISHMENTS:

Cell seeding of the acellular allografts for peripheral nerve repair.

- This methodology is being compiled as a manuscript for submission.

All test groups of animals were successfully treated using the appropriate nerve repair constructs as originally proposed. The functional outcomes of these large gap nerve repairs have been compiled and are being assessed.

CONCLUSION:

Summarize the importance and/or implications with respect to medical and /or military significance of the completed research including distinctive contributions, innovations, or changes in practice or behavior that has come about as a result of the project. A brief description of future plans to accomplish the goals and objectives shall also be included.

The ability to incorporate cells into nerve scaffold poses a research challenge. Current techniques are inadequate. The current research has tried two innovative approaches which have not been successful. This potential pitfall was recognized in the research plan and the project pursued methods to increase the permeability of the nerve epineurium. **This obstacle was overcome through an innovative combination of techniques utilizing injection of cells into the body of the nerve and increasing the porosity of the epinurium using microneedle punctures.** The increased porosity of the epineurium insures appropriate nutrition of the implanted cells via diffusion. These constructs have been demonstrated to retain viability following implantation into a nerve defect and offer improved outcomes compared to unseeded nerve allografts for segmental nerve defect repairs. These constructs will be tested in a preclinical non-human primate model.

PUBLICATIONS, ABTRACTS, AND PRESENTATIONS:

Abstract submitted to the Orthopaedic Research Society Annual Meeting in 2016 entitled: "Regeneration of large-gap peripheral nerve injuries using acellular nerve allografts plus amniotic fluid derived stem cells (AFS)".
Authors: Ma A, Marquez-Lara AJ, Martin E, Smith TL, Li Z.
To be presented at the Orthopaedic Research Society Annual Meeting in Orlando FL in March of 2016.

Abstract submitted to the Federation of American Societies for Experimental Biology annual meeting in 2016 entitled: "Regeneration of Large-gap Peripheral Nerve Injuries Using Acellular Nerve Allografts plus Amniotic Fluid Derived Stem Cells (AFS)" Authors: Xue Ma, MD, PhD, Alejandro Jose Marquez-Lara, MD, Eileen Martin, Thomas L. Smith, PhD, Zhongyu Li, MD PhD
To be presented in San Diego, Ca in April of 2016.

INVENTIONS, PATENTS, AND LICENSES:
Nothing to report

REPORTABLE OUTCOMES:
Nothing to report

OTHER ACHIEVEMENTS
Nothing to report

CHALLENGES:
Because of the extended timeline required to achieve seeding and incorporation of AFS into the nerve allografts, we anticipate that we will require a contract extension in order to complete SOW task 2.3. These non-human primates have not been acquired yet because we will not have enough time to complete the studies on them prior to the end of the contract period. They can be enrolled immediately upon extension of the contract period.

We will require Wake Forest Institutional Animal Care and Use Committee and ACURO approval to change the species of non-human primate from macaca fasciculate to vervet monkeys (Chlorocebus pygerythrus). This change is requested to reduce the acquisition costs of test subjects and expedite the enrollment of test subjects. Vervet animals are readily available on our campus and can be enrolled immediately. The are comparable in size to the Cynomologous monkeys originally proposed for use in these studies. This request will be submitted in this quarter.

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APPENDICIES: (attached)
Orthopaedic Research Society Annual Meeting 2016 abstract submitted
Federation of American Societies for Experimental Biology annual meeting 2016 abstract submitted

COLLABORATIVE AWARDS:
Dr. Z Li : CO-PI

Regeneration of Large-gap Peripheral Nerve Injuries Using Acellular Nerve Allografts plus Amniotic Fluid Derived Stem Cells (AFS)

Xue Ma, MD, PhD, Alejandro Jose Marquez-Lara, MD, Eileen Martin, Thomas L. Smith, PhD, Zhongyu Li, MD PhD
Department of Orthopedic Surgery, Wake Forest University School of Medicine, Winston-Salem, NC 27157

Introduction: Surgical reconstruction of peripheral nerve lesions in the extremities is challenging and often results in impaired functional recovery. The "gold standard" for successful nerve repair is a primary tensionless epineural repair which often is not possible. Nerve guidance tubes as well as acellular nerve allografts (ANA) have been developed to provide repairs comparable to those obtained with autografts. In order to promote nerve regeneration across large nerve gaps, regenerating axons are capable of extending the gap distance for nerve recovery when in the presence of Schwann cells. Tissue engineering strategies have attempted to mimic this cell environment by adding other supportive types of cells such as stem cells to the nerve allograft.
Hypothesis: We hypothesized that acellular nerve allografts (ANA) can be seeded with amniotic fluid-derived stem cells (AFS) to promote and accelerate nerve regeneration. The presence of the AFS provides support for the regenerating axons without the requirement of becoming Schwann cells.

Methods: *In vitro* study: $1X10^6$ "Off the shelf" AFS cells were injected underneath the epineurium of the ANAs using a 26 G syringe. Seeded grafts were placed vertically at the bottom of a small centrifuge tube covered with DMEM containing 20% FBS for overnight then transferred to a 48 well plate for additional 48 hours. *In vivo* study: ANA with AFS cells for long gap nerve repairs were studied using Lewis Rats. A large gap nerve injury (1.5 cm) was created in the sciatic nerve, and the gap was repaired immediately with an ANA construct alone (Group 1), an ANA construct with AFS cells (Group 2), or with an autograft (Group 3). Outcome assessments include walking track analysis (DigiGait Imaging system, Figure 1) to document the return of motor control at 1 month and 2 months post- injury.

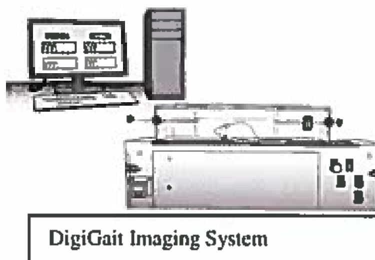
Results: *In vitro* AFS cells seeding to ANA: DAPI staining on longitudinal and cross sections of ANAs showed cells spread evenly through the nerve fibers (Figure 2.) *In vivo* gait analysis of 23 parameters of the autograft, ANA and ANA plus AFS cells groups at 2 months post-injury indicated that there were no significant differences in stride, stance/swing ratio, paw area at peak stance, stance factor, midline distance, % swing/stride, % brake/stride, % propel/stride, % stance/stride, %brake/stance, % propel/stance, % hind limb shared stance, step angle degree, stride length, MAX dA/dT and MIN dA/dT among groups.The autograft group showed greater stance width, overlap distance, axis distance, paw angle and paw drag compared to the ANA and ANA plus AFS cell groups.($p<0.01$ in all indices, Figure 3) ANA plus AFS cell group showed reduced swing time, %swing/stride at the end of 2 months compared with 1 month time point (1 month vs. 2 months: 0.17±0.01s vs.0.14±0.02s; 37.76±3.97% vs. 33.37±4.78%; $p<0.01$, $p<0.05$) In addition, ANA plus AFS cell group demonstrated a more robust motor function recovery compared to ANA alone group (paw angle and paw drag value are close to autograft group), indicating AFS cells facilitated the nerve regeneration 2 months following injury. We will keep tracking the motor function recovery as well as the histological outcomes till the end of 4 months following injury.

Discussion: We have developed an effective and consistent method to seed the ANA with AFS cells. The cells are viable 72 hours after seeding and spread through the entire ANA evenly. The seeding method could potentially prolong the time of the AFS cells staying in the ANA thus support and enhance the host nerve regeneration.

Significance: The findings of the study may have a direct impact on the future of stem cell therapies to facilitate nerve regeneration in patients who sustain peripheral nerve injuries.

Acknowledgements: The study is funded by Department of Defense USAMRAA (W81XWH-13-1-0310)

Figure 1.



DigiGait Imaging System

Figure 2.

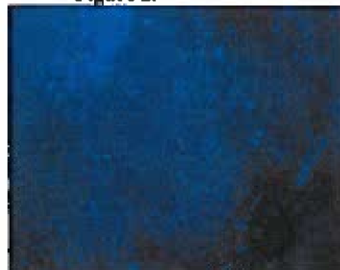


Figure 2. Cross section of a sciatic nerve allograft seeded with $1X10^6$ AFS cells -DAPI staining showed AFS cells nuclei appeared bright blue. Magnification X100

Figure 3.

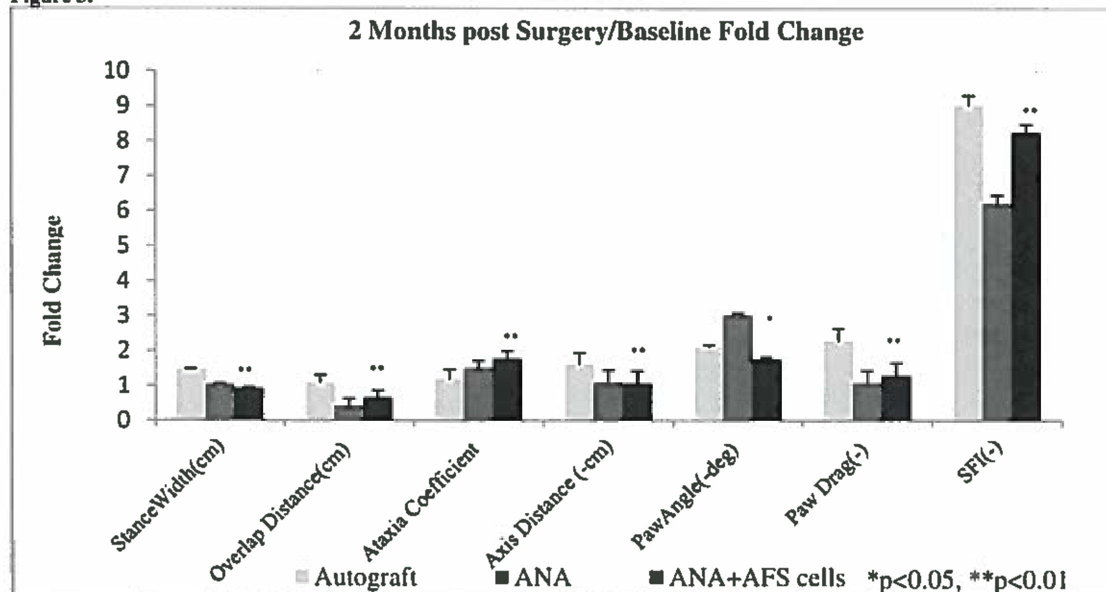


Figure 3. ANA+AFS cells group demonstrated differences in stance width, overlap distance, ataxia coefficient, axis distance, paw angle, paw drag and SFI compared with autograft group at the end of 2 months post- surgery.

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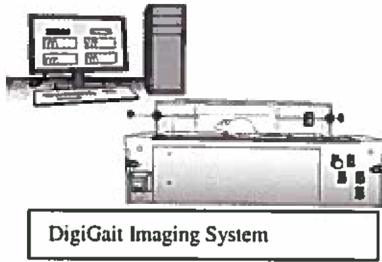

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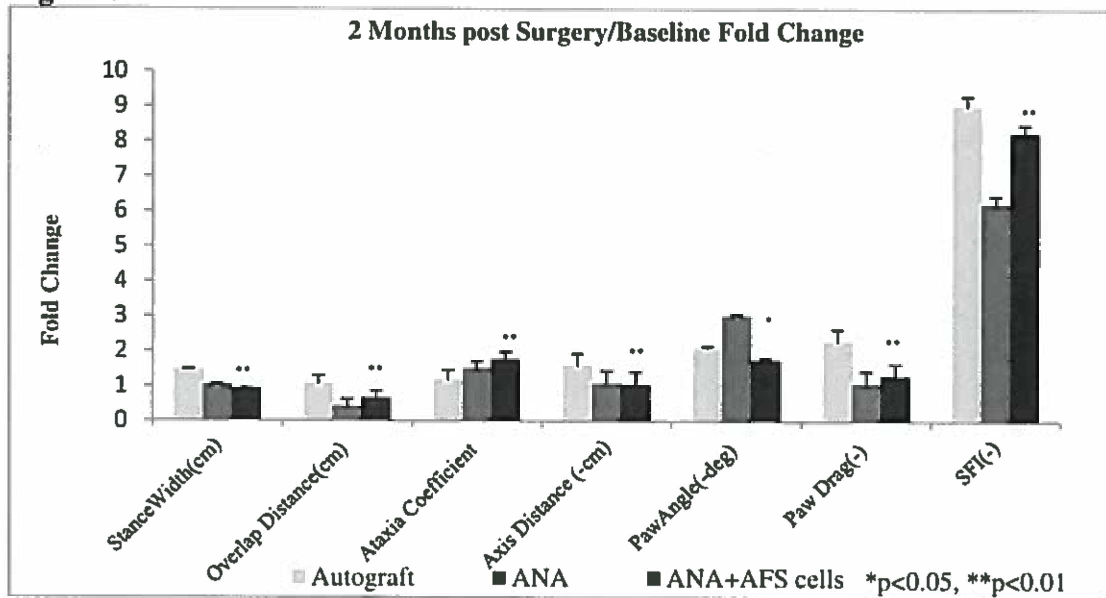

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Wake Forest™ School of Medicine

Introduction

Surgical reconstruction of peripheral nerve lesions in the extremities is challenging and often results in impaired functional recovery. The "gold standard" for successful nerve repair is primary tensionless epineural repair which often is not possible as a result of the extensive loss of nerve substance, unavoidable delays or additional injuries associated with the nerve injury. Nerve guidance tubes and acellular nerve allografts (ANA) have been developed to provide repair results comparable to those obtained with autografts. In order to promote nerve regeneration across large nerve gaps, regenerating axons are capable of extending the gap distance for nerve recovery when in the presence of Schwann cells. Tissue engineering strategies have attempted to mimic this cell environment by adding other supportive types of cells such as stem cells to the nerve allograft.

Hypothesis

We hypothesized that acellular nerve allografts (ANA) can be seeded with amniotic fluid-derived stem cells (AFS) to promote and accelerate nerve regeneration. The presence of the AFS provides support for the regenerating axons without the requirement of becoming Schwann cells.

Methods

In vivo study:

-ANA with AFS cells for long gap nerve repairs were studied using 4 Lewis Rats per group. A large gap nerve injury (1.5 cm) was created, and the gap was repaired immediately with an ANA construct alone (Group 1), an ANA construct with AFS cells (Group 2), or with an autograft (Group 3).

In vitro study:

-Off the shelf" AFS cells were seeded into ANA (AxoGen Corp) by injection.

Outcome assessments:

Walking track analysis (DigiGait Imaging System) was performed to document the return of motor control at 1 month, 2 months, and 4 months post-injury.

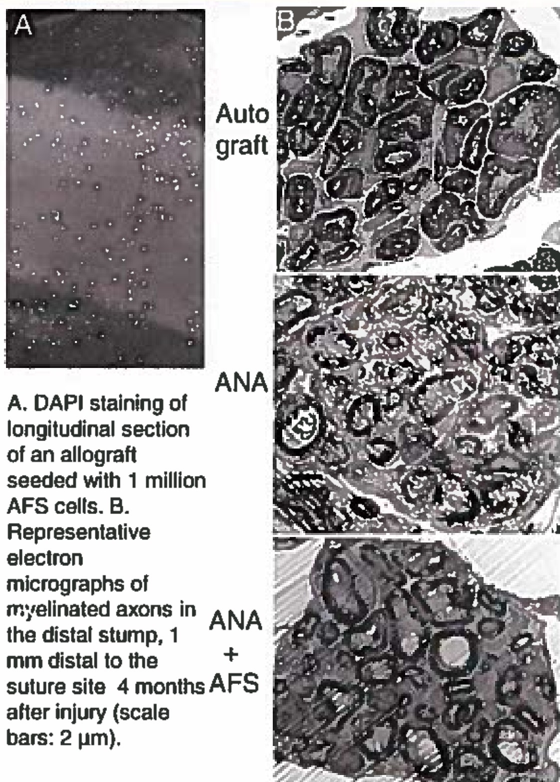
Histologic analysis of nerve recovery at the end of 4 months was performed. Axon counts on the post injury nerve segments were assessed and axon morphology was documented.

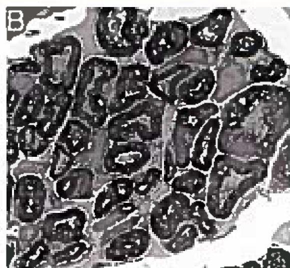
Analysis of neuromuscular junction (NMJ) density within the normal distribution of motor end plates was determined using immunohistochemistry .

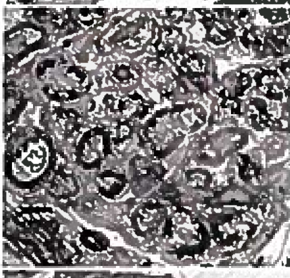
Fate of AFS in ANA's following regeneration was employed by immunohistochemistry to identify the location of the AFS cells after implantation over time.

Electromyography and muscle force generation was performed after the last walking track analysis to determine the degree of motor recovery.

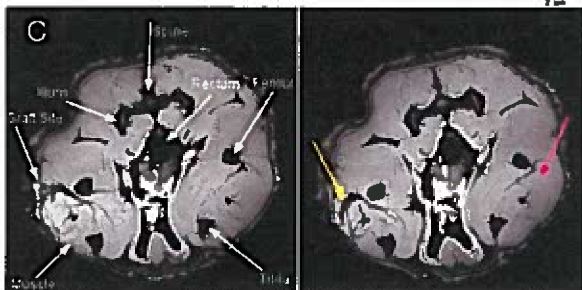
MRI tracking of AFS cells labeled with micron sized iron oxide (MPIO) particles







A. DAPI staining of longitudinal section of an allograft seeded with 1 million AFS cells. B. Representative electron micrographs of myelinated axons in the distal stump, 1 mm distal to the suture site 4 months after injury (scale bars: 2 μm).



C. MRI T2 images of AFS cells labeled with micron sized iron oxide particles (yellow arrow) 1 week following graft implantation

Results

Functional and Histological Outcomes

	Autograft	ANA	ANA+AFS
Stance/Swing Ratio	0.66 ± 0.22	0.64 ± 0.23	0.66 ± 0.22
Ataxia Coefficient	1.06 ± 0.29	1.27 ± 0.3	1.35 ± 0.23
Overlap Distance	0.79 ± 0.34	0.42 ± 0.19	0.71 ± 0.33 *
Step Angle Degree	0.9 ± 0.33	0.98 ± 0.37	0.97± 0.36
Paw Angle Degree	2.01 ± 0.25	2.88 ± 0.36	2.09 ± 0.22 **
Stride Length	1.1 ± 0.19	1.18 ± 0.28	1.16 ± 0.14
Paw Drag	1.38 ± 0.3	1.23 ± 0.38	1.08 ± 0.31 *
Stance Width	1.41 ± 0.28	1.04 ± 0.33	1.2 ± 0.21 *
Axis Distance	1.58 ± 0.25	1.13 ± 0.36	1.35 ± 0.23 *
Midline Distance	1 ± 0.22	1.25 ± 0.27	0.92 ± 0.17
SFI	9.02 ± 0.63	5.41 ± 0.63	7.29 ± 0.55 *
Wet Muscle Mass Ratio (Gastrocnemius Muscle)	0.52 ±0.02	0.50 ±0.01	0.51 ±0.05
Gastrocnemius CAMP Ratio	0.29 ± 0.05	0.27 ± 0.04	0.39 ± 0.05 *
Myelin Thickness (μm)	1.14 ± 0.22	0.69 ± 0.09	0.88 ± 0.13 **
Axon Diameter (μm)	2.29 ± 0.28	1.96 ± 0.24	2.36 ± 0.36 **
Fiber Diameter (μm)	3.93 ± 0.28	2.86 ± 0.25	3.84 ± 0.3 **
G Ratio (AD/FD)	0.58 ± 0.02	0.68 ± 0.02	0.61 ± 0.01 **

*p<0.05, **p<0.01 compared to ANA group

Conclusion

- AFS cells can be seeded directly into acellular allograft and remain viable *in vitro* and *in vivo*.
- Allograft plus AFS cells group demonstrated significantly improved functional and histological outcomes compared to allograft group alone, closely resembling the results of autograft.
- AFS cell is a suitable cell source to replace Schwann cells to support and accelerate nerve regeneration.

Funding

This study was funded by Department of Defense USAMRAA Translational Research Partnership Award-W81XWH-13-1 0310

"Acceleration of Regeneration of Large-Gap Peripheral Nerve Injuries Using Acellular Nerve Allografts plus Amniotic Fluid Derived Stem Cells (AFS)"

ERMS/Log Number - OR120157 and OR120157P1

Insert Award Number – W81XWH-13-1-0309 and W81XWH-13-1-0310

PI: Thomas Smith, PhD and Zhongyu Li, MD, PhD **Org:** Wake Forest University Health Sciences **Award Amount:** $939,786

Study/Product Aim(s)

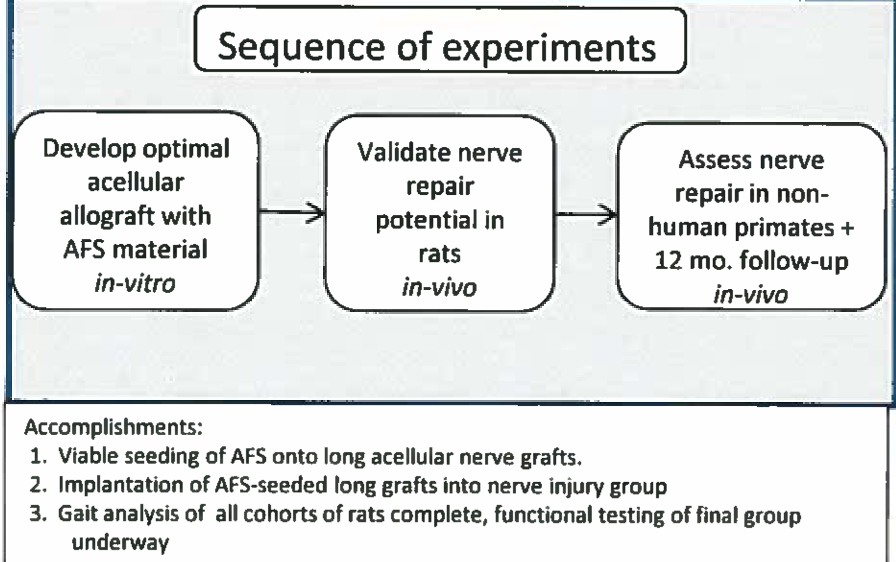
Specific Aim 1 (SA1): To demonstrate the ability to seed Acellular Nerve Allografts (ANA's) with AFS using sub-atmospheric pressure (SAP) *in-vitro*

Specific Aim 2 (SA2): To establish the feasibility of using AFS seeded ANA's in large gap nerve repairs *in-vivo.*

Approach – Partnership: Basic Scientist + Hand Surgeon

SA1: Establish the feasibility of using AFS seeded ANA's in large gap nerve repairs *in-vivo.* Cell culture techniques will be employed to seed commercially available ANA's with commercially available AFS. Both ANA's and AFS materials are FDA approved

SA2: Establish the feasibility of using AFS seeded ANA's in large gap nerve repairs *in-vivo.* Rats will be studied first to establish optimal nerve construct. Non-human primates will be studied as a pre-clinical model.



Accomplishments:

1. Viable seeding of AFS onto long acellular nerve grafts.
2. Implantation of AFS-seeded long grafts into nerve injury group
3. Gait analysis of all cohorts of rats complete, functional testing of final group underway

Timeline and Cost

Activities CY	13	14	15	16
Specific Aim 1.1				
Specific Aim 1.2				
Specific Aim 1.3, 2.1, 2.2				
Specific Aim 2.3				
Estimated Budget ($K)	**$94.5**	**$261.8**	**$147.9**	**$00**

Updated: August 31, 2015

Goals/Milestones

1.1 – Cell seeding using SAP – **Completed**

1.2 - AFS seeded onto ANA - **Completed**

1.3 – Study migration of Schwann cells onto the AFS seeded scaffold. **Completed**

2.1 – ANA with AFS studied using Lewis Rats with large nerve gaps. **Completed**

2.2 – Motor end plate preservation to increase functional recovery of rats – (USMRMC ACURO approval for additional animals pending being submitted)

2.3 – Large gap nerve repairs will be initiated in Q2 in non-human primates.

Comments/Challenges/Issues/Concerns

- – Acquisition of NHP's postponed pending extension of contract to permit testing at one year following nerve repair.

Budget Expenditure to Date

Projected Expenditure: $614,888

Actual Expenditure: $504,320.41