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14. ABSTRACT

Major accomplishments this year include the use of AFS seeded Acellular Nerve Allografts (ANA) to repair critical size nerve defects (1.5 cm) in rats. Functional recovery was monitored longitudinally using digital video gait analysis as well as electrophysiologic and histologic outcomes. The results demonstrated that the AFS seeded ANA used for nerve repair resulted in an improved functional outcome for the rats compared to ANA alone and were equivalent to those repaired using nerve autograft, the current gold standard for tension-free repair of transected peripheral nerves. Axon counts and neuromuscular junction morphology were equivalent between the AFS seeded ANA. Additional studies investigated the use of post-partum acellular materials to promote Schwann cell proliferation as well as renewed investigations into decellularization/oxidation of nerves.

The coming year will utilize these techniques for repairing large-gap (6 cm) nerve injuries in non-human primates. This pre-clinical model represents a more translational model of peripheral nerve injury and repair. In addition, preservation of neuromuscular junctions using beta 2 agonists will be studied. IACUC and ACURO approvals for these studies were renewed.

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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

<u>Specific Aim 1</u>: 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

<u>Specific Aim 2</u>: 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
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Animal Use at this site: Animals will be used at this site

Progress over the past 48 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.

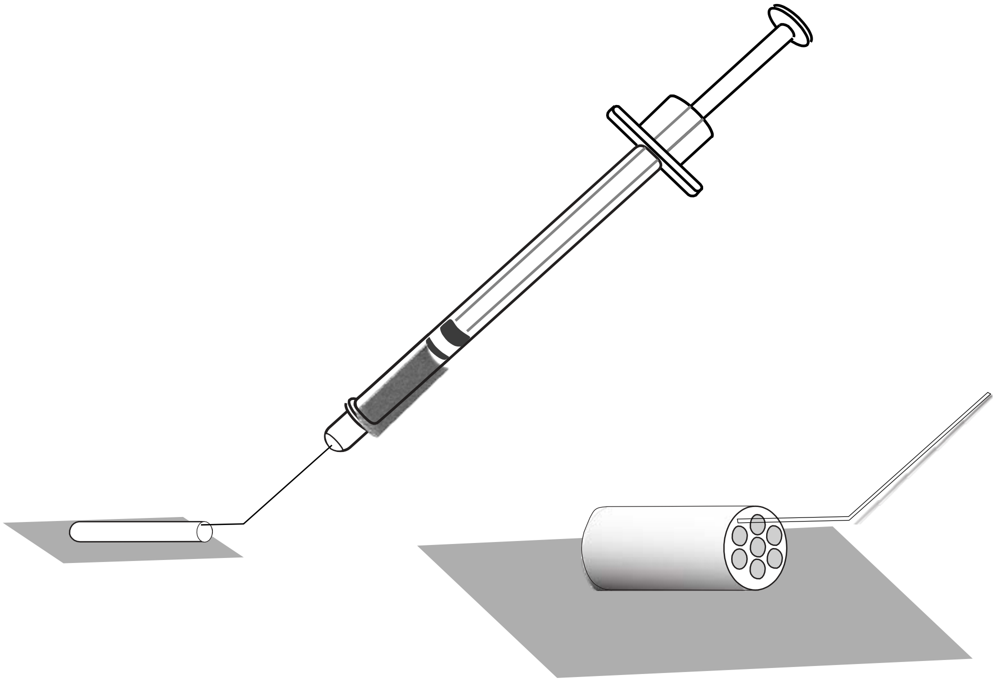
- Material transfer agreements are in place and amniotic tissues have been obtained from NuTech (26-11-2013)

- 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.

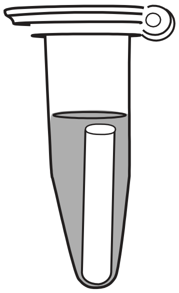
- Cell seeing of 1.5 cm long ANA was successful using an injection technique of AFS cells into the ends of the graft and beneath the epinurium of the graft near the mid-point followed by perforation of the epinurium using a microneedle array. The AFS-seeded ANA then was cultured for 72 hours. The perforation 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.

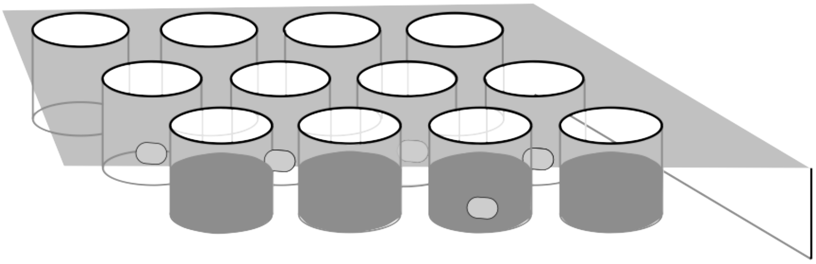


Sciaticnerve graft



Sciatic nerve graft standing vertically in media overnight

48 hours



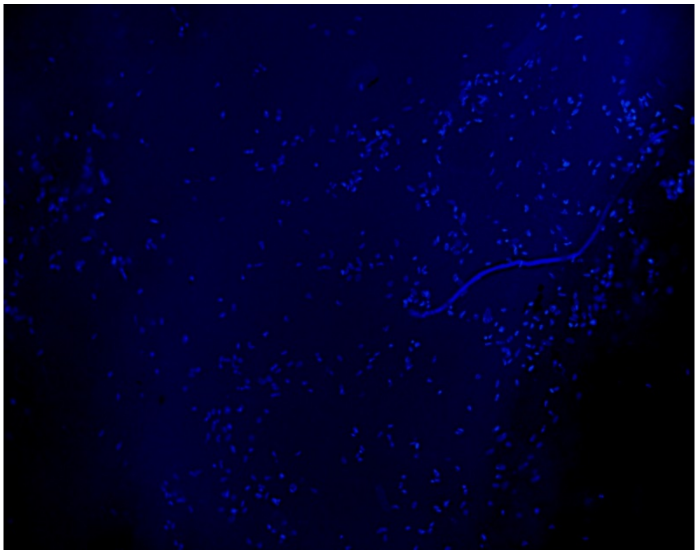
<u>Task 1.1 complete</u>

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:

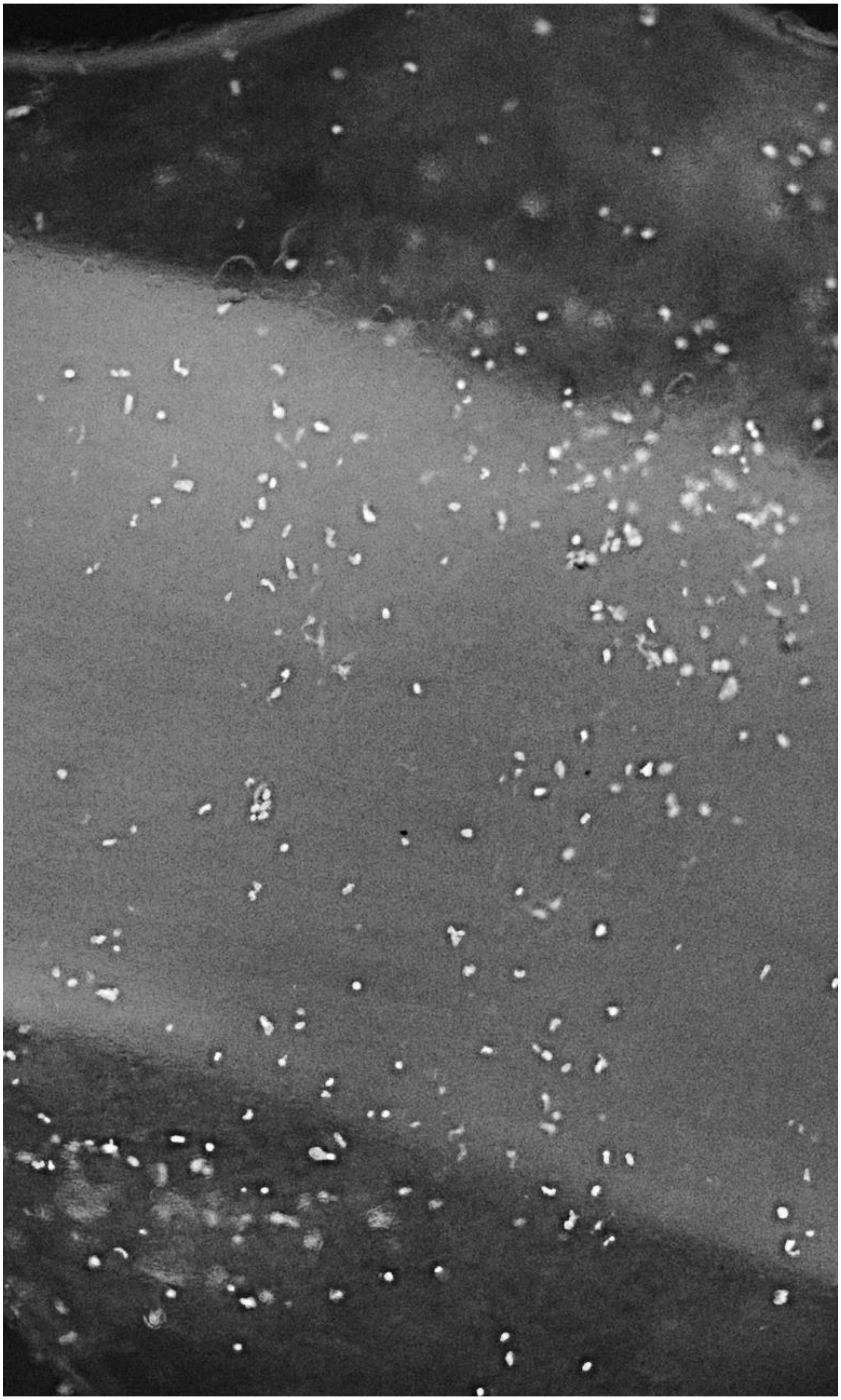
DAPI staining on longitudinal and cross sections of grafts showed cells spread evenly through the nerve fibers.



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 1.2 Complete

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 1.3 complete

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 1.4 Limited availability of commercially available decellularized nerve grafts led to the application of these decellulatization/oxidation methods on rat nerves. Careful adjustment of the decellularization/oxidation methods to process peripheral nerves led to the successful decellularization of these tissues. The accompanying oxidation improved the porosity of the epineurium. Histology documented these improvements in porosity as well as the ability to seed these scaffolds with Schwann cells.

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

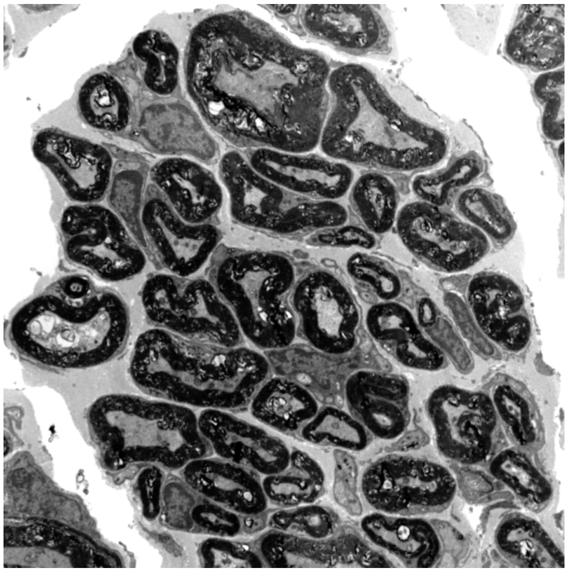
- Nerve autograft repairs of sciatic nerve injuries have been performed on the first six treadmill trained Lewis rats. These surgeries were uneventful and all animals have had their staples removed. The first animals to undergo nerve autograft repairs will be tested on the DigiGate device at 1 month 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.

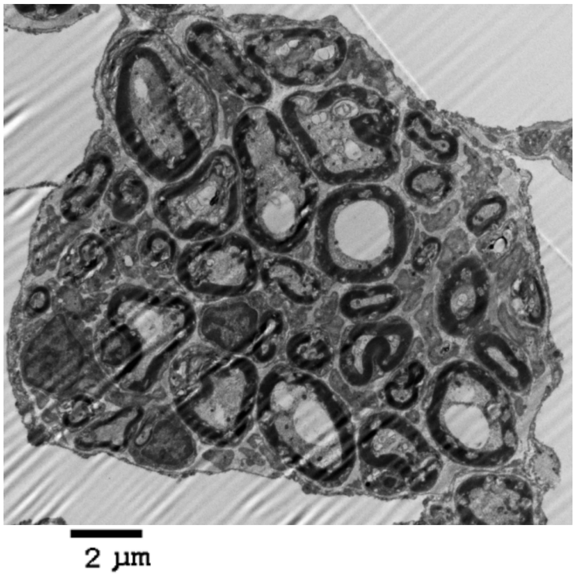
Progress Q4

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



Electron micrograph of nerve autograph

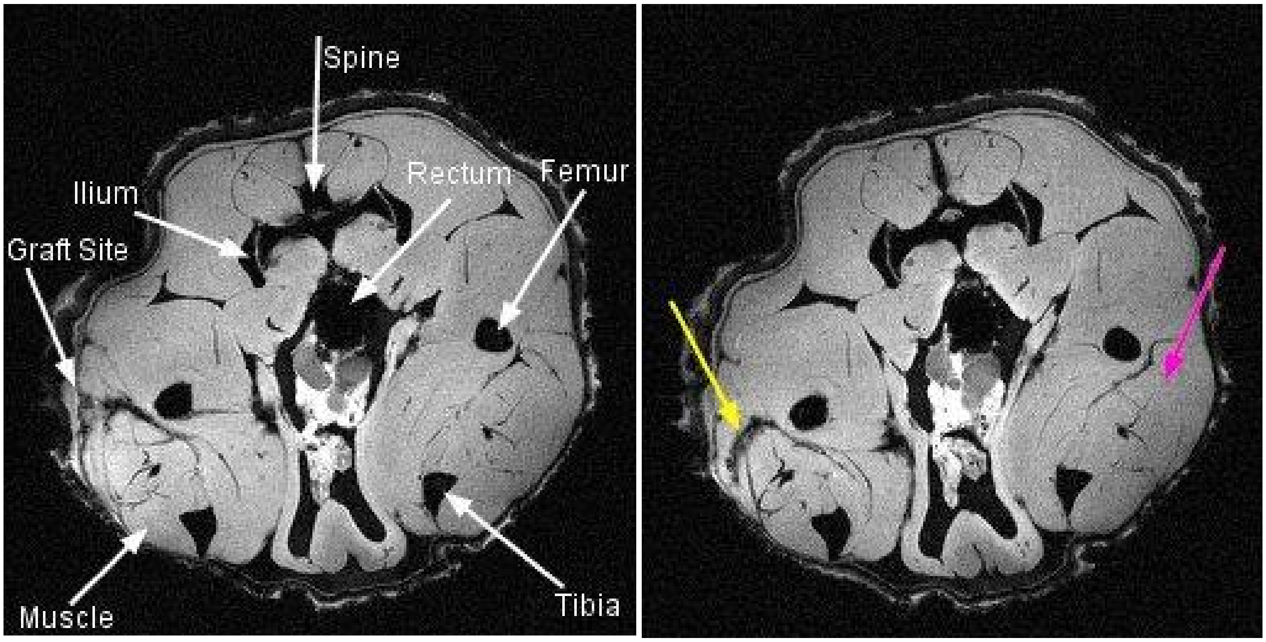
Electron micrograph of nerve allograft



Electron micrograph of nerve allograft + AFS

Figure 2.1.1 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

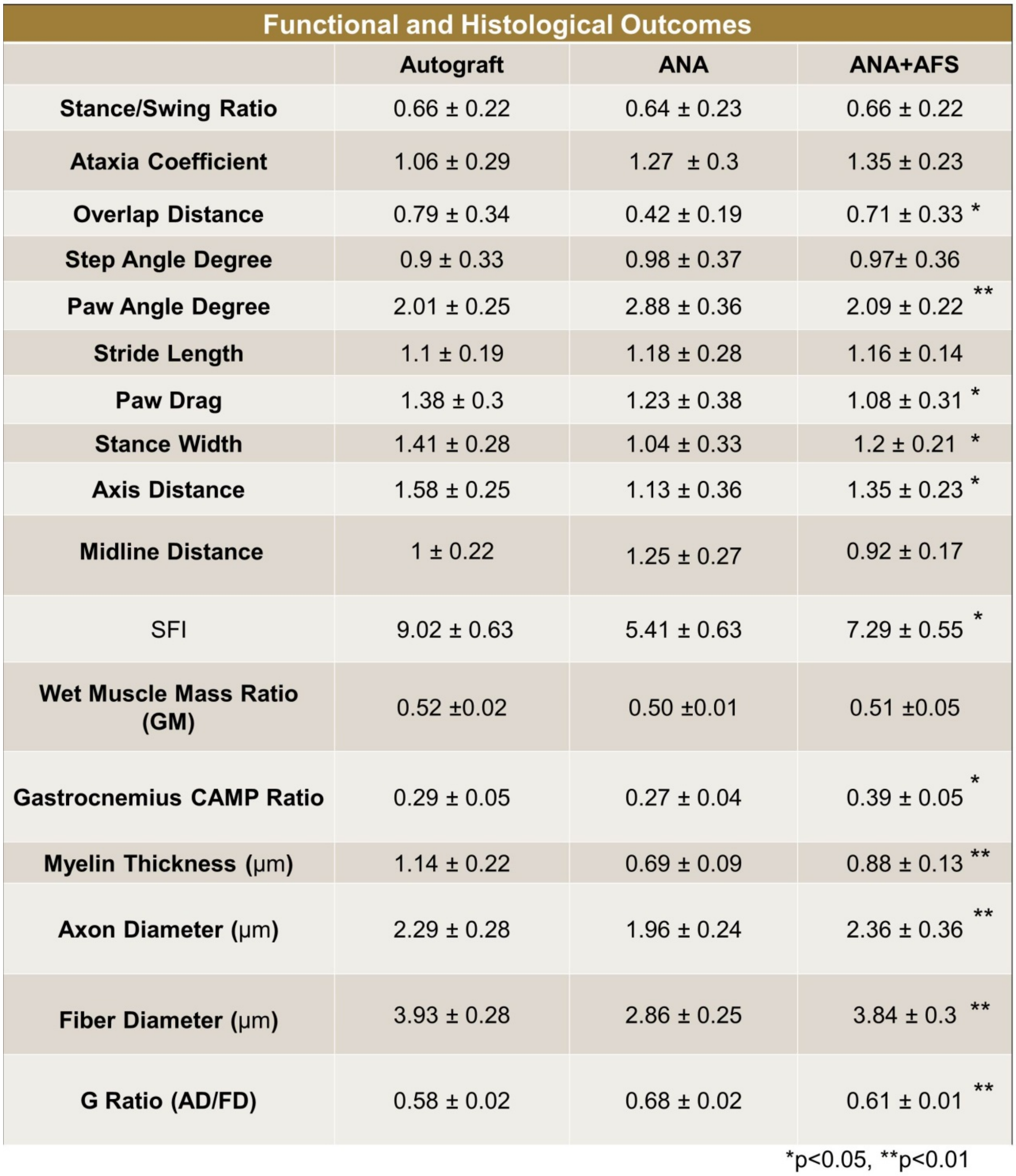


T2 images of AFS cells labled with micron-sized iron oxide particles (yellow arrow) 1 week following graft implantation into sciatic nerve defect.

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. Preliminary results of functional and histological analysis at the end of 4 months post nerve injury. ANA plus AFS cells group showed significant 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.

Progress Months 24-36

Histology :

The gastrocnemius and tibialis muscles from both the experimental and contralateral side were harvested and weighed. The ratio of the experimental and contralateral muscle weights was calculated to measure the recovery of atrophy. 14µm sections of muscle were cut and stained with α-bungarotoxin (Thermo Fisher, NY) to visualize neuromuscular junction morphology following nerve injury and repair as previously described. 10 consecutive slides per animal were analyzed for each group.

Statistical analysis

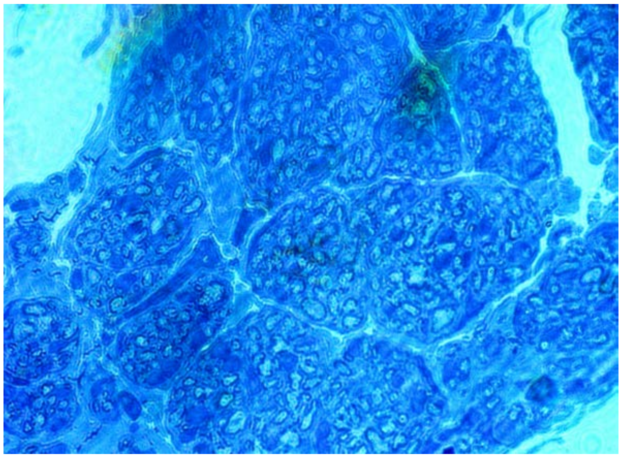
Results were reported as mean values and the standard error of the mean (SEM). One-way ANOVA test with Bonferroni multiple comparisons was used to determine the statistically significant differences between experimental groups. The following conventions were used:significant, *$p < 0.05$; very significant, **$p < 0.01$; and extremely significant, ***$p < 0.001$

Histologic results of nerve autograft v. nerve allograft plus AFS cells. Cross sections of the distal part of the regenerated nerves were evaluated by light and electronic microscopy. ANA plus AFS group showed significantly higher value of myelinated axon area per nerve, axon diameter, fiber diameter and myelin diameter compared with ANA alone, which closely resembled the outcomes obtained from autograft group. (Table 1).

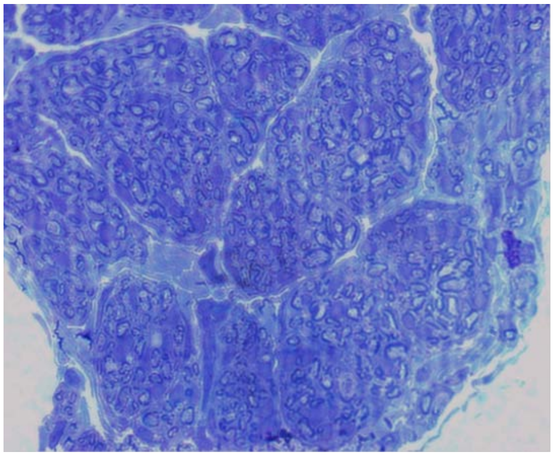
Histology of sciatic nerve graft at 4 mo post-injury/repair.

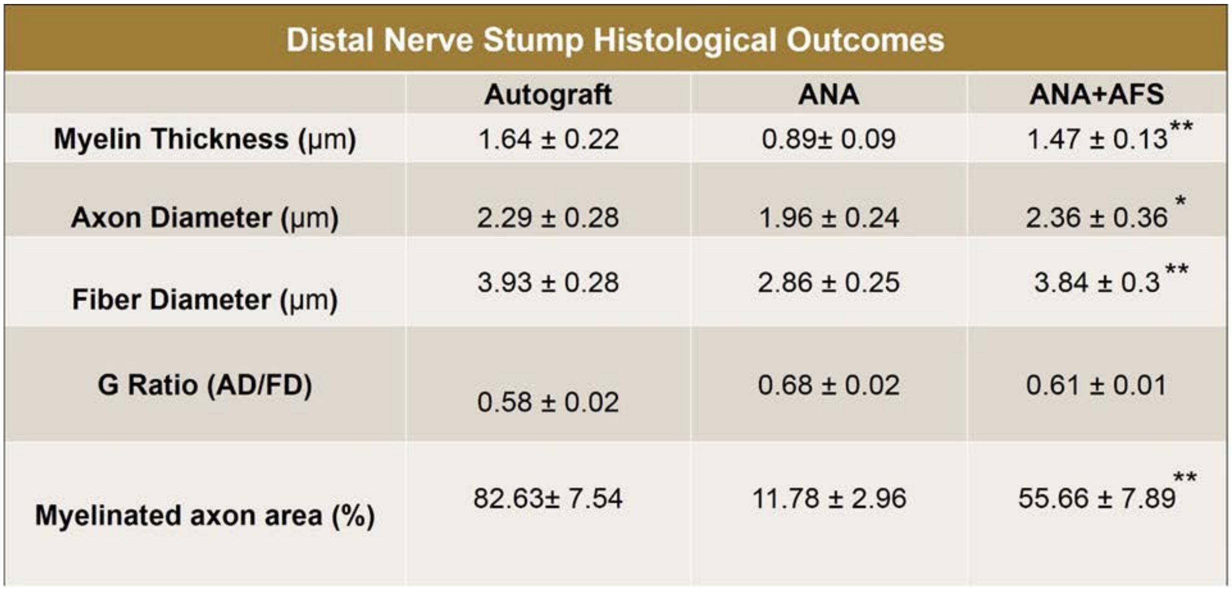
H&E stains of nerve cross sections:

Autograft –1000X at 4 mo.



AFS seeded ANA,1000X at 4 mo.





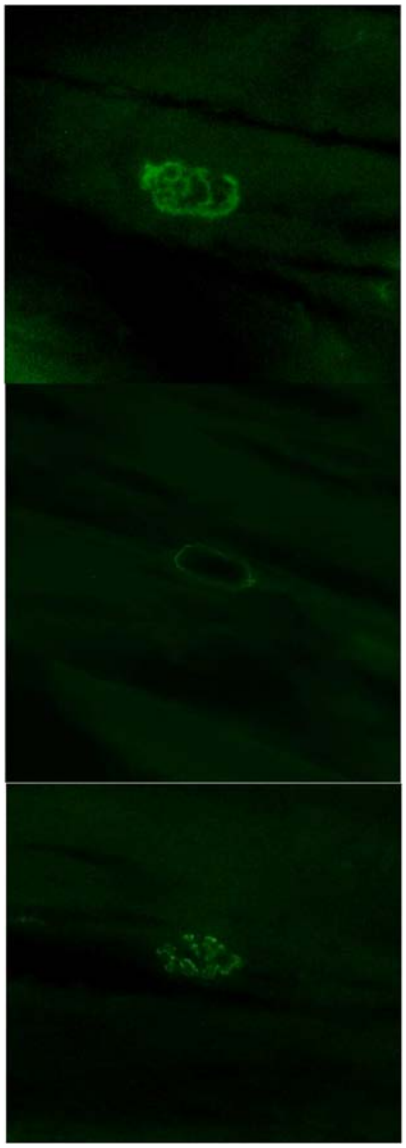
Distal Nerve Stump Histological Outcomes			
	Autograft	ANA	ANA+AFS
Myelin Thickness (µm)	1.64 ± 0.22	0.89± 0.09	1.47 ± 0.13**
Axon Diameter (µm)	2.29 ± 0.28	1.96 ± 0.24	2.36 ± 0.36 *
Fiber Diameter (µm)	3.93 ± 0.28	2.86 ± 0.25	3.84 ± 0.3 **
G Ratio (AD/FD)	0.58 ± 0.02	0.68 ± 0.02	0.61 ± 0.01
Myelinated axon area (%)	82.63± 7.54	11.78 ± 2.96	55.66 ± 7.89**

Table1. * indicated significance compared with ANA group (* P<0.05, ** P<0.01).

Electronic microscopy revealed greater myelinated axon surface and myelin thickness in ANA plus AFS cells treated group (Figure 2.1.1), indicating enhanced regenerating ability of the axons.

Neuromuscular junction morphology analysis

Cross sections of gastrocnemius and tibialis anterior muscle were assessed at the junctions where tibial and common peroneal nerves enter the muscles. There were no significant differences in the number and shape of NMJ between ANA plus AFS group and autograft group.(P= 0.69) (autograft vs. ANA+AFS vs. ANA: 45 ± 9 vs. 39 ± 9 vs. 28 ± 8, Figure 8) The NMJs of ANA group demonstrated a flat synapse outline and fewer neuromuscular junctions compared with autograft and ANA plus AFS groups.(p<0.05)



Autograft

Allograft

Allograft + AFS

Fluorescent microscopy representative pictures of neuromuscular junctions in gastrocnemius muscle. Magnification: 200X

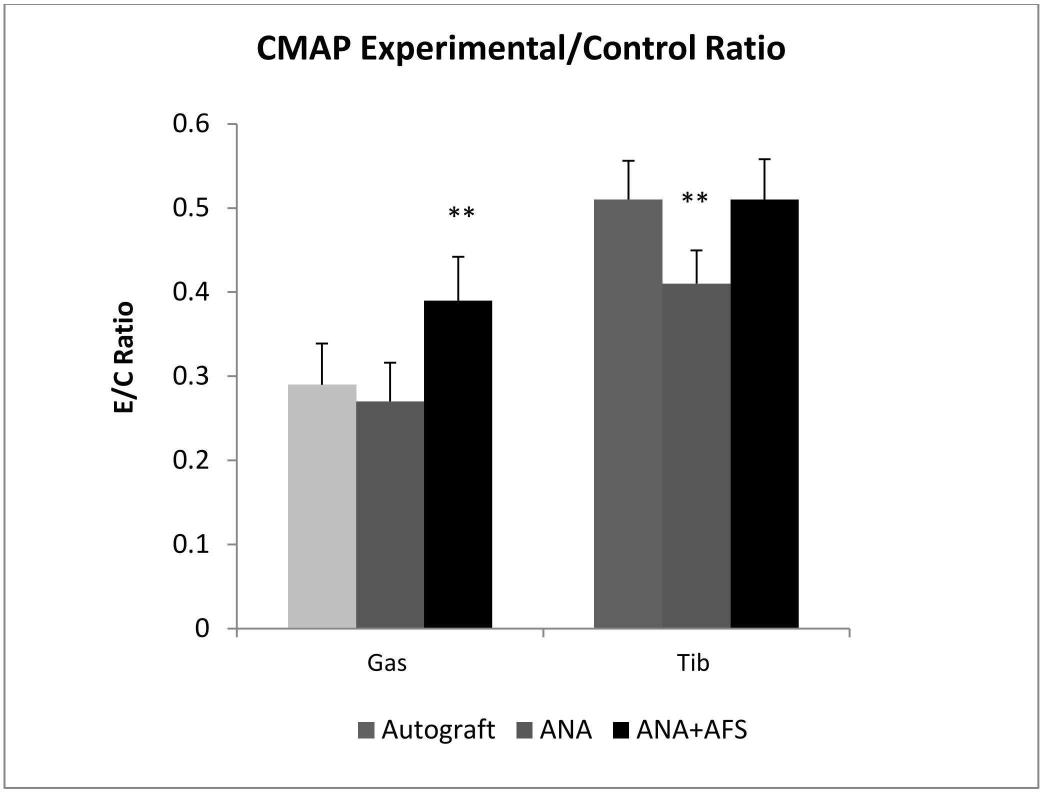
Functional recovery of the innervated muscles following nerve transection/repair using the different constructs also was evaluated by studying compound motor action potentials elicited by nerve stimulation above the repair site four months after nerve repair.

Electrophysiology analysis comparison among autograft, ANA and ANA plus AFS cells groups.

The Cadwell EMG Sienna Wave System was used for the electrophysiology testing. 12 weeks after the nerve autograft, ANA and ANA plus AFS cells implantation, rats were anesthetized with isoflurane and the regenerated sciatic nerve was exposed. Electromyographic analysis was examined by stimulating the regenerated nerve distally (suture sites were taken as referral points) with a monopolar cathodic electrode at 1mA, the anode was placed on the rat chest. Muscle contractions were recorded by electrodes placed into the gastrocnemius muscle (medial and lateral) and tibialis muscle of both experimental and control limbs.

Compound evoked muscle action potentials (CMAP) was recorded by three consecutive stimulations that were averaged for CMAP delays and amplitudes measurement.

Electrophysiological analysis of CMAP indicated that ANA plus AFS cells group had significant higher experimental/control ratio of wave potentials on gastrocnemius muscle compared with autograft and ANA groups. (Left CMAP (mv) autograft vs. ANA vs. ANA+AFS: 10.14±3.52 vs. 9.20±3.33 vs. 10.32±2.7; Right: 34.25±8.25 vs. 33.45±4.2 vs. 26.37±6.17. $p<0.01$) CMAP ratio of tibialis muscle had no significant differences between autograft and ANA plus AFS groups but was significantly higher than ANA group alone. (Left: 12.00±1.39 vs. 11.20±2.17 vs. 13.17±5.80; Right: 23.24±6.69 vs. 26.75±5.78 vs. 25.60±7.34. $p<0.01$)



Mean amplitudes of compound muscle action potential (CMAP) after stimulation of regenerating and contralateral control sciatic nerve with a monopolar electrode proximally. B. Ratio of amplitude of experimental to contralateral CMAP of gastrocnemius and tibialis muscle in ANA, ANA plus AFS and autograft groups.

Muscle atrophy after autograft, ANA or ANA+ AFS cells implantation was analyzed by excising the gastrocnemius muscle and tibialis muscle at the end of 4 months and calculating the ratio of the mass of the experimental muscle vs. the mass of the muscle in the control side (E/C ratio). There was no significant difference among autograft, ANA and ANA plus AFS groups on E/C ratio of gastrocnemius muscle and tibialis muscle. (gastrocnemius muscle weight E/C ratio, autograft vs. ANA vs. ANA+AFS: 0.51 ± 0.03 vs. 0.50 ±0.04 vs. 0.51±0.05; tibialis muscle: 0.65 ± 0.05 vs. 0.60 ± 0.06 vs. 0.6± 0.04,

Walking track analysis after 4 months recovery

Gait analysis of 24 parameters at the end of 4 months following injury indicated that there were no significant differences in stance/swing ratio, stride time, stance factor, swing stride percentage, brake stride percentage, propel stride percentage, stance stride percentage, brake stance percentage, propel stance percentage, hind limb shared stance percentage, step angle, stide length, max dA/dT among three groups.

Baseline	Autograft	ANA	ANA+AFS	4mons	Autograft	ANA	ANA+AFS
Stride(s)	0.48	0.45	0.432932	Stride(s)	0.54	0.52	0.50
Stance/Swing	2.79	2.76	2.630303	Stance/Swing	1.86	1.79	1.76
StanceWidth(cm)	2.64	3.02	2.92197	StanceWidth(cm)	3.73	3.16	3.51
Paw Area at Peak Stance in sq. cm(cm^2)	3.81	3.31	3.304318	Paw Area at Peak Stance in sq. cm(cm^2)	2.71	2.64	2.48
StanceFactor	1.02	1	1.000909	StanceFactor	0.85	0.83	0.83
Overlap Distance(cm)	1.85	1.84	1.389921	Overlap Distance(cm)	1.47	0.79	0.98
Ataxia Coefficient	0.44	0.36	0.482045	Ataxia Coefficient	0.47	0.46	0.65
Midline Distance (cm)	2.23	2.32	3.161136	Midline Distance (cm)	2.25	2.9	2.22
Axis Distance (-cm)	1.31	1.58	1.359167	Axis Distance (-cm)	2.08	1.79	1.83
%SwingStride	26.49	26.71	27.92348	%SwingStride	35.25	36.32	36.91
%BrakeStride	13.45	15.61	20.95833	%BrakeStride	17.02	18.63	24.11
%PropelStride	60.04	57.67	51.11288	%PropelStride	47.7	45.05	38.97
%StanceStride	73.51	73.29	72.07652	%StanceStride	64.71	63.68	63.08
%BrakeStance	18.3	21.44	28.5487	%BrakeStance	26.32	29.96	38.04
%PropelStance	81.7	78.69	70.73125	%PropelStance	73.68	70.16	61.95
% Hind limb Shared Stance	65.4	66.29	65.3417	% Hind limb Shared Stance	67.58	67.05	69.7
StepAngle(deg)	68.94	64.1	63.52901	StepAngle(deg)	62.37	62.92	61.73
PawAngle(-deg)	9.18	6.19	-7.89836	PawAngle(-deg)	18.48	17.87	16.53
StrideLength(cm)	12.14	11.12	10.82803	StrideLength(cm)	13.45	13.18	12.62
Paw Drag(-)	8.45	9.14	-11.2632	Paw Drag(-)	11.72	11.29	12.24
SFI(-)	4.84	7.55	5.271452	SFI(-)	43.7	40.92	38.42
MAX dA/dT (cm^2/s)	375.58	317.98	288.6579	MAX dA/dT (cm^2/s)	241.17	225.1	211.24
MIN dA/dT(-cm^2/s)	43.64	37.79	46.7363	MIN dA/dT(-cm^2/s)	23.05	20.68	33.31

The autograft group showed significant better recovery at stance width, overlap distance, ataxia coefficient, axis distance, SFI compared to ANA and ANA plus AFS groups. ANA plus AFS group exhibited better functional recovery in stance width, overlap distance, midline distance, axis distance, paw angle, paw drag than ANA group alone and didn't show significant differences from autograft group in these parameters, indicating preferred regenerating ability of AFS cells at the end of 16 weeks following a long nerve gap injury. In addition, the ratio of 4 months post-surgery to the baseline was significantly higher than allograft alone, suggesting an overall better sciatic function recovery than ANA group. (*p<0.05, **p<0.01 in all indices)

Task 2.1 complete

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.

Progress Months 24-36 - These studies were delayed pending approval of an extension of the animal care and use committee approval for this research. Protocol approval is only good for three years. These protocols were approved by the Wake Forest IACUC on 23/06/2016. The ACURO reviewed and approved this protocol on 25/08/2016.

Materials to complete this task were acquired and include: 30 osmotic minipumps with delivery rates of 0.25 microliters/hour, silastic tubing, sutures for suturing rat nerves, soft tissue, and skin. An initial cohort of 10 animals (5 experimental treatment, 5 vehicle treated controls) will be initiated in Q1 of year 4.

A no-cost extension of the award through 31/08/2018 was received on 14/08/2017 to allow completion of the proposed studies.

Progress Months 36-38 – These studies were initiated and all *in-vivo* data collection performed. Half to the test animals were initiated October 31, 2017 and the other half were initiated March 1, 2018. Currently we are awaiting histology on these tissues. These data will be available in the next quarter.

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 Chlorocebus pygerythrus 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 monkeys 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.

Progress Months 24-36 - These studies were delayed pending approval of an extension of the animal care and use committee approval for this research. Protocol approval is only good for three years. These protocols were approved by the Wake Forest IACUC on 23/06/2016. The ACURO reviewed and approved this protocol on

25/08/2016. These studies will be initiated within this quarter. A refurbished Cadwell EMG Sienna Wave System was purchased for electrophysiology testing. This will allow the investigators ready access to that equipment. The machine used previously was used by many investigators and was difficult to schedule and reconfigure between users.

A no-cost extension of the award through 31/08/2018 was received on 14/08/2017.

Progress months 36-48 – The 6 cm nerve allografts from Axogen were received and placed in -80°C freezers. The ability to seed these constructs with AFS was demonstrated. A 6 cm nerve allograft was seeded with 12 X 106 amnion derived stem cells. Grafts were incubated and then stained with DAPI to demonstrate viability. An example of a longitudinal section of this graft with fluorescently labled cells is given below (Figure 1).

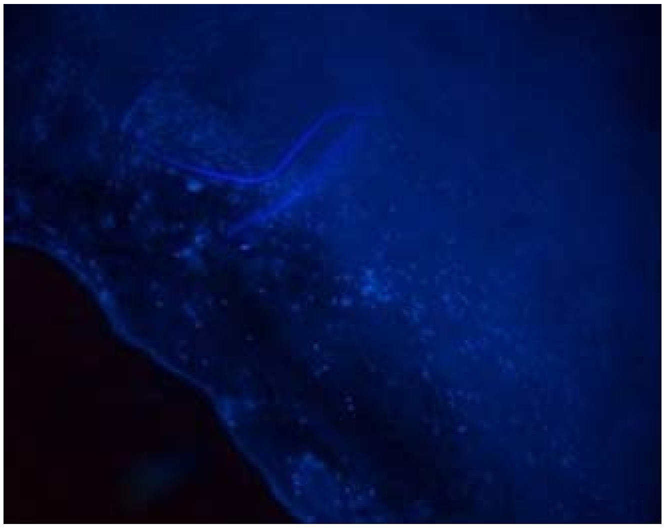


Figure 1 – A human acellular nerve allograft (6cm) seeded with amnion derived stem cells (12 X 106). Cells are stined using DAPI fluorescent staining to demonstrate cells were evenly distributed throughout the graft. It took about 2 weeks to culture the number of cells required for seeding.

Non-human Primate Surgeries

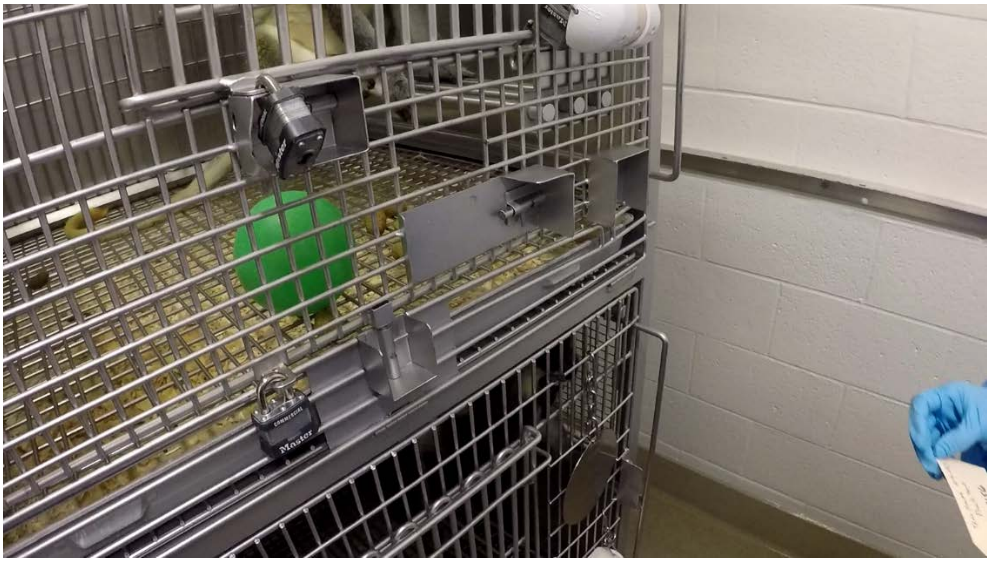
Beginning in February of 2019 surgeries were performed on the median nerves of seven Chlorocebus pygerythrus monkeys. Bilateral surgeries were performed during which a 7 cm segmental nerve graft was inserted in the median nerve between the elbow and the wrist crease. Nerve grafts consisted of either a decellularized nerve graft ANA or a decellularized nerve graft plus amnion derived stem cells (AFS). Remaining animals were instrumented in March 2019 and in June 2019. Surgeries were staggered among this group so that AFS cells could be expanded in culture in quantities sufficient to accomplish projected seeding requirements.

The initial cohort of four non-human primates (NHP's) were euthanized one year from the date of surgical implantation of the nerve grafts. Electromyography and assessments of pinch function were performed prior to euthanasia. All nerve grafts were successful with one exception. One ANA + AFS graft demonstrated neuroma formation.

The last cohort of three non-human primates (NHP's) were euthanized in June, 2020, one year from the date of surgical implantation of the allografts. No complications during surgery were observed. Electromyography and assessments of pinch function were performed prior to euthanasia.

Pinch test

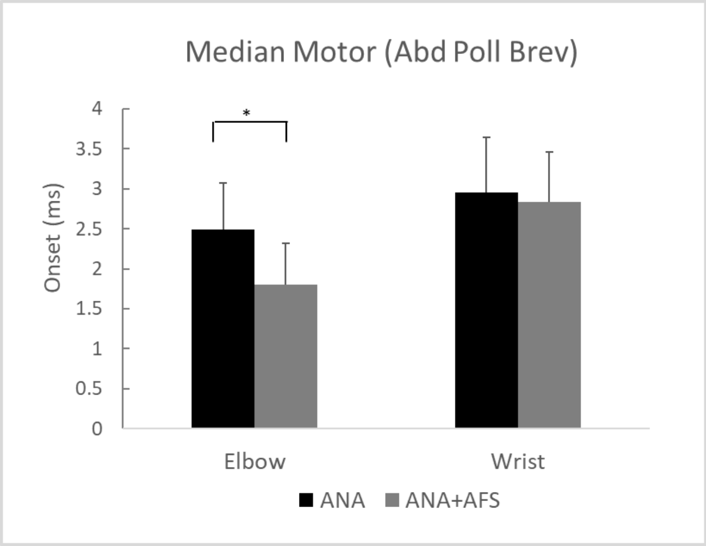
All non-human primates (NHP's) showed significant recovery on pinch function with both hands. Picking treats with thumb, index finger and middle fingers were observed in all animals expect one due to a fracture in middle finger after a fight with next cage NHP. The pinch test analysis between the ANA and ANA plus AFS cells are undergoing.

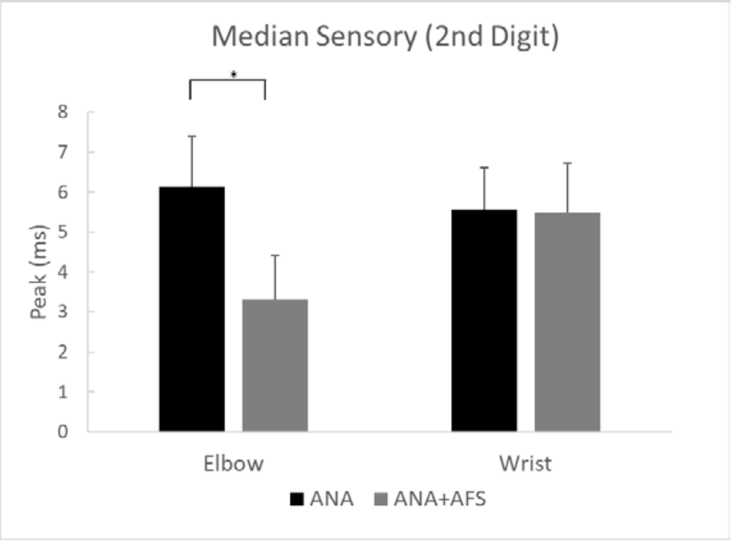


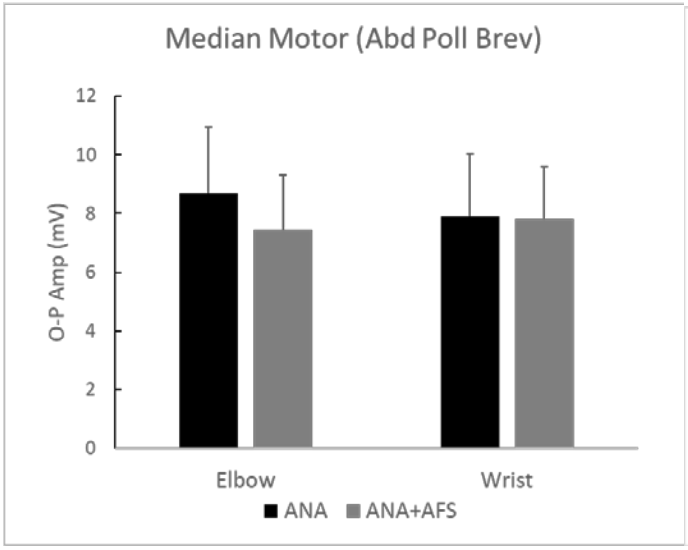
Electromyography

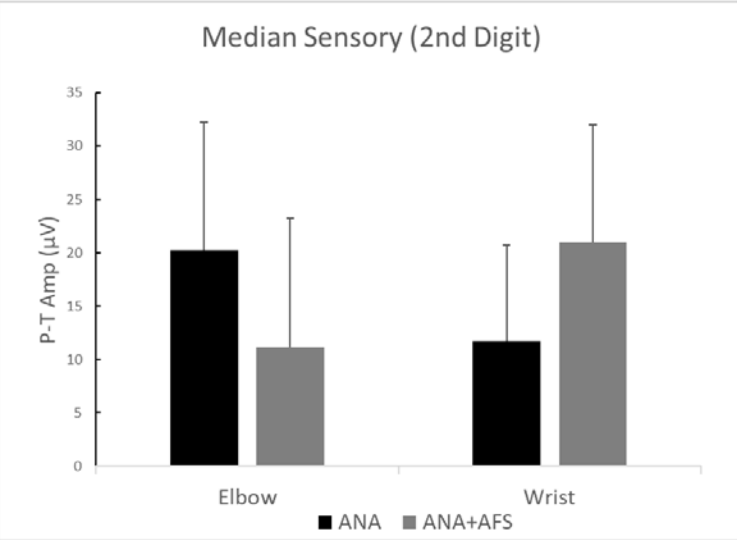
Electromyography studies at 12 months post injury and repair revealed significant improved nerve conduction onset latency in ANA plus AFS cells group. At elbow level, the onset latency in median motor nerve was 28% faster in ANA plus AFS cells group compared with ANA treatment only. This effect was also observed in median sensory function where the peak time was 48% quicker in ANA plus AFS cells group at elbow level. ($p<0.05$). There was no significant difference of motor or sensory nerve functions at wrist level.

The compound muscle action potential studies didn't show differences of O-P Amp or p-T Amp in median motor or sensory recoveries at elbow or wrist level.









Task 2.3 completed.

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 in Task 2.1 (rat studies) 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 the results are being prepared for submission for publication.

Cell Seeding of long (6 cm) nerve grafts with AFS successfully completed.

Technique for decellularization/oxidation of nerves accomplished and demonstrated in rat nerve allografts. Cell seeding of Schwann cells in these allografts was successfully demonstrated. This methodology is being prepared for publication.

Seven non-human primates (NHP's) have received bilateral 7cm median median nerve grafts. These surgeries have been successful and the outcomes following one year of recovery following these implants in the first four NHP's are being analyzed.

All implants and muscles of non-human primates (NHP's) have been harvested. Electromyography, pinch functional tests and histological outcomes are being analyzed.

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.

In-vivo assessment of these constructs was evaluated using a rat sciatic nerve model. The animals in which a nerve allograft that was seeded with AFS cells demonstrated improved recovery compared to animals receiving nerve allograft alone. This recovery was comparable to that achieved using nerve autograft, the current clinical gold standard for repairing large nerve gaps.

These constructs are being tested in a preclinical non-human primate model. Seven animals have been implanted with these constructs and the first four have been necropsied following a one-year recovery from surgery. All non- human primates (NHP's) have underwent final testing and necropsy. Electromyography, pinch functional tests and histological outcomes are being analyzed.

In addition to the techniques described above, a technique utilizing decellularization/oxidation of peripheral nerve tissue was developed. This technique improves the permeability of the epineurium so that cell seeding and diffusion of nutrients are improved. Cohorts of rats have been implanted with these new constructs and outcomes are being analyzed.

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.
Presented at the Ortohopaedic 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
Presented in San Diego, Ca in April of 2016.

In-Progress Report- Ft Detrick, MD, 04 February, 2016.

Abstract submitted to both American Association of Hand Surgery(AAHS) and the American Society of Peripheral Nerve (ASPN) "In vivo tracking of amniotic fluid derived stem cells on acellular nerve graft" has been accepted as an oral presentation at both the AAHS and ASPN 2017 annual meeting in Hawaii. Copy previously submitted.

Abstract presented at 2017 Military Health System Research Symposium for podium presentation. 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, Tianyi David Luo, MD, Eileen Martin, Thomas L. Smith, PhD, Zhongyu Li, MD PhD

Oral presentation, 2017 Military Health System Research Syposium, Aug, 2017. Regeneration of Large-gap Peripheral Nerve Injuries Using Acellular Nerve Allografts plus Amniotic Fluid Derived Stem Cells (AFS). Xue Ma, Alejandro Maquez-Lara, Thomas L. Smith, Zhongyu Li.

Abstract presented at 2017 Tissue Engineering and Regenerative Medicine International Society (TERMIS, December 2017) for Oral Presentation . 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, Tianyi David Luo, MD, Eileen Martin, Thomas L. Smith, PhD, Zhongyu Li, MD PhD

Large-gap Peripheral Nerve Repair Using Amniotic Fluid Derived Stem Cells Seeded Acellular Nerve Allografts
Xue Ma, Alejandro Jose Marquez-Lara, Tianyi David Luo, Eileen Elsner, Thomas L. Smith, Zhongyu Li
Department of Orthopaedic Surgery; Winston-Salem, North Carolina, USA
Sunderland Society, 2019 annual meeting, Oral presentation

Amniotic Fluid Stem Cell Conditioned Media Promotes Schwann Cell Proliferation and Viability
Chukwuweike Gwam MD, Rachel Bordelon, BS, Xue Ma MD, PhD, Thomas L. Smith, PhD Zhongyu Li MD PhD
Ortho Summit 2019 annual meeting, Oral presentation

Chukwuweike Gwam, Rachel Bordelon, Xue Ma. Stem Cell Conditioned Media Promotes Schwann Cell Proliferation and Viability, EOA annual meeting 2020

Acceleration of Regeneration of Large-gap Peripheral Nerve Injuries Using Acellular Nerve Allografts plus Amniotic Fluid Derived Stem Cells (AFS) Xue Ma, MD PhD1, Eileen Elsner3, Jiaozhong Cai3, Alejandro Jose Marquez-Lara, MD3, Thomas L. Smith, PhD1,2, Zhongyu Li, MD PhD1,2 Journal of nerve and muscle, submitted, in revision

Chukwuweike Gwam Ahmed K. Emara Nequesha Mohamed Johannes Plates Xue Ma Amniotic Stem Cell Conditioned Media for the Treatment of Nerve and Muscle Pathology: A Systematic Review. Submitted.

INVENTIONS, PATENTS, AND LICENSES:

Nothing to report

REPORTABLE OUTCOMES:

Nothing to report

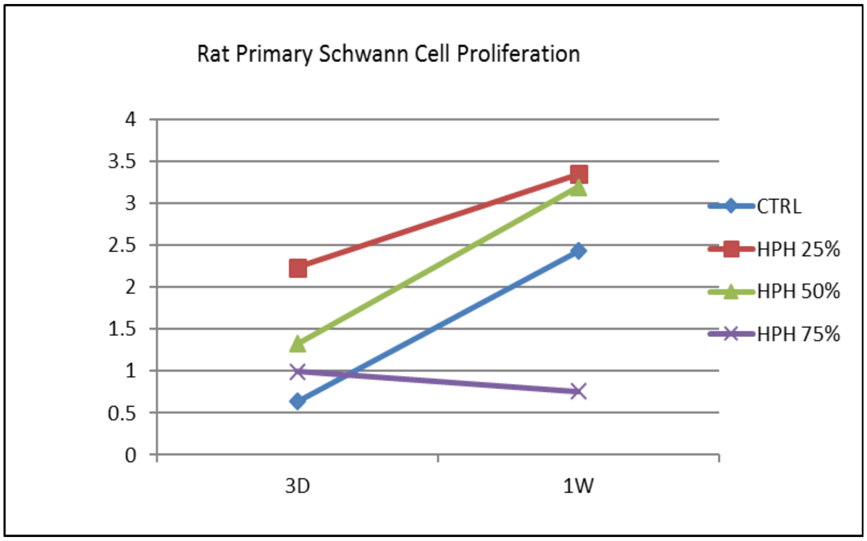
OTHER ACHIEVEMENTS

In 2017 the investigators established a research relationship with Plakous Therapeutics, a Winston-Salem based company specializing in post-partum placental materials. Plakous have supplied us with decellularized placental materials and primary Schwann cell proliferation was assessed. These results are positive, demonstrating a significant increase in Schwann cell number in the presence of these materials.

Effects of Post-Delivery Placenta Disc (HPH) on Schwann cell Proliferation

Peripheral nerve repairs utilizing amnion wraps have demonstrated excellent pre-clinical results. Both the concentrations of trophic factors contained within the amnion stroma as well as amnion’s well recognized anti-inflammatory properties may contribute to the excellent outcomes of this regenerative biologic. Even better outcomes might be achieved by loading biosorbable with higher concentrations of placental derived trophic factors and the absence of inflammatory chemokines elaborated by the amnion epithelium.

The term pregnancy, post-delivery human placenta is a rich source of trophic factors and ECM-P which orchestrate and sustain fetal development, including the complete central and peripheral nervous systems. The placental disc contains numerous cell types responsible for synthesizing, storing, and delivering trophic factors of the amniotic membrane and amniotic fluid. In this study we tested the effect of a Post-Delivery Placenta Disc (HPH) (Plakous Therapeutics, Inc), which contains high concentrations of chemokines essential to wound healing with a much lower pro-inflammatory chemokine ratio compared to term amniotic fluid on the growth rate of human Schwann cells. The efficacy of HPH in Schwann cell proliferation assay shows 50% higher proliferation than the positive control at less than 1% of the protein concentration.



The performance of HPH in a CCK-8 Schwann cell proliferation assay. Red Line: HPH 25% (1.87 mg/ml), green line and HPH 50% (3.75mg/ml) significantly accelerated the rat primary Schwann cell growth at 3 and 7 days compared to control (blue line) cell cultured in 1% FBS. (p<0.01).

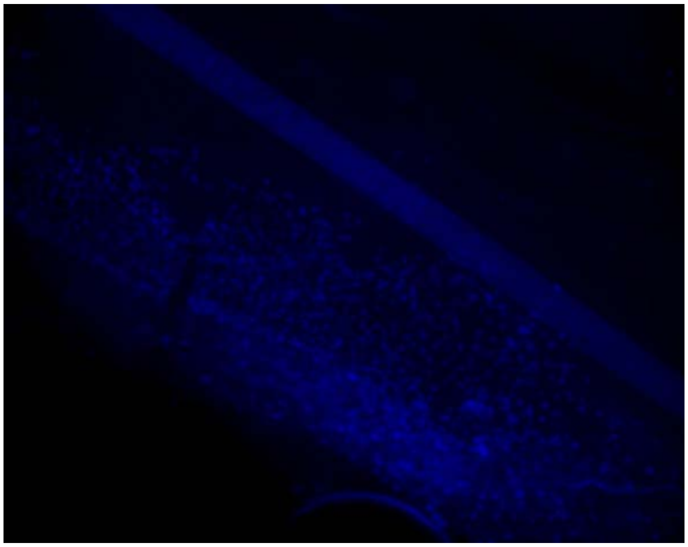
Nerve decellularization/oxidation

The decellularization/oxidation techniques originally proposed for nerve allografts were revisited after discussions with the inventors. Initial attempts had resulted in excessive breakdown of the nerve tissues. These protocols were modified and the structural integrity of the nerves was preserved. Additional studies examining the ultrastructural outcomes of this process are underway. If the results are positive, the investigators will request additional animals, at no additional cost, to assess the utility of these constructs. The increased porosity of the oxidized construct should permit improved cell seeding with amnion derived stem cells. Initial studies were performed on rat cadaveric materials from other experiments and upon chicken nerves from commercial sources.

Nerve Allograft Decellularization and Oxidation

Peripheral nerve injuries are commonly associated with extremity trauma. In order to achieve functionality following extremity reconstruction, nervous innervation must also be restored. The "gold standard" for successful nerve repair is primary tensionless epineural repair. However, due to extensive nerve substance loss caused by the injury, primary repair is often not possible. Autologous sensory nerve grafting has been developed as an alternative, when primary repair is not possible. However, this method requires harvesting graft material from a donor nerve, which is limited due to donor site morbidity and a limitation in the total number of nerves that can be harvested and used as autografts. Nerve guidance tubes have recently been developed and shown to provide repair results comparable to autografts with smaller defects. For nerve defects larger than 5 cm innovative techniques are required. Acellular nerve allografts (ANA) have been shown to restore meaningful functionality for larger nerve defects, however the functionality achieved is not equivalent to pre-injury functionality. The methodology used to produce the ANA can affect the functionality of the nerve repair. For example nerve regeneration across large nerve defects can be promoted by the presence of supporting cells around the regenerating axon. The purpose of this study was to use novel protocols to produce ANAs that could be seeded with stem cells. Sciatic nerves were harvested from six month old rats (necropsied animals from other experiments); one set of nerves underwent a protocol that involved decellularization at 4°C. The other set of sciatic nerves underwent a protocol that involved decellurization at 37°C and oxidation with 1.5% peracetic acid for 2 hours. The allografts that were seeded with schwannoma cells had cells present within the grafts. The two protocols used for the decellularization and oxidation of these nerve allografts were shown to be successful, future studies should focus on optimizing this protocol in order to increase the effectivness of cell seeding.

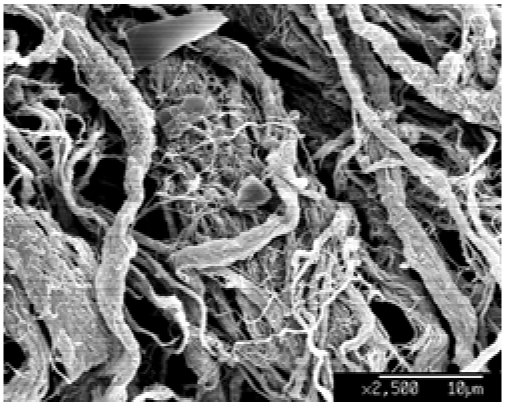
Dapi staining of decellurized rat sciatic nerve allograft . No residual cells were detected after decellurization of the graft.



Dapi staining of decelllurized rat sciatic nerve allograft seeded with 1X 10^6 human Schwannoma cells for 48 hours.

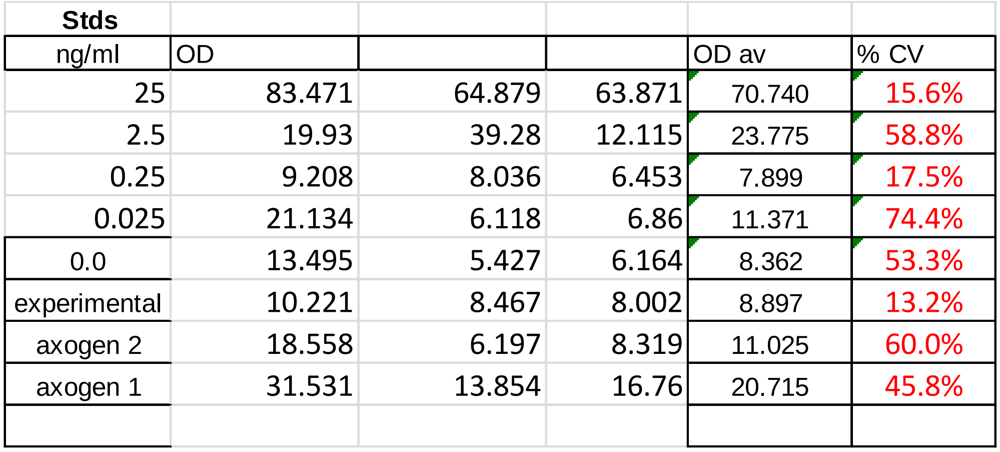
X200

Scanning electron microscopy of decellularized/oxidizes nerve reveals a more open ultrastructure when compared to non-oxidized nerve allografts.



SEM picture of decelllurized rat sciatic nerve allograft

Picogreen DNA analysis of the decellularized/oxidized nerve allograft revealed less DNA present than decellularized nerve allograft (AxoGen). Table below:



Stds					
ng/ml	OD			OD av	% CV
25	83.471	64.879	63.871	70.740	15.6%
2.5	19.93	39.28	12.115	23.775	58.8%
0.25	9.208	8.036	6.453	7.899	17.5%
0.025	21.134	6.118	6.86	11.371	74.4%
0.0	13.495	5.427	6.164	8.362	53.3%
experimental	10.221	8.467	8.002	8.897	13.2%
axogen 2	18.558	6.197	8.319	11.025	60.0%
axogen 1	31.531	13.854	16.76	20.715	45.8%

Cohorts of rats have been implanted with these decellularized/oxidized constructs to evaluate their potential for nerve graft applications for critical nerve defects. Preliminary results suggest that these new nerve constructs can restore motor function when used to repair sciatic nerve defects in rats. Analysis of these results, including histology, are pending.

IACUC and ACURO

Renewal of the IACUC and ACURO authorizations that are part of the oversight of this research were renewed by the Wake Forest University IACUC on June 5, 2019. ACURO approval of this authorization was received subsequently.

CHALLENGES:

Because of the extended timeline required to achieve seeding and incorporation of AFS into the nerve allografts, we requested and received a contract extension in order to complete SOW task 4.1. These non-human primates were acquired and implanted with the proposed nerve constructs over the past year.

The Wake Forest Institutional Animal Care and Use Committee and ACURO approved a change of species of non-human primate from macaca fasciculate to vervet monkeys (Chlorocebus pygerythrus). This change was 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. They are comparable in size to the Cynomologous monkeys originally proposed for use in these studies.

The COVID-19 pandemic has closed our laboratories since March 25, 2020. We concluded all rodent studies by that date. We still have three NHP’s on study at the Clarkson Primate Center at the Wake Forest University. We will be able to test and necropsy these animals in June of 2020 following a one-year recovery period from their surgery. We are optimistic that we will be allowed to return to our laboratories to complete our histologic work and to analyze our electromyographic data this summer. This prediction is based upon current helath trends in our community.

We were able to collect and harvest the NHP tissues in June 2020. However, the core lab and processing center were significantly delayed by staff furlough and reduction of employee density policies at medical center. The histology and image analysis were impacted by the delay as well. We are currently working on these and making slow progression. With the vaccination being administrated, we are accelerating the process and hopefully to finish up analyzing the data in the near future.

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APPENDICIES: (attached)

Orthopaedic Research Society Annual Meeting 2016 abstract
Federation of American Societies for Experimental Biology annual meeting 2016 abstract
American Association for Hand Surgery annual meeting abstract 2017
Peripheral Nerve Society Annual meeting abstract 2017
Scientific Research Grants:

1.) American Society for Surgery of the Hand – In-vivo tracking of Amniotic Fluid Derived Stem cells on Acellular Nerve Graft. PI - Xue Amy Ma, MD, PhD
2.) NuTech, Inc. Effect of Amniotic Membrane and Amniotic fluid Stem Cells on Schwann cell Neurotrophic Cytokine Production. PI- Xue Amy Ma, MD, PhD

COLLABORATIVE AWARDS:

Dr. Z Li : CO-PI