AWARD NUMBER: W81XWH-17-1-0513

TITLE: Pulsed Electromagnetic Field Therapy for Accelerating Peripheral Nerve Regeneration and Preserving Neuromuscular Junctions

PRINCIPAL INVESTIGATOR: Thomas L. Smith, PhD

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

REPORT DATE: December 2021

TYPE OF REPORT: Final

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
December 2021	Final	15Aug2017-14Aug2021

4. TITLE AND SUBTITLE

Pulsed Electromagnetic Field Therapy for Accelerating Peripheral Nerve Regeneration and Preserving Neuromuscular Junctions

5a. CONTRACT NUMBER
W81XWH-17-1-0513

5b. GRANT NUMBER
OR160258

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Thomas L. Smith, PhD

E-Mail:

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Wake Forest University Health Sciences
Medical Center Boulevard
Winston-Salem, NC 27157

8. PERFORMING ORGANIZATION REPORT NUMBER

Project #115620 (GTS #45190)

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

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

10. SPONSOR/MONITOR'S ACRONYM(S)
USARMRAA

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Major accomplishments include receipt of IACUC and ACURO approvals for all animal protocols, receipt and installation of a PEMF cage system providing commercially available PEMF waveforms, and initiation of experiments in Specific Aims 1 and 2. The PEMF cage system is installed in a dedicated room in the Wake Forest vivarium. Caging does not interfere with the PEMF waveform.

Rats were placed on study to address Specific Aims 1, 2, 3. Initial gait analysis was performed, the sciatic nerve was transected and repaired, and the animals received PEMF treatment for 4, 3, or 2 months. Animals underwent functional testing utilizing EMG and muscle force generation following their last gait analysis.

Specific Aim 3 (ability of PEMF to sustain neuromuscular junctions) was completed – Animals were place on study following nerve transection without repair. They were treated for 4 months. A second cohort was placed on study for a treatment period of 3 months.

15. SUBJECT TERMS
None listed.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRDC
Unclassified	Unclassified	Unclassified	Unclassified	25	19b. TELEPHONE NUMBER *(include area code)*

TABLE OF CONTENTS

1. INTRODUCTION:

Peripheral nerve injuries often accompany blast and shrapnel wounds. Repair of peripheral nerves requires a relatively long period of recovery. Pulsed electromagnetic field (PEMF) exposure offers an FDA approved intervention that has the potential to accelerate nerve recovery. The proposed studies will study PEMF treatments in rats with transected and repaired sciatic nerves. The rate and degree of functional motor recovery will be assessed in animals with and without treatment. In addition, the ability of PEMF to maintain neuromuscular junction integrity and function following denervation will be studied.

2. KEYWORDS:

Nerve injury, pulsed electromagnetic fields, motor nerve function, neuromuscular junctions, sciatic nerve, gait.

3. OVERALL PROJECT SUMMARY:

What were the major goals of the project?

Major goals:

Specific Aim 1- In-vivo effects of Pulsed Electromagnetic Fields (PEMF) on peripheral nerve axonal regrowth will be examined in rats subjected to sciatic nerve injury and repair.

- Major Task 1: Obtain regulatory approval for animal studies
    - Milestone Achieved November 8, 2017
- Major Task 2: Construct apparatus to deliver PEMF to test subjects –
    - Milestone achieved November 20, 2018. Second apparatus constructed by Wake Forest Center for Nanotechnology June 2018.
- Major Task 3: Perform surgeries on animals and enroll in PEMF protocol. – First cohort of 8 rats initiated in protocol on May 14, 2018. Second cohort of 8 animals was subsequently enrolled bringing the number of animals to 8 animals exposed to PEMF and 8 animals exposed to sham PEMF for 4 months

Specific Aim 2 - Assess functional outcomes of PEMF exposure on peripheral nerve injury/repair

- Major Task 4: Perform gait analysis on animals in Specific Aim 1. – First cohort of 8 rats initiated on May 1, 2018. These animals came off PEMF exposure on September 14, 2018. Second cohort initiated 5/23/2019 and completed exposure 9/25/2019.

Specific Aim 3 – Determine whether PEMF exposure has the ability to sustain neuromuscular junction morphology following denervation of muscle.

- Major task 1: subject exposed to PEMF/sham PEMF following denervation of muscle. – Initiated 5/09/2019 and completed 6 month exposure 11/9/2019
- Major task 2: Harvest tissues and perform histological analysis-completed 11/2021

What was accomplished under these goals?

Specific Aim 1- In-vivo effects of Pulsed Electromagnetic Fields (PEMF) on peripheral nerve axonal h will be examined in rats subjected to sciatic nerve injury and repair.

- Major Task 1: Obtain regulatory approval for animal studies
    - Milestone Achieved November 8, 2017
    - Replacement IACUC protocol initiated in May 2020.
- Major Task 2: Construct apparatus to deliver PEMF to test subjects –
    - Milestone achieved November 20, 2018.
- Major Task 3: Perform surgeries on animals and enroll in PEMF protocol. – First cohort of 8 rats initiated in protocol on May 14, 2018
    - These aseptic surgeries consist of transecting the sciatic nerve followed by immediate primary repair. Rats then are pair housed within the PEMF exposure cages and are subjected to specific PEMF waveforms for 6 hours per day, five days per week.

Specific Aim 2 - Assess functional outcomes of PEMF exposure on peripheral nerve injury/repair

- Major Task 4: Perform gait analysis on animals in Specific Aim 1. – First cohort of 8 rats initiated on May 1, 2018. These animals came off 4 months of PEMF exposure on September 25, 2018. Second cohort initiated 5/23/2019 and completed exposure 9/25/2019.
    - Gait analysis was performed prior to nerve injury/repair using a DigiGait system for gait analysis. Rats were subsequently studied at 10 weeks, and 4 months post-injury. Gait analysis determined that while there was impairment following nerve transection and repair, there was no significant difference in gait parameters between PEMF exposure and Sham-exposure treated groups of rats.(Fig 1,2, &3).

Figure 1. Gait parameters at baseline (prior to PEMF[dark bars] /Sham PEMF [lighter bars], also included as an attachment)

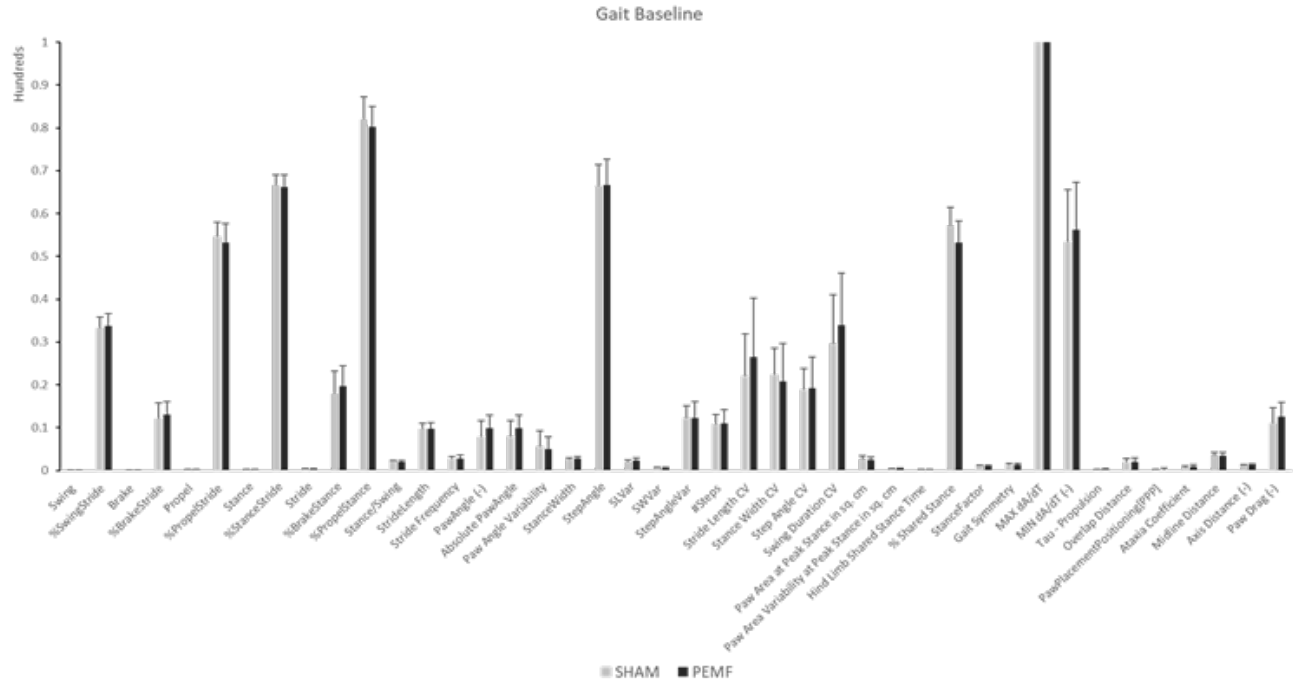


Figure 2. Gait at 3 months of PEMF/Sham PEMF

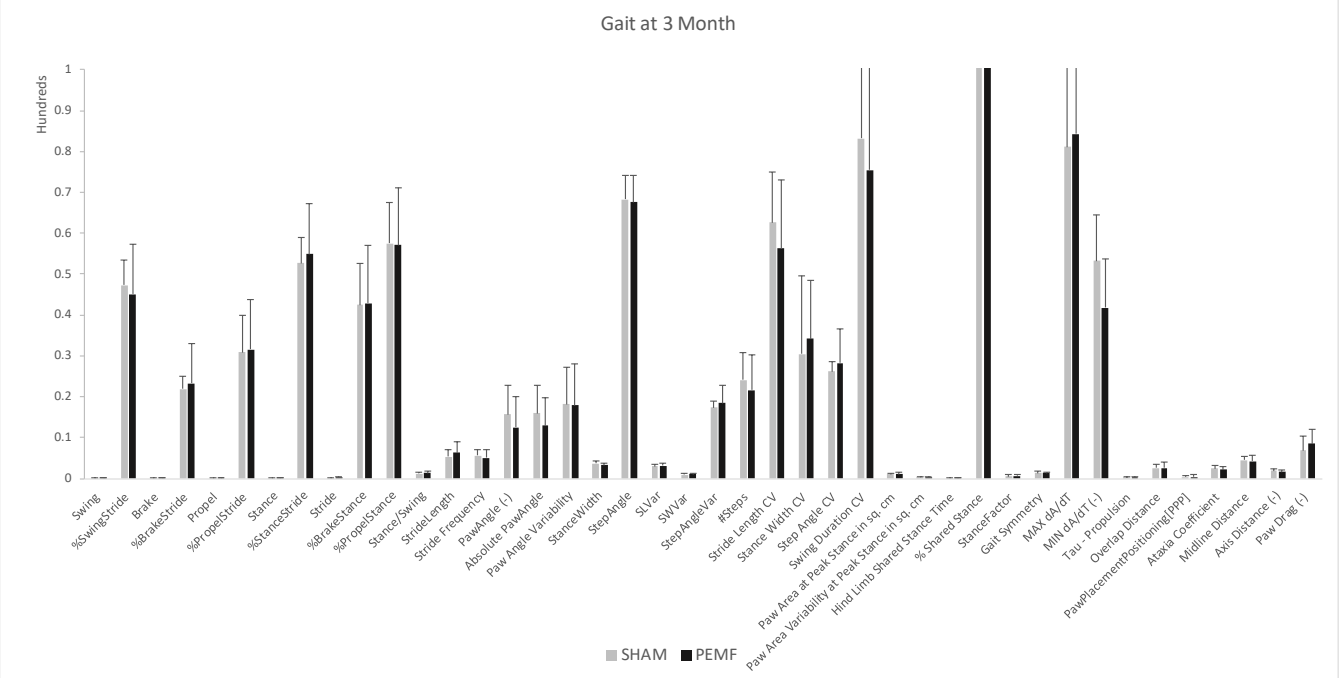
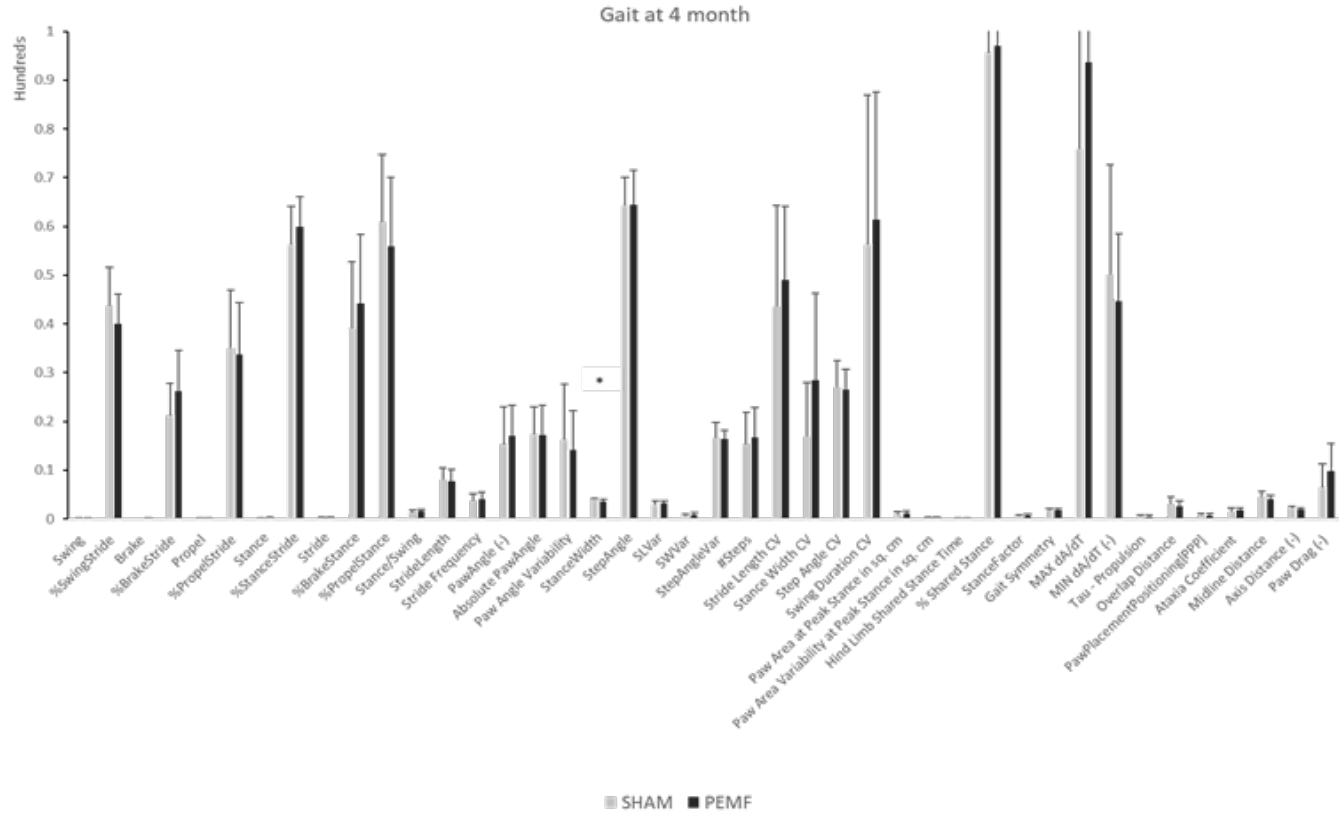
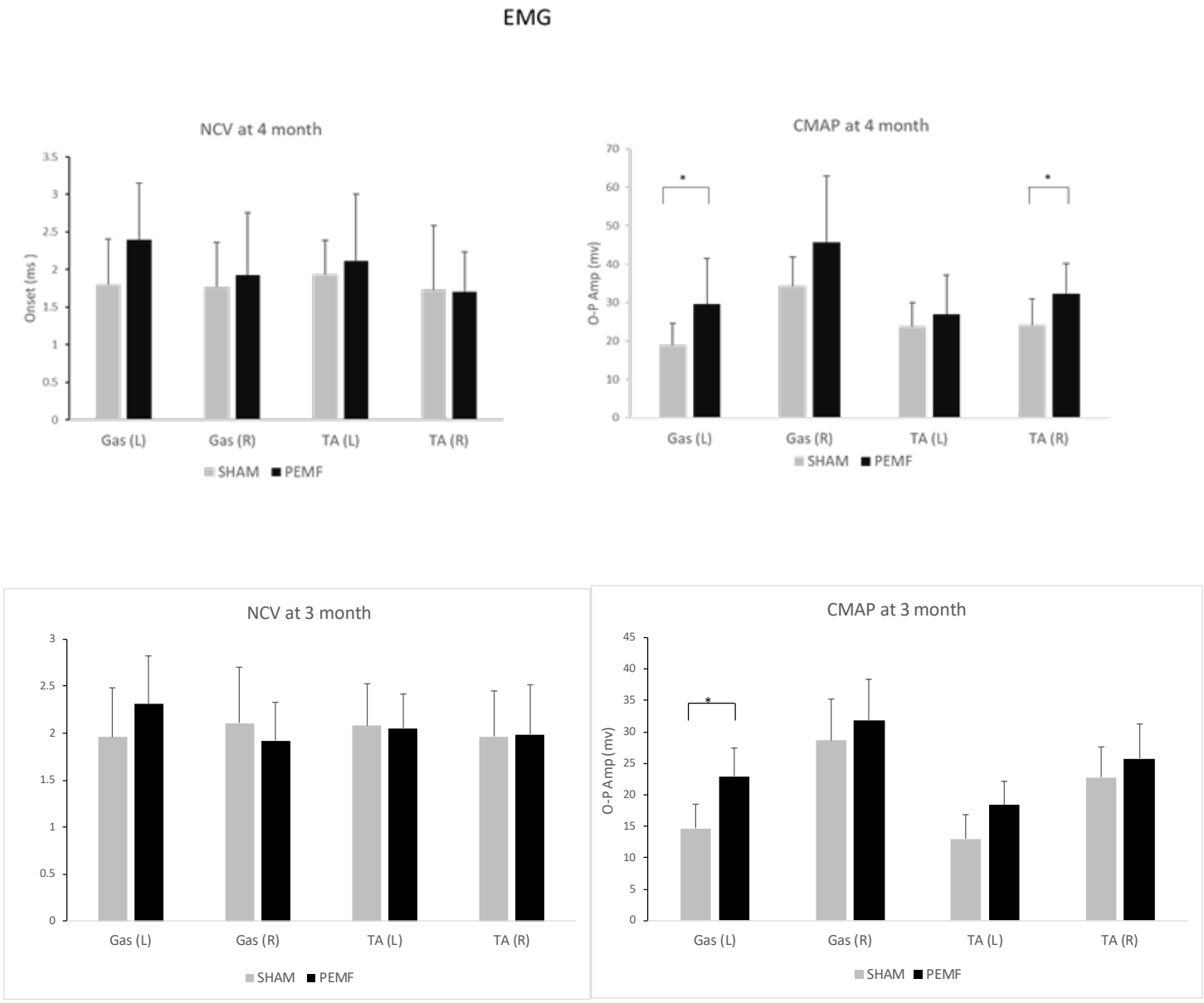


Figure 3. Gait at 4 months of PEMF/Sham PEMF



After 4 months of treatment, electromyography was performed on all test subjects. Nerve conduction velocity (NCV) and compound motor action potentials (CMAP) were obtained and are represented below in figure 4.

Figure 4. Electromyographic data collected following 4 months and 3 months of PEMF/Sham PEMF exposure in rats subsequent to sciatic nerve transection and repair.



These data are interesting in that compound motor action potentials are elevated in the PEMF –treated animals in both the gastrocnemius and tibialis anterior muscles.

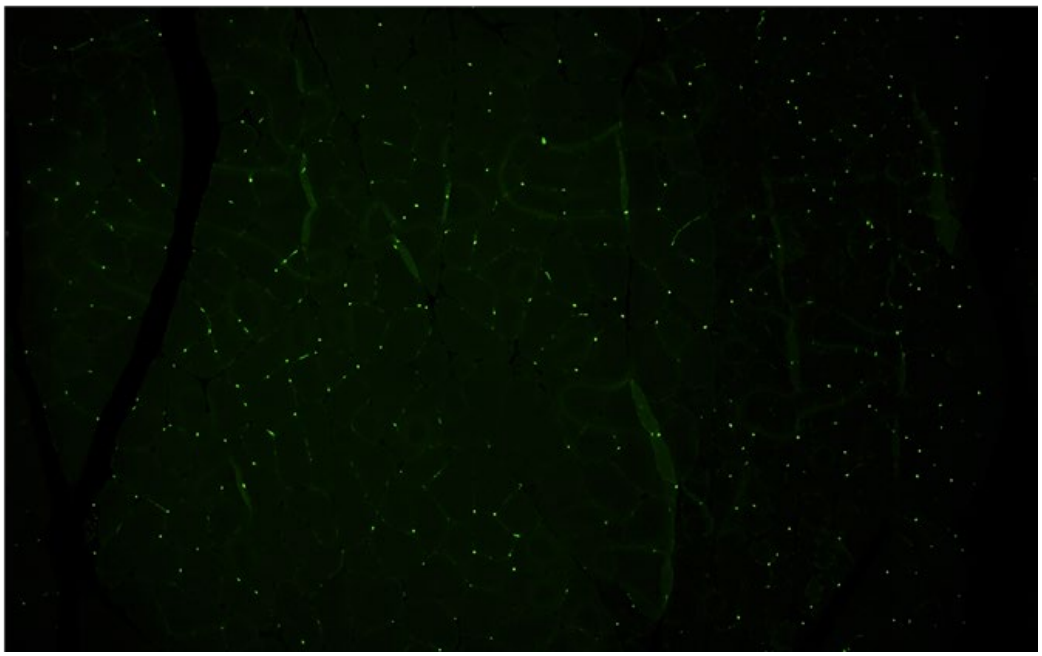
In interpreting these results, it is important to remember that the nerve repairs employed in these studies are the current "gold standard" clinically; i.e. a tension-free end-to-end anastomosis. Repairs were performed by individuals with extensive experience. Therefore, similar outcomes in gait between PEMF treated and Sham PEMF treated are not unexpected. PEMF apparently did not accelerate recovery between treated groups because there are not significant differences between 10 week treated groups or 4 month treated groups. The difference in electromyographical results are surprising because they were not accompanied by comparable changes in gait. However, these EMG differences are consistent with the observations of NMJ changes in the innervated muscles, which are described below.

Specific Aim 3 – Determine whether PEMF exposure has the ability to sustain neuromuscular junction morphology following denervation of muscle.
Major task 1: subject rats to PEMF following denervation of muscle. – Initiated in 5/19/2019.
- Exposure completed 11/02/2019.
- Animals euthanized for recovery of Tibialis anterior and gastrocnemius to assess neuromuscular junction morphology and muscle mass.

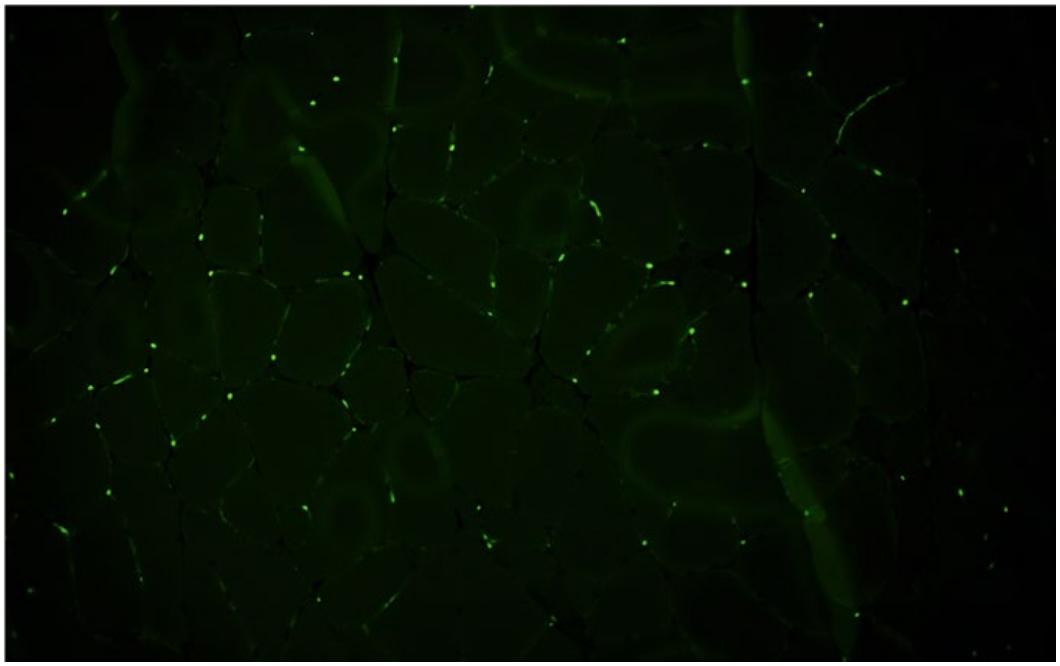
Histology is completed for 4 months and 6 months of PEMF or Sham exposure. Representative images are included below.

Panel A. 6 months of Sham PEMF treatment following complete denervation of the gastrocnemius muscle. Cross section of muscle with the neuromuscular junctions stained with a fluorescent marker. Images a 10 and 20 X magnification.

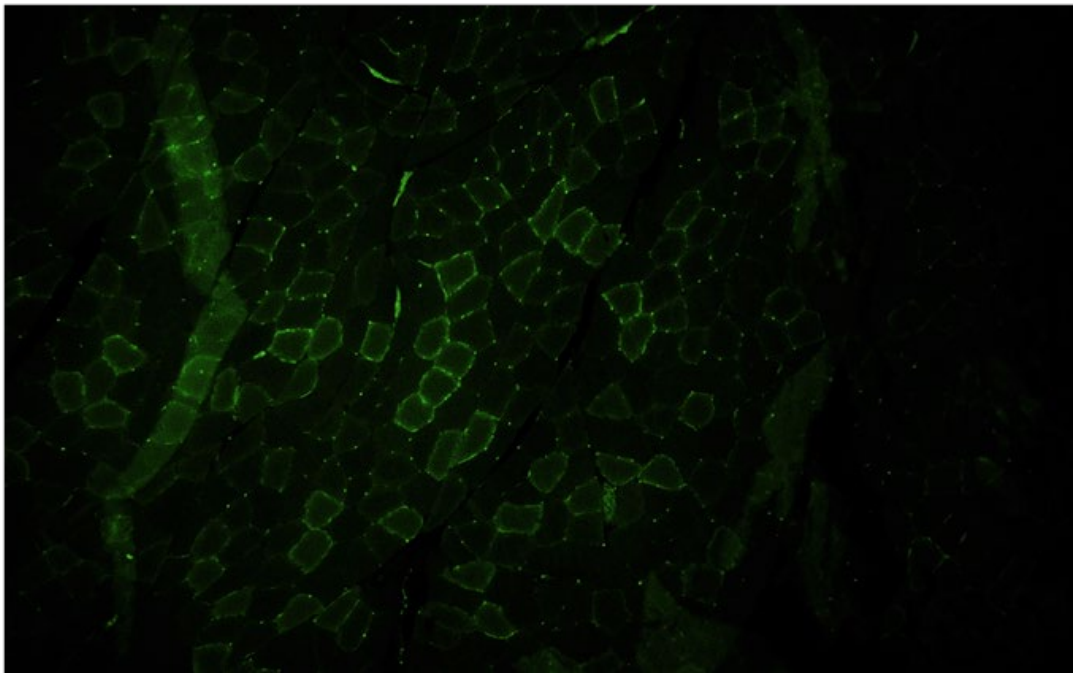
PEMF Gas 6 month 10X



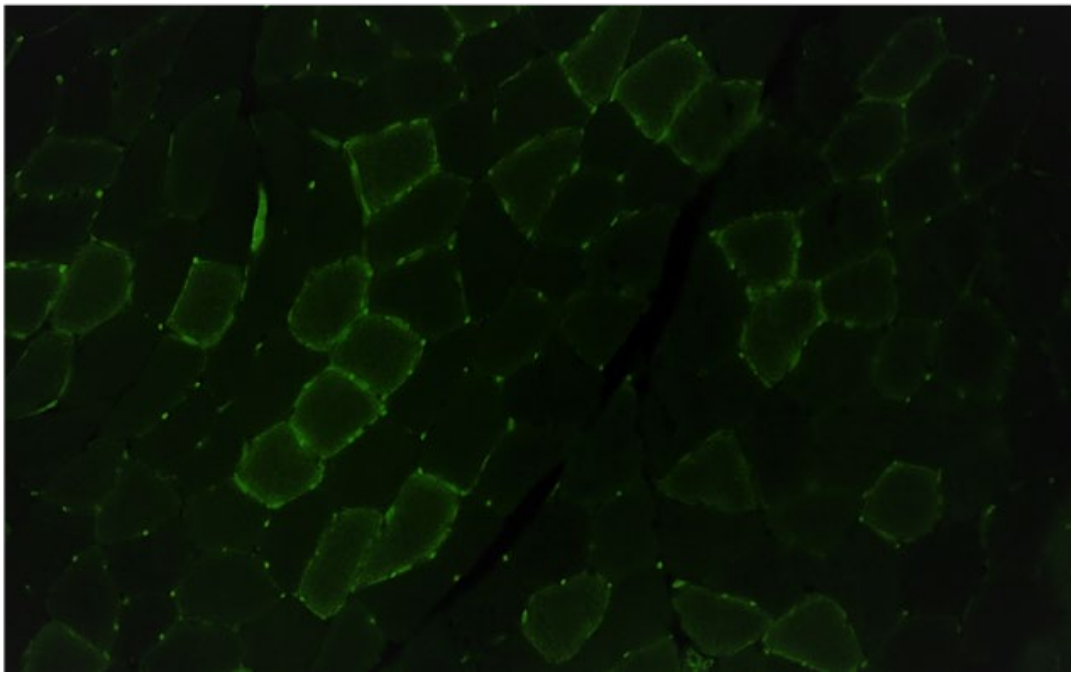
PEMF Gas 6 month 20X



Sham Gas 6 month 10X

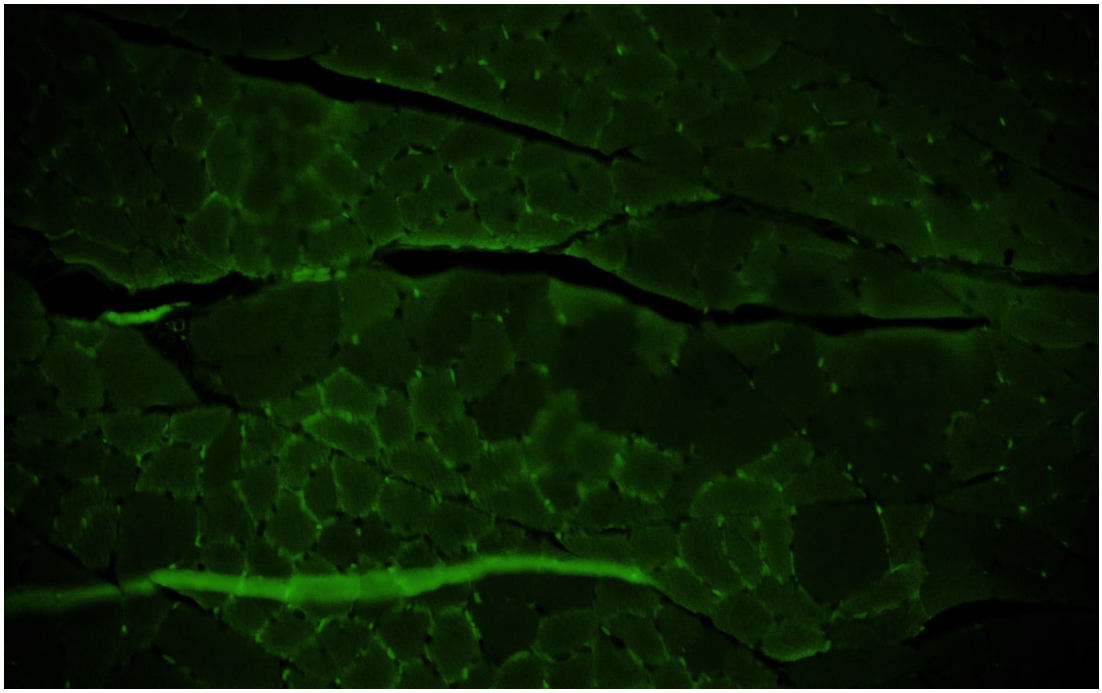


Sham Gas 6 month 20X

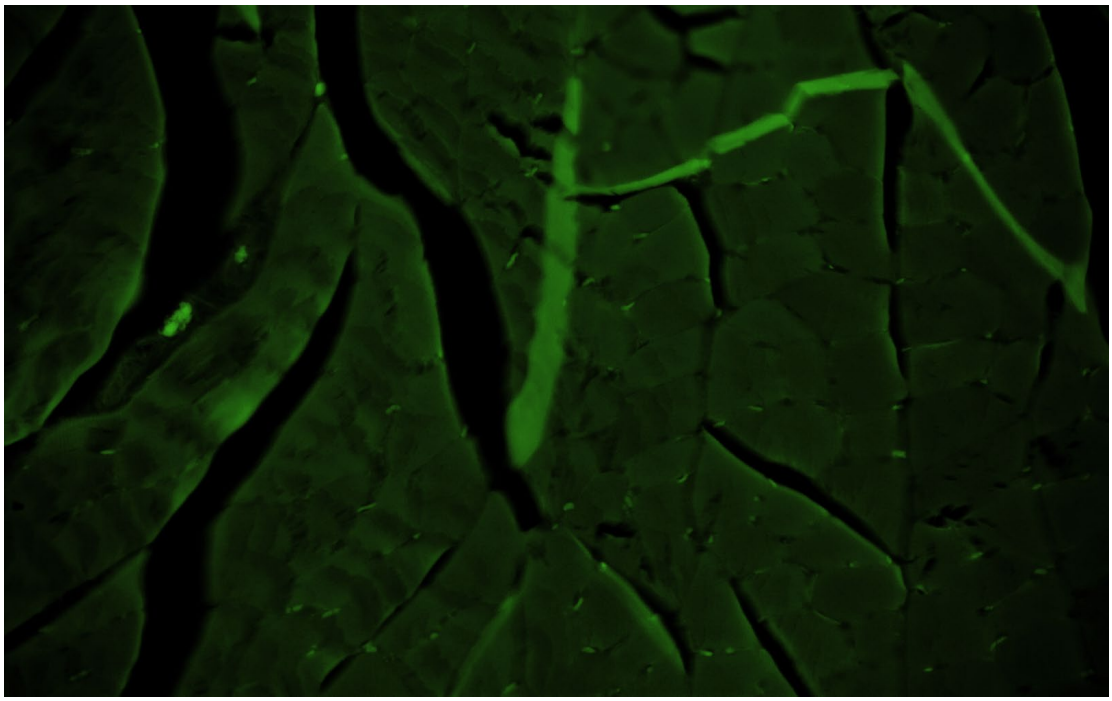


Panel B. 4 months of Sham PEMF treatment following complete denervation of the gastrocnemius muscle. Cross section of muscle with the neuromuscular junctions stained with a fluorescent marker. Images a 20 X magnification.

PEMF Gas 4 months 20X

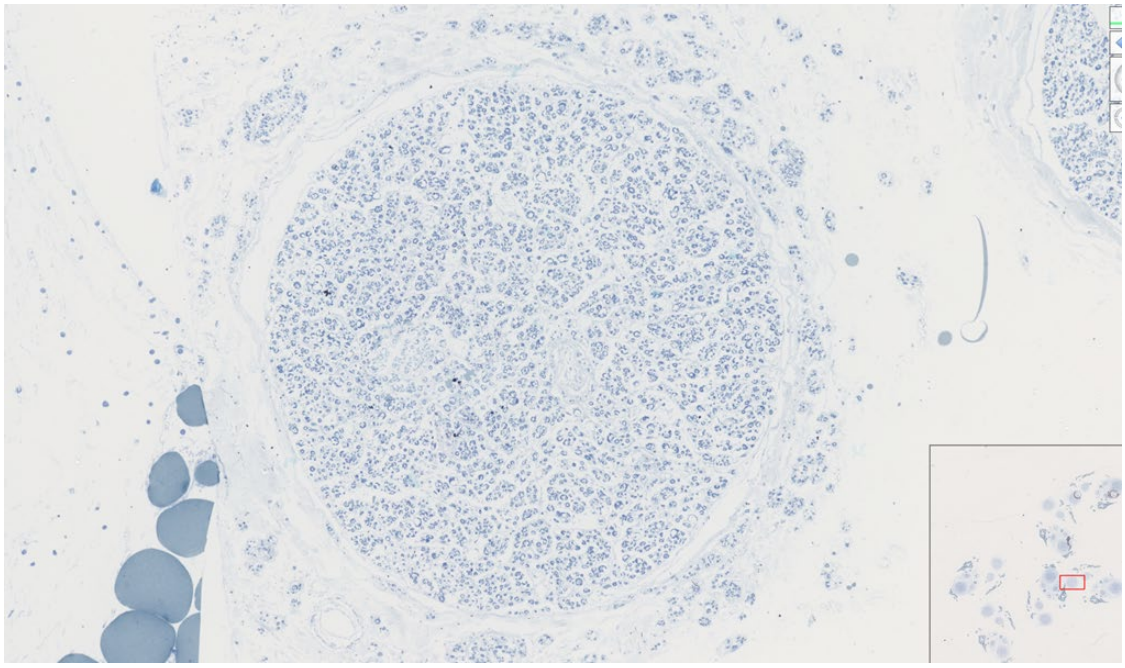


Sham Gas 4 month 20X

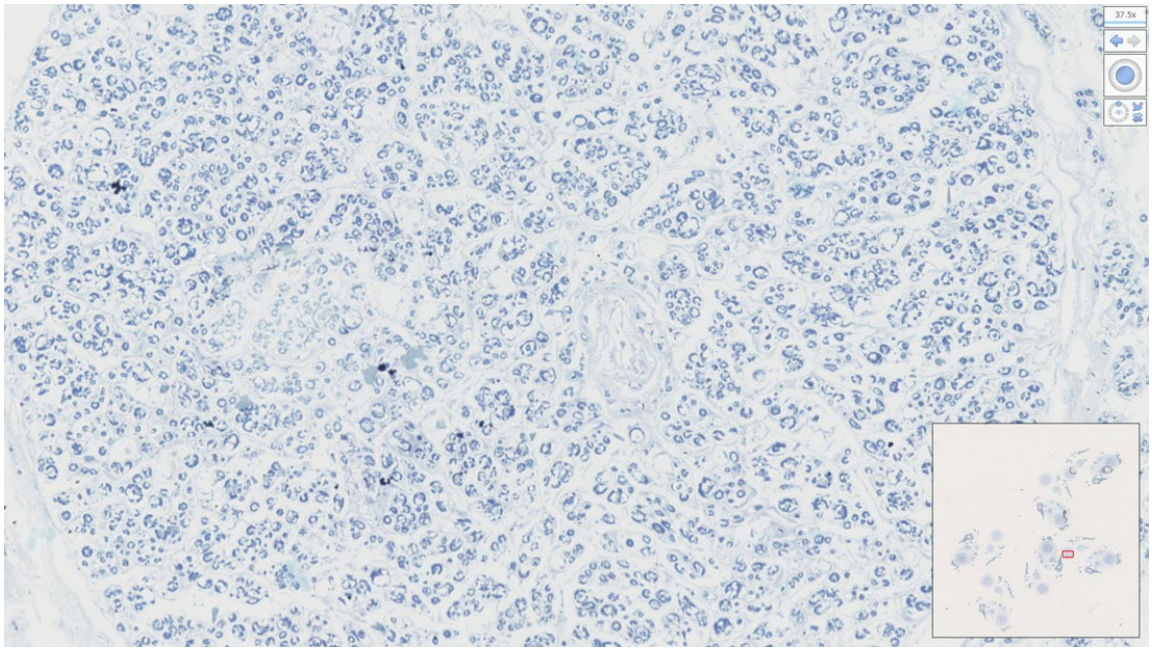


Panel C. Axon counts in left sciatic nerve of 4 months of PEMF treatment following primary repair of sciatic nerve transection.

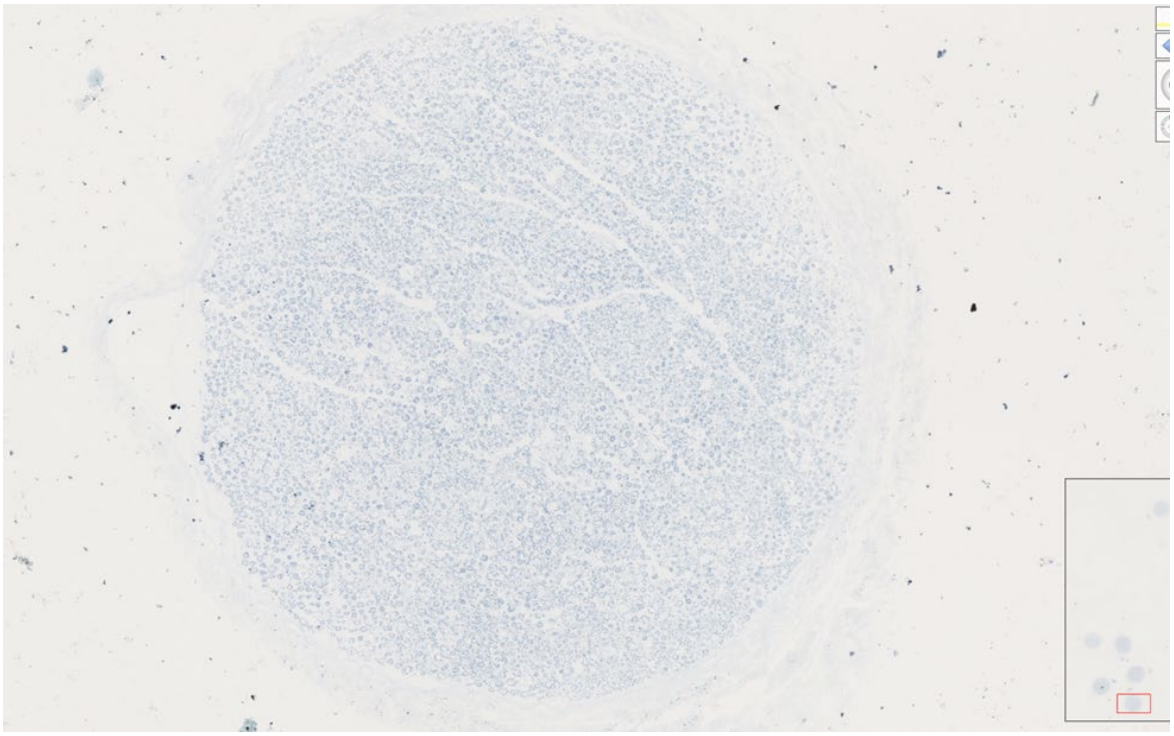
PEMF L sciatic X10



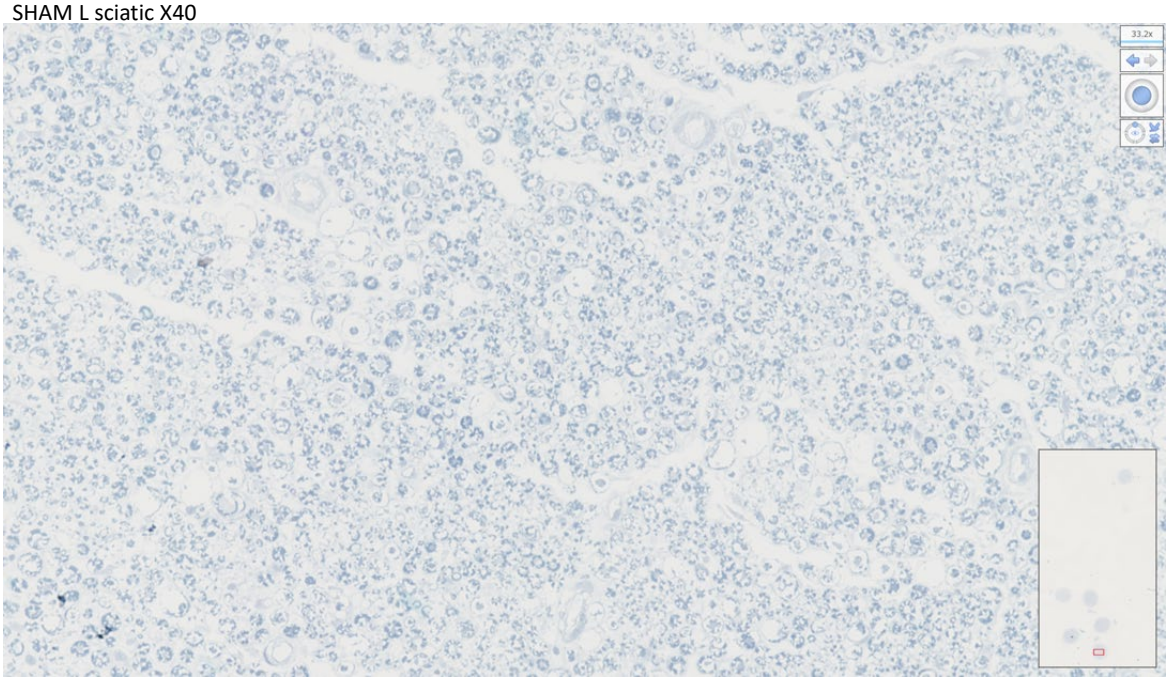
PEMF L sciatic X40



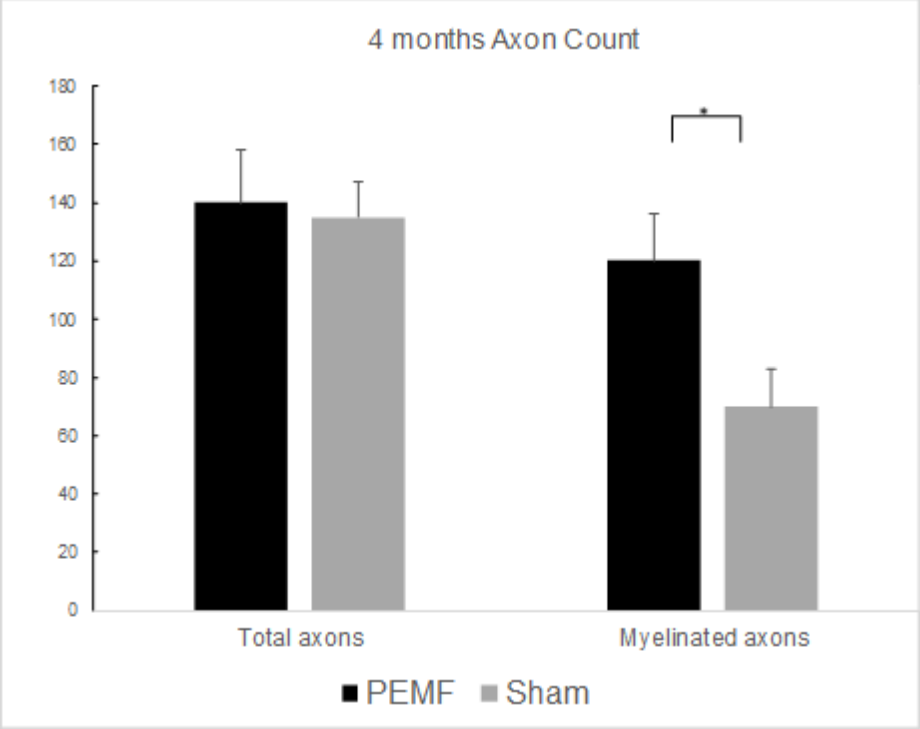
SHAM L sciatic X10

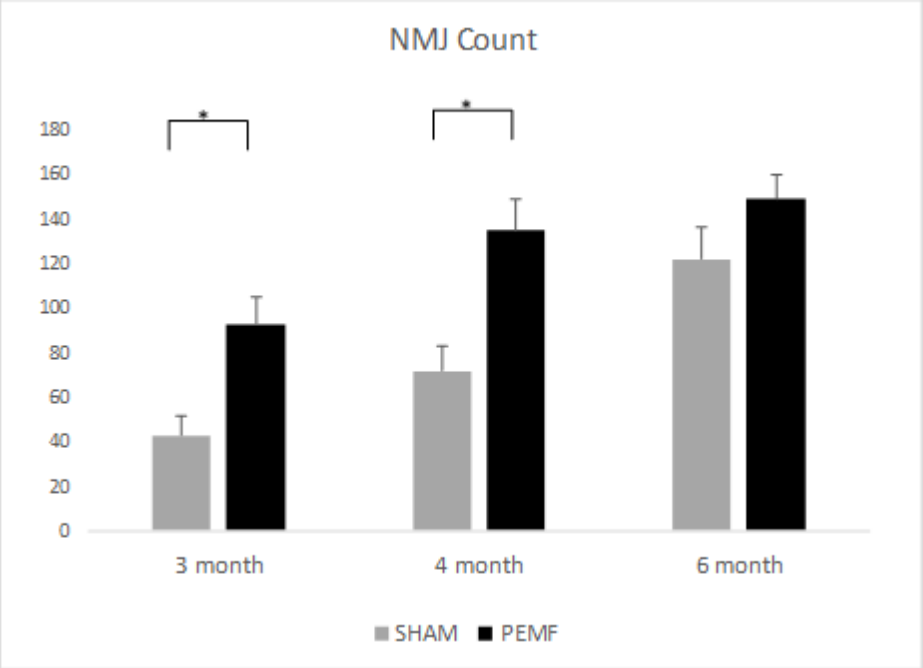


SHAM L sciatic X40



Panel D. Axon and NMJ counts in gastrocnemius muscles of 4 months of PEMF treatment following sciatic nerve transection and complete denervation of the gastrocnemius muscle.





4 months nerve analysis showed similar axon amount in PEMF and Sham group but myelinated axon was significant higher in PEMF group.(total axons: PEMF vs. sham: 140±10 vs. 135± 11; myelinated axons PEMF vs. sham: 120±5 vs. 70± 3, $p<0.01$). Neuromuscular junction (NMJ) count in gastrocnemius muscle showed PEMF group had significant more NMJ at 3 months and 4 months compared with sham group. (3 months: 93±12 vs. 43±9; 4 months: 135±10 vs. 70± 9, $p< 0.05$), At 6 months, NMJ counts were not significantly different between the groups. (6 months: 149±14 vs. 122±11, $p=0.09$) All counts were the average of 6 ROIs then normalized by the area. These data were consistent with EMG results that PEMF had higher compound muscle action potential at 4 months and 3 months in gastrocnemius muscle.(Gas op-Amp (mv), PEMF vs. sham: 4 months 29.6± 10 vs. 18.9 ± 5 , 3 months 22.9±4.5 vs. 14.6±3.8, $p<0.01$).

What opportunities for training and professional development has the project provided?

Nothing to report

How were the results disseminated to communities of interest?

Abstract of results of initial 4 months of PEMF exposure was submitted to MHSRS and SOA.

What do you plan to do during the next reporting period to accomplish the goals?

Specific Aims 1 and 2 – Enroll rats on PEMF exposure protocols of shorter duration.

Specific Aim 3 – These studies to examine the potential for PEMF to maintain neuromuscular junction integrity is completed and the histology analyzed.

4 .KEY RESEARCH ACCOMPLISHMENTS:

What was the impact on the development of the principal discipline(s) of the project?

Present results suggest no improvement in gait with PEMF exposure following sciatic nerve injury and repair at 3 months and 4 months post treatment. The technique employed in these studies yields optimal results and is considered the "Gold Standard" for peripheral nerve repair. Improvements to these outcomes would be clinically important because they could further optimize recovery of function in this patient population. The application of PEMF therapy post-operatively is non-invasive and cost effective and could be readily implemented because PEMF therapy currently is FDA approved.

Experimental electromyography findings of increased muscle force potential following 3 months and 4 months exposure to PEMF is of interest because it may suggest that the muscle responds to PEMF treatment. This could be through preservation of neuromuscular junctions following treatment with PEMF after denervation accompanying nerve injury and repair. This concept is reinforced by preliminary histology of muscle fibers in the two treatment arms of the experiment in which muscle is denervated and the animal is treated with PEMF or Sham PEMF. The neuromuscular junction counts in PEMF group was significantly higher than that of sham group at both 3 months and 4 months' time points, indicating the denervated muscles were better preserved with PEMF exposure.

The axons count in the sciatic nerves following primary transection then PEMF or sham treatment for 4 months revealed interesting findings. The total axon count was not significantly different between the PEMF and sham groups but the myelinated axon numbers were remarkably higher in PEMF group compared with sham. This finding was in consistent with the EMG results of increased compound muscle action potential in PEMF group, suggesting PEMF may accelerate nerve regeneration by reversing myelin sheath degeneration following nerve transection injury.

What was the impact on other disciplines?

Nothing to report

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

Nothing to report

5. CONCLUSIONS

We have completed the specific aims 1-3 and data interpretation and manuscript writing are in progress.

12. **PRODUCTS:**

- **Publications, conference papers, and presentations**

Journal publications.

Manuscript writing is in progress as we just got the histology analyzed with the shorter PEMF exposure time. Draft is shown in appendices.

Books or other non-periodical, one-time publications.

Nothing to report

Other publications, conference papers and presentations.

Abstract of results of initial 4 months of PEMF exposure was submitted to MHSRS and SOA annual meetings 2019.

- **Website(s) or other Internet site(s)**

Not applicable

- **Technologies or techniques**

Nothing to report

8. Inventions, patent applications, and/or licenses

Nothing to report

- **Other Products**

Nothing to report

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: Thomas L. Smith, PhD
Project Role: Principal Investigator
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 1.2 calendar months
Contribution to Project: Dr. Smith developed the animal model for use in this application and completed all animal surgeries. He has worked closely with his team to review, analyze, and interpret experimental results.
Funding Support: W81XWH-17-1-0513

Name: Zhongyu Li, MD, PhD
Project Role: Co-Investigator
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 0.24 calendar months
Contribution to Project: Dr. Li's role for this project has been to provide expertise as needed with respect to experimental design, nerve injury and repair surgeries, and data interpretation.
Funding Support: W81XWH-17-1-0513

Name: Xue (Amy) Ma, MD, PhD
Project Role: Co-Investigator
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 6 calendar months
Contribution to Project: Dr. Ma's role for this project has been to assist Dr. Smith in nerve injury and repair as well as functional testing at specific time points. She also is the primary person responsible for histologic preparation of the tissues being studied.
Funding Support: W81XWH-17-1-0513

Name: Eileen Elsner
Project Role: Laboratory Technician
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 6 calendar months
Contribution to Project: Ms. Elsner has assisted Dr. Smith in acquisition of animals and general animal husbandry. She has prepared the surgical suites and ensured appropriate supplies have been available for animal surgeries. She has also monitored the animals during daily PEMF exposure.
Funding Support: W81XWH-17-1-0513

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report

Zhongyu Li, MD, PhD
No changes in active support

Xue (Amy) Ma, MD, PhD

What other organizations were involved as partners?

Organizational name: Orthofix
Location of Organization: 345 Plano Parkway
Lewisville, TX 75056
Partner's contribution: Orthofix, a major supplier of PEMF equipment in the medical device industry, graciously loaned Wake Forest School of Medicine a complete, functional cage system for delivering the PEMF waveform approved in our grant. This system utilizes solenoid coils for generation of the PEMF signal. This system compliments but does not replace the PEMF system constructed by the Wake Forest Center for Nanotechnology and Molecular Materials.

- Collaboration : Jane Jacob, PhD, Vice President for Research and Clinical Affairs
  - Erik Waldorf, PhD, Principal Scientist and Research Manager
    - Dr. Waldorf oversaw the transportation and installation of the Orthofix PEMF system

8. OTHER ACHIEVEMENTS/SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

9. CHANGES/PROBLEMS:

Changes in approach and reasons for change

No major changes in approach were experienced. A minor change was made to utilize a solenoid-typed PEMF coil rather than a Helmholz-type coil. This change was made upon the recommendation of Orthofix, a major supplier of medical instrumentation for PEMF therapy. By utilizing a solenoid coil around the animal's home cage, a more consistent PEMF field was obtained, insuring uniform exposure of the animal to the correct waveform intensity, regardless of where they are located within their cage.

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Challenges have arisen with collecting gait data in trained rats. Although all rats enrolled in the studies have provided gait data, some animals have refused to walk or run on the treadmill after undergone extensive PEMF exposure. A variety of methods have been employed to obtain gait information from these animals, but some animals could not provide gait data.

The COVID-19 pandemic has significantly impacted the timeline of the current studies. The research endeavors at our teaching hospital were halted by our administration on March 17, 2020. This suspension continued until June 1, 2020. Furloughs for some personnel remained in place, however, for an addition few months. Renewal of our animal care and use committee approval for this research protocol was required and was initiated 5/08/2020. Because of furloughs, final approval of this protocol was not achieved until 8/11/2020, at which time the protocol was forwarded to the ACURO for review. Further research was suspended pending their approval.

In year 2021 we are still experiencing delayed animal order and histology processing due to short of staff in core lab and vivarium renovation. The histological process and analysis for the second cohort of 3 months of PEMF and PEMF/sham treatment group was finished in November 2021. Manuscript writing is still in progress.

Changes that had a significant impact on expenditures

Suspension of research activities due to the COVID-19 pandemic has had a negative impact on our expenditures. In year 2021 we have experienced delay in histology processing and staining in core lab due to staff shortage and animal order delay due to vivarium renovation.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

Not applicable

Significant changes in use or care of vertebrate animals

Nothing to report

Significant changes in use of biohazards and/or select agents

Nothing to report

10. REFERENCES

(1) Cho MS, Rinker BD, Weber RV, Chao JD, Ingari JV, Brooks D, et al. Functional outcome following nerve repair in the upper extremity using processed nerve allograft. J Hand Surg Am 2012 Nov;37(11):2340-9.

(2) Smith TL, Wong-Gibbons D, Maultsby J. Microcirculatory effects of pulsed electromagnetic fields. J Orthop Res. 2004 Jan;22(1):80-4.

(3) De Pedro JA1, Pérez-Caballer AJ, Dominguez J, Collía F, Blanco J, Salvado M. Pulsed electromagnetic fields induce peripheral nerve regeneration and endplate enzymatic changes. Bioelectromagnetics. 2005 Jan;26(1):20-7.

(4) Andersen RC, Fleming M, Forsberg JA, Gordon WT, Nanos GP, Charlton MT, Ficke JR. Dismounted Complex Blast Injury. J Surg Orthop Adv. 2012 Spring;21(1):2-7.

(5) Ma J, Shen J, Garrett JP, Lee CA, Li Z, Elsaidi GA, Ritting A, Hick J, Tan KH, Smith TL, Smith BP, Koman LA.Gene expression of myogenic regulatory factors, nicotinic acetylcholine receptor subunits, and GAP-43 in skeletal muscle following denervation in a rat model. J Orthop Res. 2007 Nov;25(11):1498-505.

(6) Walsh SK, Kumar R, Grochmal JK, Kemp SW, Forden J, Midha R. Fate of stem cell transplants in peripheral nerves. Stem Cell Res. 2012 Mar;8(2):226-38.

(7) Apel PJ, Ma J, Callahan M, Northam CN, Alton TB, Sonntag WE, Li Z Effect of locally delivered IGF-1 on nerve regeneration during aging: an experimental study in rats. Muscle Nerve. 2010 Mar;41(3):335-41

(8) Delo DM, Olson J, Baptista PM, D'Agostino RB, Jr., Atala A, Zhu JM, et al. Non-invasive longitudinal tracking of human amniotic fluid stem cells in the mouse heart. Stem Cells Dev 2008 Dec;17(6):1185-94.

(9) Tung TH, Mackinnon SE. Nerve transfers: indications, techniques, and outcomes. J Hand Surg Am. 2010 Feb;35(2):332-41.

(10) Isaacs J. Treatment of acute peripheral nerve injuries: current concepts. J Hand Surg Am 2010 Mar;35(3):491-7.

(11) Lundborg G. A 25-year perspective of peripheral nerve surgery: evolving neuroscientific concepts and clinical significance. J Hand Surg Am 2000 May;25(3):391-414.

11.APPENDICES:

Pulsed Electromagnetic Field Therapy Effect on Muscle Function Following Nerve Injury and Repair
Daniel Lara MD, Xue Ma, MD PhD, Zhongyu li, MD PhD, Thomas Smith, PhD, Wake Forest University School of Medicine, Winston Salem, NC 27157

MHSRS Annual Meeting 2019; SOA annual meeting 2019

Background: The most common injuries sustained by soldiers in Iraq and Afghanistan result from blast and shrapnel wounds caused by Improvised Explosive Devices (IEDs). These injuries are associated with a high probability of nerve damage to the extremities. Although IED injuries typically are survivable, injured soldiers face a lengthy rehabilitation course and the possibility of long-term functional impairments. In order to achieve functionality, nervous innervation must also be restored. Surgical reconstruction of peripheral nerve lesions in the extremities are challenging and often result in impaired functional recovery. Even with surgical intervention, patients who undergo repair of transected nerves may experience long term problems including sensory loss, functional impairment, pain, cold intolerance, and hyperesthesia. A major factor limiting recovery of muscle function following motor nerve repair is alteration of the motor end plate with loss of post-synaptic acetylcholine receptor (ACh) function. Following denervation, irreversible changes in post-synaptic neuromuscular junction (NMJ) motor end plate volume and surface area and in the ratios of ACh receptor subtypes can occur in the denervated muscle. These changes can occur when the distance required for re-innervation is so great that the regenerating axons take too long to reach the innervation muscle target. They also can occur if there is too long of a delay in repairing the nerve, which can occur in multi-trauma situations where life-threatening injuries in the patient must be addressed as a priority.

A potential intervention that holds promise in addressing both of these challenges in peripheral nerve repair is the use of pulsed electromagnetic fields (PEMF) therapy. PEMF has been used successfully clinically to treat fracture non-unions. Basic science studies have found that PEMF can affect biological tissues at the cellular level and their effects may be modulated through alteration of specific receptors. In studies of crush injuries to the sciatic nerve, PEMF has been demonstrated to significantly increase the rate of axon regeneration. PEMF also has been shown to increase Schwann cell proliferation in-vitro as well as in-vivo.

Methods: 8 female Lewis rats underwent sciatic nerve transection and immediate repair on their left hind leg. Following surgery the rats were randomly assigned to one of two groups. Group 1 (n=4): Received 6hrs/day of PEMF therapy 5 days/week for 4 months, in a cage apparatus that emitted The PhysioStim® waveform (Orthofix, McKinney Texas) across the cage. Group 2 (n=4): Placed in a similar cage apparatus but did not receive exposure to PEMF and was used as the control group. After 4 months the rats underwent walking track analysis as well as Electromyography (EMG) testing.

Results: Walking track analysis showed that the rats that received PEMF therapy following nerve transection and repair had a greater average stride length (4.8cm) than the control group (4.1cm), however this difference was not statistically significant ($p= 0.058$). EMG test results showed that the rats that underwent PEMF therapy had a greater response in both the gastrocnemius and tibialis anterior following sciatic nerve stimulation proximal to the repair site versus the control group, however neither difference was statistically significant($p=0.33$ and $p=0.61$).

Conclusions: The rats that received PEMF therapy following nerve transection and repair had a greater average stride length, as well as improved EMG readings when compared to the control group. Even though these results were not statistically significant, given the small number of animals tested, these results show that PEMF

therapy following nerve injury may indeed help with the recovery of the repaired nerve as well as the potential for better limb functionality following recovery. A greater number of animals will be tested to further determine if our results are significant. Future studies will involve the histological analysis of the muscles and nerves, to examine the NMJs and axons.

Pulsed Electromagnetic Field Therapy Effect on Muscle Function Following Nerve Injury and Repair

Xue Ma, MD PhD, Daniel Lara MD, Eileen Elsner, Jacob Hamby, Zhongyu li, MD PhD, Thomas Smith, PhD, Wake Forest University School of Medicine, Winston Salem, NC 27157

Key words: Nerve injury, pulsed electromagnetic fields, motor nerve function, neuromuscular junctions, sciatic nerve, gait.

Introduction: The most common injuries sustained by soldiers in Iraq and Afghanistan result from blast and shrapnel wounds caused by Improvised Explosive Devices (IEDs). These injuries are associated with a high probability of nerve damage to the extremities. Although IED injuries typically are survivable, injured soldiers face a lengthy rehabilitation course and the possibility of long-term functional impairments. In order to achieve functionality, nervous innervation must also be restored. Surgical reconstruction of peripheral nerve lesions in the extremities are challenging and often result in impaired functional recovery. Even with surgical intervention, patients who undergo repair of transected nerves may experience long term problems including sensory loss, functional impairment, pain, cold intolerance, and hyperesthesia.(1-4) A major factor limiting recovery of muscle function following motor nerve repair is alteration of the motor end plate with loss of post-synaptic acetylcholine receptor (ACh) function. Following denervation, irreversible changes in post-synaptic neuromuscular junction (NMJ) motor end plate volume and surface area and in the ratios of ACh receptor subtypes can occur in the denervated muscle. These changes can occur when the distance required for re-innervation is so great that the regenerating axons take too long to reach the innervation muscle target. They also can occur if there is too long of a delay in repairing the nerve, which can occur in multi-trauma situations where life-threatening injuries in the patient must be addressed as a priority.

A potential intervention that holds promise in addressing both of these challenges in peripheral nerve repair is the use of pulsed electromagnetic fields (PEMF) therapy. PEMF has been used successfully clinically to treat fracture non-unions.(5) Basic science studies have found that PEMF can affect biological tissues at the cellular level and their effects may be modulated through alteration of specific receptors.(6-8) In studies of crush injuries to the sciatic nerve, PEMF has been demonstrated to significantly increase the rate of axon regeneration. (7) PEMF also has been shown to increase Schwann cell proliferation in-vitro as well as in-vivo. (8-10)

In this study the potential of PEMF to improve both the rate of axonal growth following nerve injury and repair as well as preservation of the post-synaptic NMJ morphology following denervation were examined using a rat model. Functional gait outcomes, electromyography of compound muscle action potential (CMAP) in denervated muscles, regeneration of injured nerves and the preservation of the post-synaptic NMJ were assessed with the use of externally applied PEMF to the extremity of rats.

Material and Methods:

Surgery Procedure and PEMF treatment

All animal use was approved by the Animal Care and Use Committee (ACUC) of Wake Forest University Health Sciences.4 groups of 12 female Lewis rats per group underwent left sciatic nerve transection and primary repair surgery. The rat was anesthetized using isoflurane (1.5-2.5 volume %), initially in an induction chamber. Anesthesia was maintained via a nosecone at 1.5 volume%. The posterior aspect of the left hind limb was shaved with clippers, cleansed with betadine scrub, and disinfected with betadine solution. Using aseptic technique, a posterolateral incision was made and the sciatic nerve was exposed by dissecting the muscle plane.

A transection was created in the nerve and immediately repaired using 10-0 nylon and standard microsurgical technique. Following nerve repair, the muscle was approximated using interrupted sutures of 5-0 coated vicryl. The skin was closed by approximating the cut edges using stainless steel surgical wound clips and subdermal sutures 5-0 coated vicryl. The rat was given buprenorphine for post-surgical analgesia (0.01 mg/kg, SC) at the end of surgery. Following surgery the rats were randomly assigned to one of four groups. Group 1 (n=12): Received 6hrs/day of PEMF therapy 5 days/week for 4 months, in a cage apparatus that emitted The PhysioStim® waveform (Orthofix, McKinney Texas) across the cage. Group 2 (n=12): Placed in a similar cage apparatus but did not receive exposure to PEMF and was used as the sham group. Group 3 (n=12): Received 6hrs/day of PEMF therapy 5 days/week for 3 months, in a cage apparatus that emitted The PhysioStim® waveform across the cage. Group 4 (n=12): Placed in a similar cage apparatus but did not receive exposure to PEMF and was used as the sham group. For the fifth and sixth group of 12 female rats, the sciatic nerve was transected and the two free ends were buried then sewn in the conjunct muscles. Group 5 (n=12): Received 6hrs/day of PEMF therapy 5 days/week for 6 months, in a cage apparatus that emitted The PhysioStim® waveform across the cage. Group 6 (n=12): Placed in a similar cage apparatus but did not receive exposure to PEMF and was used as the sham group.

Walking track analysis

Gait recovery is an indicator of return of motor control. The DigiGait Imaging system (MouseSpecifics Inc. MA) was used to test the motor function recovery of the allograft reconstruction following sciatic nerve defect in Lewis rats. The DigiGait system imaged the underside of the running rat with a high speed digital video camera continuously (188 frames/second) and generates digital paw prints which can be translated to dynamic gait signals of the temporal record of paw placement relative to the crystal treadmill belt. The return of motor control at 3 months and 4 months after sciatic transection and primary repair were documented (Figure 1.) Each animal was compared to their pre-injury walking track values. This technique permits use of the highly sensitive repeated measures analysis of variance for these animals and is capable of detecting slight differences between groups. 24 parameters at the end of 4 months following injury were analyzed.

Electrophysiology analysis

Cadwell EMG Sienna Wave System was used for the electrophysiology testing 3 and 4 months after the nerve injury and repair, Group 1-4 rats were anesthetized with isoflurane and the regenerated and contralateral sciatic nerves were exposed. At first, tibial and peroneal branches distal to the regenerated gap were briefly stimulated to test for plantar flexion and foot eversion. After the viability of the nerve was assessed, electromyographic analysis was examined by stimulating the regenerated nerve proximally (suture sites were taken as referral points) with a monopolar cathodic electrode at 1mA, the reference anode was placed on the rat chest. The stimulating–recording electrode distance was verified visually using a ruler. 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.

Tissue harvesting and histomorphometric analysis

Nerve

The animals were euthanized with intracardiac injection of saturated potassium chloride following electrophysiological testing. The left sciatic nerves were harvested 2mm distal to the suture point as a reference. The nerve samples were fixed in 4% paraformaldehyde or 2% osmium tetroxide, dehydrated and later

embedded in paraffin or resin. Serial 5µm sections were cut 1 mm distally to the suture inserting point to assess the regenerating nerve fibers penetrating to the nerve stump. The slides were stained with toluidine blue and examined under Zeiss light microscopes (Thornwood, NY) at 100X and 400X final magnifications. The images were analyzed using VisioPharm software (Hoersholm, Denmark) to measure regenerated axons. The number of axons was counted and the outlines of myelinated axons and total axons were manually traced. The cross section area, the number of myelinated fibers (n), myelin thickness (MT), average axonal diameter (AD) and fiber diameter (FD) were assessed using ImageJ software (Wayne Rasband).The G ratio was calculated as AD/FD and the fiber density calculated as number of fibers/mm2. The axonal area (_ πAD/2)2 and the fiber area (_π FD/2)2 were obtained assuming the circularity of the nerve fiber area. The myelinated area was measured as the difference between fiber area and axonal area. Axon areas were counted at minimum of 5 areas for each transverse section and 15 sections per animal were analyzed for the different experimental groups.

Muscle

The gastrocnemius and tibialis anterior muscles from both the experimental and contralateral side were harvested. (Figure 3). 10µm sections of muscle were cut and stained with α-bungarotoxin (Thermo Fisher, NY) to visualize neuromuscular junction replenishment following nerve injury and repair.

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, ***$p < 0.001$.

Results

Walking track analysis

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 between PEMF (group 3) and sham (group 4) groups.

The PEMF group (group 1) showed significant enhanced recovery at stance width and axis distance compared to sham group (group 2) at 4 months. (*$p<0.05$, **$p<0.01$ in all indices.)

Electrophysiology analysis

Electrophysiological analysis of CMAP indicated that PEMF group had significant higher of wave potentials on gastrocnemius muscle compared with sham groups. PEMF had higher compound muscle action potential at both 4 months and 3 months in denervated gastrocnemius muscles.(Gas op-Amp (mv), PEMF vs. sham: 4 months 29.6± 10 vs. 18.9 ± 5 , 3 months 22.9±4.5 vs. 14.6±3.8, $p<0.01$) PEMF also showed significant beneficial effects on CMAP of contralateral uninjured gastrocnemius and tibialis anterior (TA) muscles at 4 months post treatment. (Gas op-Amp (mv), PEMF vs. sham: 4 months 45.6± 7.2 vs. 34.2 ± 6.63; TA op-Amp (mv), PEMF vs. sham: 4 months 32.3± 6.79 vs. 24 ± 5.94, $p<0.05$)

Histomorphological analysis

Evaluation of cross sections through the distal part of the regenerated nerves was conducted by light and microscopy. PEMF group demonstrated remarkably increase number of myelinated axon, compared to sham group at 4 months post injury. On light microscopy, the PEMF group showed well aligned and regenerated nerve fibers, whereas the fibers of sham group had an overall disrupted endoneurium architecture of myelinated axons. 4 months nerve analysis showed similar axon amount in PEMF and Sham group but myelinated axon was significant higher in PEMF group.(total axons: PEMF vs. sham: 140±10 vs. 135± 11; myelinated axons PEMF vs. sham: 120±5 vs. 70± 3, $p<0.01$). Immunohistochemistry analysis also showed PEMF group had significant increased number of neuromuscular junction (NMJ) with more complexed morphology compared with sham groups at 3 months and 4 months post injury and repair. (3 months: 93±12 vs. 43±9; 4 months: 135±10 vs. 70± 9, $p< 0.05$), At 6 months, NMJ counts were not significantly different between the groups. (6 months: 149±14 vs. 122±11, $p=0.09$) All counts were the average of 6 ROIs then normalized by the area.

Discussion: in progress