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TITLE: Membrane Fusion Nerve Repair to Improve Limb Transplant Function

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14. ABSTRACT

Background: Limb transplantation has dramatically improved the lives of servicemen and civilians alive. Those who have had their limbs restored through this innovative surgery have seen improvement in their body image, decreased reliance on prosthetics and increased independence. The outcomes of these transplants are highly dependent on nerve regeneration. Nerve regeneration is time consuming and relies on intense rehabilitation. A new and exciting technique to connect cut nerve fibers using polyethylene glycol (PEG) can improve nerve function following transplant.

Methods: Using a Lewis rat model, we studied the effect of PEG membrane fusion on the sciatic nerve in nerve repair and limb transplantation. To verify and quantify the results, nerve conduction studies, histomorphometry, retrograde cell labeling, and gait analysis were performed.

Results: Improvements could be seen in nerve regeneration with nerve conduction, and histomorphometry. On analysis at 1 week and 20 weeks, the sciatic nerves treated with PEG/membrane fusion protocol had a higher count of distal axons than nerves without treatment. Functional improvements were noted in the simple repair groups but limb contractures made it impossible to measure function recovery in transplanted hind limbs.

Conclusion: PEG membrane fusion nerve repair improved outcomes in nerve repair and showed promise in hind limb transplantation although the demonstration of functional recovery was hampered by limitations of the model. Another group at our institution has shown good results in an alternative model. Future work will involve translational large animal models to verify and refine the techniques prior to use in humans.

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NONE LISTED

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1. **INTRODUCTION:** This project investigated the use of a new technique for re-innervation of limb transplants in order to dramatically speed rehabilitation and improve reconstructive transplant function. In its relatively short history over the last two decades, reconstructive transplantation of the upper extremity has dramatically improved the lives of servicemen and civilians alike who have had their limbs restored through this innovative surgery. The pace and overall success of rehabilitation depends on the re-innervation of the graft by the slow process of recipient nerve fibers growing into the graft. A new and exciting technique to connect cut nerve fibers using membrane fusion has the potential to repair nerves rather than allowing them to slowly regenerate. This technique has shown promise and the goal of this work was to determine whether nerve fusion can improve the outcome of nerve repair and physiological function of limb transplants in an animal model.

2. **KEYWORDS:** Nerve repair, Polyethylene Glycol, membrane fusion, limb transplant

3. **ACCOMPLISHMENTS:**

	complete
<u>Aim 1</u> **a) Optimize membrane fusion nerve repair techniques in the transplant model.** **b) Determine differences between cut/repair and transplant nerve fusion**	
Major task 1: Prepare for experiments	
- Prepare animal protocols for local IACUC and ACURO	100%
- Coordinate data sharing and sample exchange protocols. Train personnel	100%
- local IACUC approval	100%
- ACURO approval	100%
Major task 2: Optimize fusion conditions for transplant	
- Begin transplants with standard repair and membrane fusion	100%
- Vary experimental variables in fusion conditions; time, concentration, temperature, perfusion solution	100%
- Milestone achieved: Determine optimal fusion conditions for use in transplant and publish findings.	**90%**
<u>Aim 2</u> **a) Quantify the functional outcomes of limb rehabilitation using physiologic testing,** **b) Histology and** **c) Diffusion tensor magnetic resonance nerve imaging**	
- Determine rate and extent of functional rehabilitation using physiologic parameter testing.	***50%***
- Complete histology and diffusion-tensor magnetic resonance nerve imaging	*50%*
- Compile and analyze data/compare groups	*50%*
- Milestone achieved: publish and present final finding at national meeting	*N/A*

Aim 1

a) **Optimize membrane fusion nerve repair techniques in the transplant model.**
b) **Determine differences between cut/repair and transplant nerve fusion**

Major task 1: Prepare for experiments

- **Milestone achieved: Prepare animal protocols for local IACUC and ACURO:**

The start of the project involved writing an animal protocol which detailed the experimental groups required to achieve the outcomes postulated by our statement of work. These groups defined the tests that needed to be performed and their requirements to assure the compliance with the Animal Welfare Act regulations and Public Health Service (PHS) Policy. A comprehensive and stringent review was required to ensure experimental animals received the highest quality care and to protect the health of the project members working with and around the animals.

- **Milestone achieved: Coordinate data sharing and sample exchange protocols. Train personnel**

Starting with an introductory meeting with the collaborators at Vanderbilt University to introduce the different members of both parties. This was followed by discussion of the experiments that would be performed, and the roles and responsibilities of each party. Established protocols were exchanged, and run-throughs were attempted to identify any issues that may arise with specimen transfer, as Vanderbilt would be processing and analyzing the histomorphometry of nerve samples. During the first four months, the lab fellow at JHU started to train in animal surgical models and testing including:

- General anesthesia maintenance and post-operative care
- Nerve repairs
- Microscopy training
- Hind-limb transplantation
- Forelimb transplantation
- AD instruments electrophysiological testing
- Catwalk sciatic functional index analysis
- Grip strength testing
- PEG nerve fusion technique

- **Milestone achieved: local IACUC and ACURO approval**

After submission and review of the animal protocols approval was obtained from the IACUC, and this was followed by submission and approval by ACURO.

Major task 2: Optimize fusion conditions for transplant

- **Milestone achieved: Begin transplants with standard repair and membrane fusion**

Sciatic transection and repairs were performed with both standard suture technique and PEG membrane fusion repairs. This was initially done in non-transplant conditions to simplify the model during the initial phase of the experiments. Outcomes measures were developed which are described in the description of Aim2 below which were then used to assess the success of the nerve regeneration under various conditions. These included nerve conduction studies, histomorphometry and retrograde axonal labeling. To be able to optimize the fusion condition, two measures had to be quantified. The first being the number of axons being fused, and the second being functional recovery. By use of retrograde labelling we were able to prove we could use axons as tracers could only travel up an intact axon to reach the cell body in the Dorsal root ganglia (DRG) in sensory axons and spinal dorsal cells for motor axons. Changes in the compound action potential (CAP) is the most immediate way of establishing whether axonal fusion using PEG was achieved. We were able to visualize a return in the CAP which correlated with the literature.

- **Milestone achieved: Vary experimental variables in fusion conditions; time, concentration, temperature, perfusion solution**

Conditions were then varied including times of the various steps and changes in the concentration of methylene blue used. Maximal fusion was found in the cut and repair model. Syngeneic transplantation was then performed from inbred Lewis rats to obviate the need for immunosuppression. Transplantation

techniques were successfully modified to allow for the incorporation of conventional suture and PEG fusion nerve repairs into the transplant model. At this point difficulties were encountered with the functional outcome testing as described below. This made the completion of this milestone impossible and work on this milestone was concluded before the final Milestone of "Determine optimal fusion conditions for use in transplant and publish findings:" was completed. In lue of publication, our results were shared with the scientific community by being presented at local and national meetings as described below.

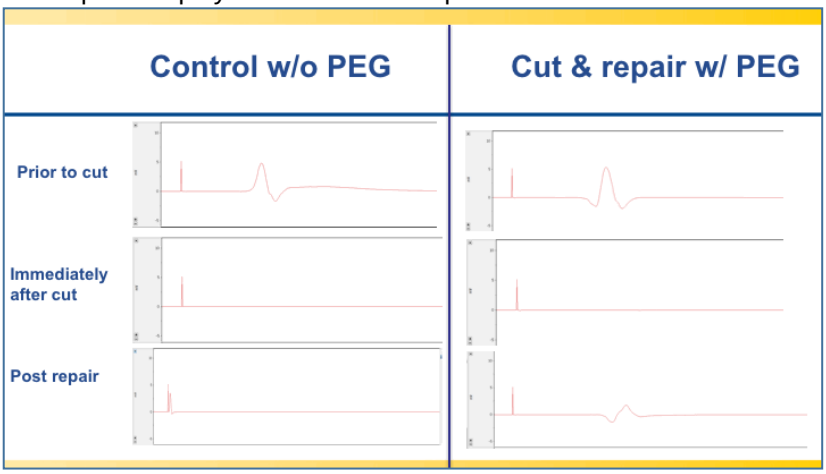
Aim 2

a) **Quantify the functional outcomes of limb rehabilitation using physiologic testing,**
b) **Histology and**
c) **Diffusion tensor magnetic resonance nerve imaging**

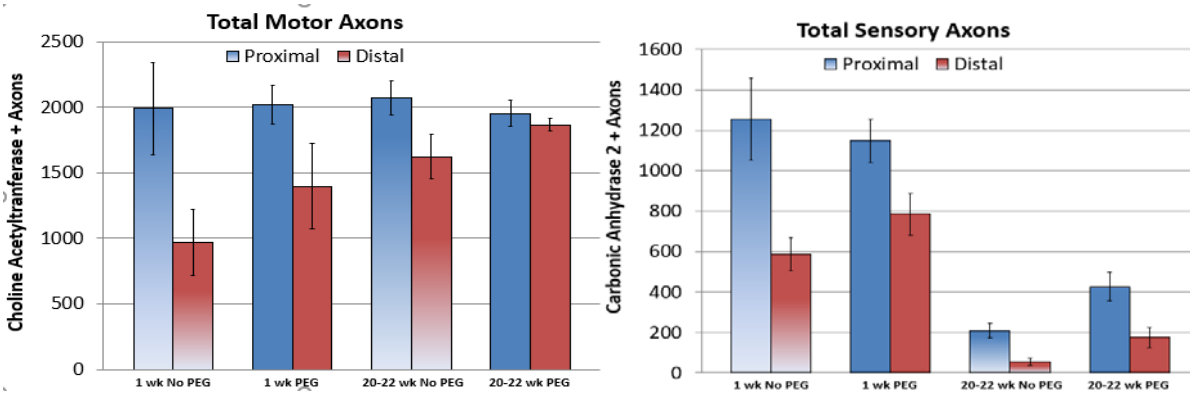
Major task 3: Measure nerve regeneration and functional recovery/rehabilitation

- **Milestone Achieved: Determine rate and extent of functional rehabilitation using physiologic parameter testing**

The goal of the second aim was to assess the improvements in nerve regeneration using PEG fusion nerve repair on the physiologic outcomes measure following nerve repair in the setting of limb transplantation. The techniques employed included intraoperative neve conduction and Electromyography (EMG). These efforts have resulted in success optimizing EMG testing of nerve repair using the standard repair technique and demonstrating improvement using PEG membrane fusion (Please see figure left). This technique provides immediate results at the time of nerve repair and membrane fusion. This has allowed us to optimize conditions necessary for nerve fusion using nerve conduction studies as an outcome measure as described in Aim 1 above. To determine the effect of reconstituting these axons with PEG fusion on nerve architecture following nerve repair we utilized quantified histomophomertry. This was done in conjunction with our collaborators at the University of Vanderbilt. (see below). This has demonstrated that PEG fusion nerve repair leads to preservation of nerve fibers with a decrease loss of axons at 1 weeks and the effect is maintained at 20-22 weeks (at which time our prior work has shown maximal functional recovery in standard suture repair models). This effect is seen in both motor and sensory axons although the effect is more prominent in motor fibers.

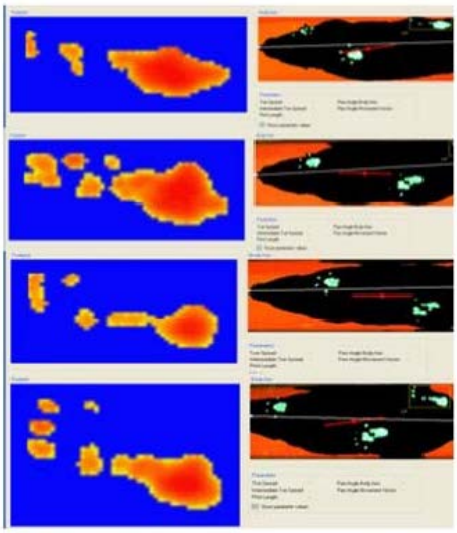


Intraoperative nerve conduction study demonstrating return of signal following PEG fusion



Histomorphometry data demonstrating improvement of motor and sensory axons after PEG fusion

To determine whether the fused nerve fiber were functional or if there was nonfunctional nerve fibers resulting from abnormal nerve fusion required the use of a functional outcome measure measuring a physiologically relevant parameter. Our prior studies have utilized gait analysis for the purpose. Functional assessment of the hind limp has been successfully evaluated using the Catwalk (see figure right) computerized gait visualization/analysis system. Gait analysis using the catwalk system was able to confirm the improvements seen in the histology of the nerve correlated with improvements in restoration of gait in the nerve cut and repair model. However, when we attempted to use this tool to follow functional recovery in hind limb transplant recipients we encountered a significant obstacle. We consistently found that even at terminal time points (>22 weeks) animals in all groups failed to place the transplanted foot on the ground during ambulation. A clear footfall is necessary to determine the sciatic functional index. This post-transplant deformity made it impossible to quantify functional recovery in the hind limb. It is not clear whether this deformity is a consequence of the additional nerves which are cut (and not repaired) during a transplant or due to additional trauma such as ischemia reperfusion injury or transection and repair of the thigh muscles and femur.



Catwalk Gait analysis demonstrating improvement over time

The decision was made to transition the study of PEG membrane fusion nerve repair in an alternate functional recovery model, that of the rat forelimb. A research group at our institution has developed this model utilizing extrinsically stimulated grip strength as a highly sensitive measure of functional recovery after nerve injury and repair. We sought out the expertise of this group in order to use this model for the final milestones of this award. Unfortunately, through this process we determined that this group was already conducting work on the use of membrane fusion nerve repair in the forelimb transplant model through a study funded through an alternative funding source. In order to prevent redundant research and potential conflict, the difficult decision was made to conclude the current project without completing the final milestone nor utilizing the remainder of the funds in the award. Despite this setback, our work has contributed the science of both nerve regeneration and limb transplantation. The work from this grant was presented at local and national conferences and published in abstract for. The work on PEG membrane fusion in limb transplantation by our group and others continues and holds great promise to dramatically improve the outcomes in human limb transplantation.

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

4. Mentorship was provided during the course of the funding period to the individuals involved in the research including;
    - Research study design and execution
    - Skills acquisition:
        - Small animal surgery including general anesthesia maintenance and post-operative care
        - Nerve repairs
        - PEG nerve fusion technique
        - Microscopy training
        - Hind-limb transplantation
        - AD instruments electrophysiological testing
        - Catwalk sciatic functional index analysis
        - Forelimb transplantation
        - Grip strength testing

- Professional development opportunities included:
    - Participation in and presentation at weekly lab meetings and departmental Grand Rounds conferences
    - Travel to and presentation at local Departmental research conferences
    - Travel to and participation in National meetings
- Research fellows included:
    - Amr Mirdad 2017-18
    - Irene Aran Chang 2018-19
    - **How were the results disseminated to communities of interest?**
        - Presentations at local and national meetings as well as through personal communications with researchers in the fields of limb transplantation and nerve repair.
    - **What do you plan to do during the next reporting period to accomplish the goals?**
        - Nothing to Report.

5. **IMPACT:**
    - **What was the impact on the development of the principal discipline(s) of the project?**
        - The techniques and findings from this research have been disseminated through presentation at meetings and through the collaborations of others in the field. This has had an impact through the continued development of these techniques which are continuing through several research programs at our institution and elsewhere. These projects will continue to push the concept of membrane fusion nerve repair closer to use in human limb transplantation through continued refinements in basic science understanding as well as translational studies aimed at eventual human use.
    - **What was the impact on other disciplines?**
        - Nothing to Report.
    - **What was the impact on technology transfer?**
        - Although not directly involving technology transfer, this project has paved the way for continued studies directed to large animal translational models.
    - **What was the impact on society beyond science and technology?**
        - Nothing to Report.

6. **CHANGES/PROBLEMS:**
    - **Changes in approach and reasons for change / Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**
        - As described in the narrative above, the project had difficulty when attempting to quantify the functional recovery following transplantation. Our prior studies have utilized gait analysis for the purpose. However, when we attempted to use this tool to follow functional recovery in hind limb transplant recipients we encountered a significant obstacle. We consistently found that even at terminal time points (>22 weeks) animals in all groups failed to place the transplanted foot on the ground during ambulation. A recording of the foot step is necessary to determine the sciatic functional index. This post-transplant deformity made it impossible to quantify functional recovery in the hind limb. The decision was made to change to the rat forelimb model. A research group at our

institution has developed this model utilizing extrinsically stimulated grip strength as a highly sensitive measure of functional recovery after nerve injury and repair.

- **Actual or anticipated problems or delays and actions or plans to resolve them**

  The project was significantly delayed by two significant events other than the ones discussed above. Firstly, during the 2nd quarter of the award period there was a national shortage of medical grade methylene blue. This is a critical reagent for the process of Polyethylene glycol mediated membrane fusion. During this shortage alternative formulations were investigated but ultimately the supply shortage was ameliorated and experiments were continued. The second even was the transition of research personnel. Due to career goals and time constraints, Dr. Amr Mirdad finished his time working on the project. The work was transitioned to Research technician Irene Aran Chang but there was a delay associated with the training of the new personnel and a no cost extension was requested and granted to continue the work past the original end by date.

- **Changes that had a significant impact on expenditures**

  - We determined that another research group at Johns Hopkins was already conducting work on the use of membrane fusion nerve repair in the forelimb transplant model through a study funded through an alternative funding source. In order to prevent redundant research and potential conflict, we decided to conclude the current project without completing the final milestone nor utilizing the remainder of the funds in the award.

7. **PRODUCTS:**

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

1. A Mirdad, M.B.B.S., I.A. Chang, B.S., V. Malek, M.B.B.S., M Chicco, M.B.B.S., DS Cooney, M.D., PhD. Membrane Fusion Nerve Repair to Improve Limb Transplant Function. Presented at 9th annual JHU / UMD Plastic Surgery Research Symposium; 2017 June 8; Baltimore, USA.
2. A Mirdad, M.B.B.S., I.A. Chang, B.S., V. Malek, M.B.B.S., M Chicco, M.B.B.S., DS Cooney, M.D., PhD. Membrane Fusion Nerve Repair to Improve Limb Transplant Function. Poster session at: American association of hand surgery; 2018 Jan 9-13; Phoenix, USA
3. I.A. Chang, A Mirdad, M.B.B.S., I.A. Chang, B.S., V. Malek, M.B.B.S., M Chicco, M.B.B.S., DS Cooney, M.D., PhD. Membrane Fusion Nerve Repair to Improve Limb Transplant Function. Presented at 10th annual JHU / UMD Plastic Surgery Research Symposium; 2018 June; Baltimore, USA.

8. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

- **What individuals have worked on the project?**

Name:	*Damon Cooney, M.D., Ph.D.*
Project Role:	*Principal Investigator*
Researcher Identifier (e.g. ORCID ID):	*0000-0002-2786-287X*
Nearest person month worked:	*1CM*
Contribution to Project:	*Project oversight, study design and implementation, animal protocol approval at JHU and ACURO approval, oversight over the Fellow*

Name:	*Gerald Brandacher, M.D.*
Project Role:	*Investigator*
Nearest person month worked:	*1CM*
Contribution to Project:	*Laboratory oversight, study implementation, animal protocol approval at JHU and ACURO approval, oversight over the Fellow*

Name:	*Amir Mirdad*
Project Role:	*Research Fellow*
Nearest person month worked:	*12CM*
Contribution to Project:	*Maintained study progress, performed surgeries and assays, working with PI on study implementation, presented findings.*
Funding Support:	*Saudi Arabian Cultural Ministry agreement IO 80033090*

Name:	*Irene Aran Chang*
Project Role:	*Laboratory Technician*
Nearest person month worked:	*2.7 CM*
Contribution to Project:	*Maintained study progress, working with PI on study implementation, presented findings*

Name:	*Wesley Thayer, M.D.,Ph.D*
Project Role:	*Principal Investigator for subaward Vanderbilt University Medical Center*
Nearest person month worked:	*0.3CM*
Contribution to Project:	*Maintained study progress, supervised work at subaward site*
Funding Support:	*Subaward*

- **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.

    - **What other organizations were involved as partners?**

        - Nothing to Report.

8. **SPECIAL REPORTING REQUIREMENTS**

    - Nothing to report

9. **APPENDICES:**

    - None