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TITLE: Adjunctive Therapy to Improve Functional Recovery after Limb Ischemia Reperfusion Injury

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14. ABSTRACT

Acute traumatic extremity ischemia remains a significant cause of limb loss and late limb dysfunction on the battlefield. Even with restoration of blood flow, the period of ischemia initiates a metabolic cascade that will lead to severe dysfunction of the leg and loss of the extremity in some cases. Even in cases when the blood flow is restored, additional injury to the muscle occurs, which is known as ischemia-reperfusion injury. Pyruvate is a naturally occurring metabolite that offers the promise of controlling the metabolic cascade of ischemia-reperfusion injury. This study will test whether ethyl pyruvate is protective in a pig model of limb ischemia with reperfusion. Ethyl pyruvate is one of the few agents that has shown positive results in a rodent model when administered in a clinically relevant post-ischemia protocol, e.g. the agent is effective even when administered after the leg has become ischemic. Given that small animal studies have been performed and indicate that it will likely be useful for human applications, we will combine the use of Ethyl Pyruvate with a model of perfusion-reperfusion injury in a large animal model that better simulates the way the human body responds to treatment of this important injury process.

15. SUBJECT TERMS

Ischemia-reperfusion injury, ethyl pyruvate, animal models of human disease

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TABLE OF CONTENTS

1. INTRODUCTION:

Acute traumatic extremity ischemia remains a significant cause of limb loss and late limb dysfunction on the battlefield. Even with restoration of blood flow, the period of ischemia initiates a metabolic cascade that will lead to severe dysfunction of the leg and loss of the extremity in some cases. Even in cases when the blood flow is restored, additional injury to the muscle occurs, which is known as ischemia-reperfusion injury. Pyruvate is a naturally occurring metabolite that offers the promise of controlling the metabolic cascade of ischemia-reperfusion injury. This study will test whether ethyl pyruvate is protective in a pig model of limb ischemia with reperfusion. Ethyl pyruvate is one of the few agents that has shown positive results in a rodent model when administered in a clinically relevant post-ischemia protocol, e.g. the agent is effective even when administered after the leg has become ischemic. Given that small animal studies have been performed and indicate that it will likely be useful for human applications, we will combine the use of Ethyl Pyruvate with a model of perfusion-reperfusion injury in a large animal model that better simulates the way the human body responds to treatment of this important injury process.

2. KEYWORDS:

Ischemia-reperfusion injury, ethyl pyruvate, animal models of human disease

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Task 1: Animal use approval and facility set-up. (0-4 months)

1a: Animal use approval.
Milestones achieved: Local Institutional Animal Care and Use Committee (IACUC), University of Maryland Baltimore (UMB) IACUC deferral and DoD Animal Care and Use Review Office (ACURO) (1-3 months)

1b: Model validation experiments. 6 Sus Scrofa swine to be used for model validation and set-up at UMB. (3-4) months.

Milestones Achieved: Standardized model protocol

All work related to Task 1 were completed.

The Statement of Wok (SOW) was modified and adjusted for the first year of the no cost extension period to accommodate for the completion of the remaining tasks of this project.

Note: Below timeline is based on the first 1-12 months (Sep 2018 – Sep 2019) of the No Cost Extension period for the award BA150585

Specific Aim 1: To evaluate the effects and dose-response curves of EP used as a pharmacological adjunct in the treatment of severe IRI in a clinically relevant swine model.

Task 2: Determine the optimal dose of EP at 4.5 and 6 hours after induction of ischemia. Endpoints will be biochemical, histological, and electromyography. N=46 adult Sus scrofa swine. (1-8 months)

2a: Drug dosage experiments. Two different doses of EP will be tested, 75 mg/Kg, 125 mg/Kg (x dose)

Milestone(s) Achieved: Determination of the best dose of EP for treatment of IRI. (1-3 Months)

2b: Dose response experiments. Based on the injury description from the model, two time points will be tested, (1) 4.5 hours of ischemia, (2) 6.0 hours of ischemia.

Milestone(s) Achieved: Dose response curve of EP effectiveness for treatment of IRI. Optimal dosage of EP therapy determined. (4-6 months)

2c: Statistical analysis of Subtask 2a and 2b. (5-6 months)

Specific Aim 2: To evaluate the effects of combination therapy (EP with and without controlled reperfusion) on the acute neuromuscular, functional, biochemical and histological outcome in severe limb IRI of the lower extremities in this same, clinically relevant, swine model.

Task 3: Evaluate the effectiveness of EP as a combination therapy with controlled reperfusion. (7-11 months)

3a: Controlled reperfusion experiments: N = 34 adult Sus scrofa swine (15-19 months)
*Milestone(s) Achieved: Optimal therapy (*7-11 months)

3b: Statistical analysis of Subtask 3a. (10-11 months)

Task 4: Final Reporting. (11-12 months)

4a: Draft Final Report (11-12 months)

Milestone(s) Achieved: Final report (12 months)

4b: Obtain Scientific and Technical Information (STINFO) clearance. (11-12 months)

Milestone(s) Achieved: STINFO clearance. (12 months**)**

The Statement of Wok (SOW) was modified for a second time and adjusted for the second year of the no cost extension period to accommodate for the completion of the remaining tasks of this project.

Note: Below timeline is based on the second 1-12 months (Sep 2019 – Sep 2020) of the No Cost Extension period for the award BA150585

Task 1:

To evaluate the effects of 125 mg/Kg EP at 4.5 hours after induction of ischemia used as a pharmacological adjunct in the treatment of severe IRI in a clinically relevant swine model. Endpoints will be biochemical, histological, and electromyography. N=12 adult Sus scrofa swine. (1-10 months)

Milestone(s) Achieved: Determination of the effect of 125 mg/Kg EP at 4.5 hours after induction ischemia for treatment of IRI. (1-10 Months)

Task 2: Statistical analysis of Task 1. (10-11 months)

Task 3: Final Reporting. (11-12 months)

3a: Draft Final Report (11-12 months)

Milestone(s) Achieved: Final report (12 months)

3b: Obtain STINFO clearance. (11-12 months)

Milestone(s) Achieved: STINFO clearance. (12 months)

What was accomplished under these goals?
For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

During the project period, we were able to:

1. Used 6 Sus Scrofa swine for model validation and set-up at UMB. This was completed after modifications in the animal protocol as well as change in the animal vendor.
2. Changed PI of the project as the original PI left UMB.
3. Modified IACUC protocols and obtained ACURO approvals for the modified protocols before commencement of any animal work.
4. Our initial experiments with the positive control group animal resulted in death of the animal as it failed to recover fully from the severe ischemia. Due to this, after consultation with the UMB veterinarian, the animal had to be euthanized.
5. We then performed one more experiment but due to technical complications in the anesthesia machine, the animal under surgery suffered severe hypoxia resulting in death.
6. Our original IACUC protocol 0416003 expired in April, 2019 and we submitted a new animal protocol to the UMB IACUC for review and approval. We obtained the final approval of this protocol 0419017 on 06/03/2019.
7. This protocol was subsequently submitted to ACURO for review and approval on 06/04/2019. We received final approval from ACURO on 09/13/2019.
8. We trained new surgical staff in the surgical procedure needed for this study.
9. Performed 3 more successful animal surgeries and harvested tissues for histological and molecular biological analyses.
10. Unfortunately, work on this project had to be suspended due to COVID-19 research restrictions put in place from February 2020 here at UMB.

During the period of this grant, we encountered two major complications that we believe should be communicated to the scientific community. Prior knowledge of these complications and their solutions may be helpful in mitigating complications arising in other future in vivo research using the swine limb ischemia model.

- The first complication that we encountered was due to the Malignant Hypothermia (MH), also known as Porcine stress syndrome (PSS). During the completion of task 1, we were able to successfully complete the surgical procedure in multiple animals but were unable to recover the animals from anesthesia due to the MH/PSS. Following the induction of anesthesia and during the surgical procedure, multiple animals demonstrated high carbon dioxide (CO2) levels in spite of adequate mechanical ventilation. These animals began developing acidosis and then fever and ultimately did not survive. After consultations with our University of Maryland veterinary resources staff and other investigators with experience in porcine models, it was concluded that the animals were experiencing MH/PSS, which is a known complication in surgery under general anesthesia in swine. MH is a condition that results in hyperthermia, muscular rigidity (not observed by us), and hypercarbia due to uncontrolled and uncoupled metabolic activity secondary to exposure to neuromuscular blocking agents (not administered in this case) or inhaled anesthetics (such as isoflurane, which was administered). This condition is due to a genetic mutation that is much more prevalent in swine than in human beings and may also be triggered in swine by significant physiologic stress, such as occult pre-existing

disease, which gives the condition its alternative name, Porcine Stress Syndrome (PSS). The condition is known to be occasionally present in Yorkshire breed swine.

To mitigate this problem, we first adjusted the accepted weight range upward to use more mature animals that are likely to have a lower rate of symptomatic MH/PSS. One animal with a greater body mass (56kg) was successfully utilized, and while there were some initially mildly elevated CO2 levels, this rapidly resolved with some adjustment to the ventilator and anesthetic. The remainder of the surgical procedure and recovery were completed without issue with the animal demonstrating no other definitive symptoms of MH/PSS. It was further determined that as MS/PSS is due to presence of specific mutations in the genome of the animals, it would be recommended that changing the commercial animal supplier to a different one would eliminate the possibility of having the same mutations in a separate breed of animals. Thus, the commercial supplier of animals was changed and in the subsequent set of animals undergoing surgery, no MH/PSS related complications were observed.

- The second complication encountered was extreme limb ischemia during the task 2 in animals undergoing complete ligation and resection of the external iliac artery. We were able to successfully complete the surgery, but during the recovery from anesthesia, the animal was not able to use the hindlimbs to move to a upright position and showed clear signs of pain and distress. After consultation with the veterinary resources staff, analgesics were administered to mitigate the situation. But even after multiple doses of analgesics, the animal continued to show signs of pain and distress and unable to move the operated hindlimb and the animal was subsequently euthanized for humane reasons. We concluded that in our model, complete ligation and resection of the external iliac artery creates a severe state of limb ischemia that the animals cannot recover from. We subsequently modified our animal use protocol and surgical technique to lessen the degree of ischemia in our study.

We were only able to successfully complete 3 animal surgeries that underwent ischemia reperfusion and treatment with ethyl pyruvate or vehicle control. One of the animals received the ethyl pyruvate treatment whereas the other 2 animals were treated with vehicle control. We have harvested tissues from these animals for histological as well as molecular biological work. The ‘n’ is too small to discern any effect of ethyl pyruvate treatment from this group of animal experiment. We plan to use the histological samples obtained to stain for morphological characterization of ischemia (ischemic limb versus the non-ischemic limb) using H&E, we plan to also use other staining procedures such as tetrazolium blue etc. to characterize the ischemic tissue. These sections will be use as reference sections for future studies.

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

This project has provided opportunities in training for general surgery residents by vascular surgery fellows and attending surgeons. Techniques of carotid exposure, vascular control, retroperitoneal dissection, and arteriotomy closure in particular were developed over time with

direct supervision and input from senior surgical staff during the training phase of this project and continue to be refined with independent study and practice by trainees.

How were the results disseminated to communities of interest?

Nothing to report.

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

Nothing to report, this is the final technical report.

4. **IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

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

Nothing to report.

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. **CHANGES/PROBLEMS**

Changes in approach and reasons for change

Nothing to report.

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

Throughout the duration of this project, we have faced significant roadblocks and delays that resulted in limited progress towards completion of the specific aims of this project. These was also a major suspension of work on the last year of this project due to the COVID-19 related restrictions.

Changes that had a significant impact on expenditures

Delays in beginning our surgical series due to various unforeseen reasons resulted in reductions in spending over the years from the initial spending estimates. The remaining unspent funds will be sent back to the respective authorities.

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

Nothing to report.

Significant changes in use or care of human subjects

Nothing to report.

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.

6. **PRODUCTS:** List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

- **Publications, conference papers, and presentations**
  Nothing to report.

  Journal publications.

  Nothing to report.

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

  Nothing to report.

  Other publications, conference papers and presentations.

  Nothing to report.

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

  Nothing to report.

- **Technologies or techniques**

Nothing to report.

- **Inventions, patent applications, and/or licenses**

  Nothing to report.

- **Other Products**

  Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: Rajabrata Sarkar, MD, PhD
Project Role: PI/Surgical Attending
Researcher Identifier (ORCID ID):
Nearest person month worked: 1
Contribution to Project: Supervision of laboratory analysis, protocol revisions, and equipment purchases.

Name: Charles Drucker, MD
Project Role: Surgical Resident/Research Coordinator
Researcher Identifier (ORCID ID): 0000-0002-5846-2027
Nearest person month worked: 2
Contribution to Project: Prepared and revised animal protocols, prepared animal protocol amendments and documentation, obtained instruments and prepared equipment sets, established instrument sterilization plan and protocol, completed surgical procedure, completed animal pre-operative and post-operative care and medication administrations, managed acquisition of tissue and blood samples, coordinated with veterinary resources staff, and arranged for equipment purchases.

Name: Brittany O. Aicher, MD
Project Role: Surgical Resident/Post-doctoral fellow
Researcher Identifier (ORCID ID):
Nearest person month worked: 4
Contribution to Project: Prepared and revised animal protocols, prepared animal protocol amendments and documentation, completed surgical procedure, and coordinated with veterinary resources staff.

Name: Subhradip Mukhopadhyay, PhD
Project Role: Post-Doctoral Research Fellow
Researcher Identifier (ORCID ID):

Nearest person month worked: 4
Contribution to Project: Provided assistance with preparation of animal data analytic protocols, development of therapeutic agent preparation protocol and prepared agents for blinded administration, supported preparation and sterilization of operative instruments, arranged for tissue and blood sample acquisition/storage/analysis, and assisted with equipment selection and acquisition.

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

COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to **https://ers.amedd.army.mil** for each unique award.

QUAD CHARTS: If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.

9. APPENDICES:

Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.