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TITLE: Exosome Therapy for Stabilization of Extremity Injury

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CONTRACTING ORGANIZATION: Augusta University Research Institute
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14. ABSTRACT

Cellular therapies have tremendous potential for the successful treatment of major extremity wounds in the combat setting; however, the challenges associated with transplanting stem cells in the prolonged field care (PFC) environment are a critical barrier to progress in treating such injuries. Our goal is develop a new strategy utilizing extracellular vesicles (EVs) secreted by stem cells that can resolve many of these issues. Aim 1 of the project is to determine the optimal dosage and storage conditions of lyophilized extracellular vesicles (EVs) for enhancing cell survival in an in vitro model of muscle ischemia. Aim 2 will determine the impact of EV treatment on tissue preservation and recovery utilizing in vivo models of hindlimb ischemia-reperfusion injury. The proposed research, by advancing stem cell EV therapy, will therefore serve the public purpose by addressing the healthcare needs of not only active duty military personnel, their families, and veterans, but also civilians for whom ischemic injury is a major cause of morbidity and mortality

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INTRODUCTION

Cellular therapies have tremendous potential for the successful treatment of major extremity wounds in the combat setting; however, the challenges associated with transplanting stem cells in the prolonged field care (PFC) environment are a critical barrier to progress in treating such injuries. These challenges include not only production and storage but also transport and handling issues. Our goal is to develop a new strategy utilizing extracellular vesicles (EVs) secreted by stem cells that can resolve many of these issues. Specific Aim 1 of the project is to determine the optimal dosage and storage conditions of lyophilized extracellular vesicles (EVs) for enhancing cell survival in an in vitro model of muscle ischemia. Specific Aim 2 will determine the impact of EV treatment on tissue preservation and recovery utilizing in vivo models of hindlimb ischemia-reperfusion injury. The proposed research, by advancing stem cell EV therapy as a novel approach for treating ischemic injury, will therefore serve the public purpose by addressing the healthcare needs of not only active duty military personnel, their families, and veterans, but also civilians for whom ischemic injury is a major cause of morbidity and mortality

KEYWORDS

Adipose-derived stem cells; Exosomes; Lyophilization; Muscle Ischemia; Tissue Preservation

ACCOMPLISHMENTS

This annual report reflects work completed over the last three years of the funded project. We have completed **Milestone 1**: Determine the impact of storage temperature and storage duration on the potential of stem cell-derived EVs for promoting myotube viability and energy production in anoxic conditions. We also reached **Milestone 2**, which is to determine the potential of lyophilized (freeze-dried) EVs as well as other FDA-approved molecules to promote myotube survival in anoxic conditions. As described in our previous quarterly report, our in vitro studies demonstrated that the mitochondrial transition pore inhibitor NIM-811 was as or more effective than lyophilized exosomes for preventing ischemia-reperfusion injury in cultured myoblasts. Our in vivo studies confirmed these findings, and showed that NIM-811 reduced muscle inflammation, serum markers of inflammation, and muscle oxidative stress after ischemia and reperfusion.

Activities over the last 12 months (year 3) focused on Major Task 3: Employ a mouse model of ischemia-reperfusion injury to determine the optimal dosing strategy (e.g, conditioning/pre-conditioning) for improving tissue viability following ischemia. These experiments are directed at reaching **Milestone 3**: Determine the effects of EVs on tissue recovery from ischemic injury utilizing small animal model. We performed in vivo studies using tail vein injection of EVs in the fourth quarter of year 3. The experimental design is shown below in Figure 1.

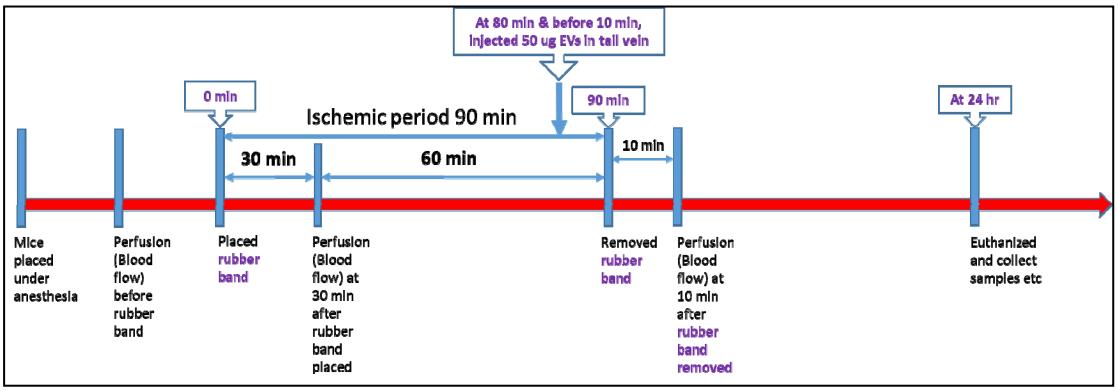

Figure 1. *Experiment design for in vivo experiments using lyophilized exosomes to prevent hindlimb ischemia-reperfusion injury.*

We continue to collect and analyze results from these experiments that were performed in May, 2021. Results obtained thus far indicate that there was no significant effect of the EVs on reperfusion capacity (Fig. 2A). This finding contrasts with our results using NIM-811, which indicated improved reperfusion with drug treatment. In addition, we previously found that NIM-811 reduced circulating (serum) levels of the inflammatory cytokine IL-1 alpha. In contrast, EV treatment did not reduce serum IL-1 alpha following ischemia and reperfusion (Fig. 2B).

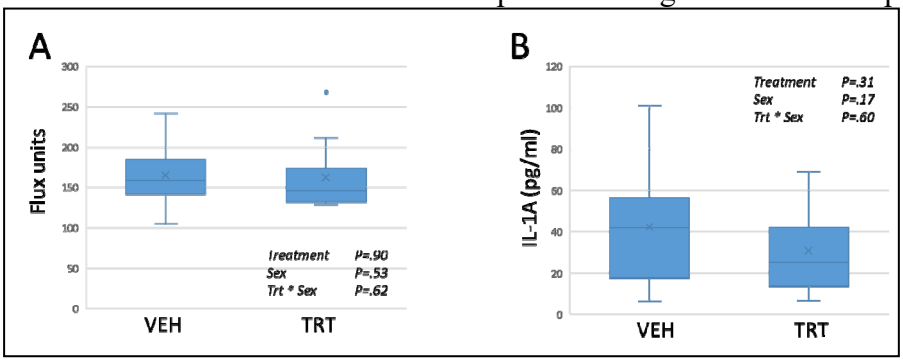

Figure 2. *A. Measurement of hindleg blood perfusion following tail vein exosome injection. Blood perfusion is measured using laser Doppler imaging. VEH=vehicle (control) mice, TRT=exosome treated mice. B. Serum IL-1 alpha levels measured using multiplex cytokine array. P-values are from ANOVAs.*

Our previous study also demonstrated that NIM-811 reduced levels of IL-1alpha, IL-6, and MCP-1 in skeletal muscle from the ischemic limb following reperfusion. Our data using tail injection of lyophilized EVs demonstrated no significant effects of EV treatment on these cytokines in skeletal muscle (Fig. 3). There is a trend toward decreased IL-1 alpha with EV treatment, which is significant in the male mice.

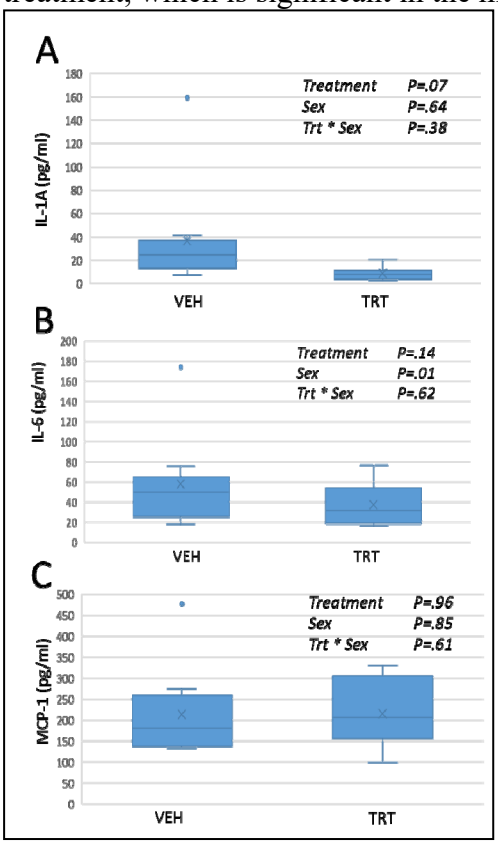

Figure 3. *Measurement of inflammatory cytokine levels in the gastrocnemius muscle from the ischemic limb following reperfusion in mice. VEH=vehicle (control) mice, TRT=exosome treated mice. Cytokines include A. Il-1 alpha, B. IL-6, and C. MCP-1. P-values are from ANOVAs.*

- **What opportunities for training and professional development has the project provided?**
  - ***"Nothing to Report."***
- **How were the results disseminated to communities of interest?**
  - ***If there is nothing significant to report during this reporting period, state "Nothing to Report.".***
- **What do you plan to do during the next reporting period to accomplish the goals?**

Our data collected thus far suggest that the mitochondrial pore inhibitor NIM-811 is more effective than stem cell-derived exosomes for attenuating tissue damage with limb ischemia-reperfusion injury. To confirm these preliminary findings we plan to repeat the study shown in Figure 1 using a higher dose (100 μg) of exosomes in August, 2021. We expect the data from that study to be complete by September-October, which will allow us to plan for **Major Task 4:** use a porcine model of hindlimb ischemia-reperfusion injury to determine the impact of EV treatment on the recovery of limb function. If the higher exosome dose is not effective in the mice then we may pursue the porcine study using NIM-811.

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?** The findings have a significant impact on the development of EVs as novel therapies. Specifically, we have shown that EVs from primary human adipose stem cells can promote survival of ischemic muscle cells. We have also shown that specific crypreservation strategies can enhance the stability of these EVs when they are freeze-dried (lyophilized) for long-term storage. Finally, we have shown that the mitochondrial pore and Cyclophilin D (CypD) inhibitor NIM-811 can reduce ischemic damage to the limb. This presents a significant opportunity to utilize CypD inhibitors in the combat setting to prevent injuries to the limbs resulting from ischemic injury.

- **What was the impact on other disciplines?**
  - ***"Nothing to Report."***
- **What was the impact on technology transfer?**
  - ***"Nothing to Report."***

CHANGES/PROBLEMS: The postdoctoral fellow working on the project, Dr. Khairat Bahagt El Baradie, had to return to Egypt in August, 2020, to renew her VISA. She was not paid off the grant during this time and Bharati Mendhe performed the work generating exosomes. Given the funds for personnel available on the NCE Khairat will not return to the US to work on this project.

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

- ***Nothing to report.***

PRODUCTS:
Publications, conference papers, and presentations

Our abstract titled "Optimization of stem cell-derived exosomes for therapeutic application in the prolonged field care environment" was presented at the 2019 MHSRS meeting in Orlando, FL.

The manuscript emanating from this work titled "**Freeze-dried extracellular vesicles from adipose-derived stem cells prevent hypoxia-induced muscle cell injury**" was published in the special issue of *Frontiers in Cell and Developmental Biology* on "Exosomes as Therapeutic Systems": https://www.frontiersin.org/articles/10.3389/fcell.2020.00181/full

A second manuscript for a Special Issue of *Connective Tissue Research* on "Cross-talk with skeletal muscle and its nexus with regenerative rehabilitation" titled "**Therapeutic Application of Extracellular Vesicles for Musculoskeletal Repair & Regeneration**" was published Jun 30:1-16. https://www.tandfonline.com/doi/full/10.1080/03008207.2020.1781102

Our abstract titled "The mitochondrial permeability transition inhibitor NIM811 increases muscle cell survival with hypoxia in vitro and reduces the inflammatory microenvironment with limb ischemia-reperfusion in vivo" was accepted for the 2020 MHSRS meeting in Orlando, FL.

The manuscript resulting from this work titled "**The cyclophilin inhibitor NIM-811 increases muscle cell survival with hypoxia in vitro and improves gait performance following ischemia-reperfusion in vivo**" was published in *Scientific Reports*: https://www.nature.com/articles/s41598-021-85753-x

A fourth manuscript titled "**Targeting the mitochondrial permeability transition pore to prevent age-associated cell damage and neurodegeneration**" was published in a special issue of *Oxidative Medicine and Cellular Longevity*: https://www.hindawi.com/journals/omcl/2021/6626484/

Our most recent abstract titled "Targeting the mitochondrial permeability transition pore (mPTP) for repair and recovery of traumatic musculoskeletal injuries" has been accepted for the 2021 MHSRS meeting in Orlando, FL.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**
  - ***Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change."***

Name: Mark Hamrick
Project Role: PI
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 3.0
Contribution to Project: Provided oversight for staffing, ordering, and experimental design and statistical analysis.

Name: Sadanand Fulzele
Project Role: Co-I
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 2.4
Contribution to Project: Ran in vitro experiments using EVs derived from adipose stem cells, supervised all cell culture work.

Name: Yutao Liu
Project Role: Co-I
Research Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 1.2
Contribution to Project: Provided oversight and assistance with nanoparticle tracking analysis (ZetaView instrument) and EV characterization.

Name: Bharati Mendhe
Project Role: Research assistant
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 6.0
Contribution to Project: Assisted with in vitro experiments, maintain cell cultures, purchasing reagents.

Name: Ling Ruan
Project Role: Research associate
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 7.2
Contribution to Project: Assisted with lab management and oversight, troubleshooting, and optimization of cell viability and toxicity assays.

Name: Khairat Bahagt El Baradie
Project Role: Visiting scientist
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 1.5
Contribution to Project: Assisted with trehalose crypreservation and exosome isolation and characterization.

- **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."***

SPECIAL REPORTING REQUIREMENTS

- **COLLABORATIVE AWARDS:** ***For collaborative awards, independent reports are required from BOTH the Initiating 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.***