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PRINCIPAL INVESTIGATOR: Mark W. Hamrick

CONTRACTING ORGANIZATION: Augusta University Research Institute, Augusta, GA

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Mark W. Hamrick, Ph.D.

E-Mail: mhamrick@augusta.edu

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Augusta University Research
Institute, Inc.
AURI
1120 15th Street #CJ3301
Augusta, GA 30912-0004

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14. ABSTRACT

Ischemia-reperfusion (I/R) injury is a complication impacting multiple organs and tissues in the trauma setting. We tested the hypothesis that lyophilized extracellular vesicles derived from adipose stem cells could serve as an "off-the-shelf" treatment modality for I/R injury. Our in vitro studies showed that these vesicles reduced markers of I/R injury in cultured primary human muscle cells. Our in vivo (rodent) studies indicate that EVs significantly increase reperfusion; however, the increased reperfusion was also associated with a marked decrease in muscle structural proteins such as dystrophin, plectin, and obscurin. Circulating inflammatory cytokines TNF-alpha and IL-6 were increased with EV treatment, and serum TNF-alpha showed a significant, positive correlation with reperfusion level. These findings suggest that, while EVs may enhance reperfusion, the increased reperfusion can negatively impact muscle tissue and possibly remote organs. Alternative approaches, such as targeting mitochondrial permeability, may be more effective at mitigating I/R injury.

15. SUBJECT TERMS
None listed.

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1. 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. The major tasks for this aim involved determining the impact of lyophilization, storage conditions, and storage duration on EV bioactivity *in vitro* and comparing the effects of EV treatment on cell survival with molecules previously determined to attenuate ischemia-reperfusion injury. 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 major tasks for this aim utilized a mouse model of ischemia-reperfusion injury to determine the optimal dosing strategy (e.g, conditioning/pre-conditioning) for improving tissue viability following ischemia. A large animal (porcine) model was also to be used to further validate the approach. 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

2. KEYWORDS:

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

3. ACCOMPLISHMENTS:

What were the major goals of the project?

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. Milestone 2: Determine the potential of lyophilized EVs as well as other FDA-approved molecules to promote myotube survival in anoxic conditions. Milestone 3: Determine the effects of EVs on tissue recovery from ischemic injury utilizing small animal model. Milestone 4: Determine the effects of EVs on tissue recovery from ischemic injury utilizing a large animal model. All milestones were completed with the exception of milestone 4. We did not pursue the large animal model due to the muscle injury and inflammation observed in the rodent model.

What was accomplished under these goals?

The studies completed under milestones 1 and 2 are described in detail in the first manuscript of the appendix (*Front Cell Dev Biol*. 2020, 8:181). Our work revealed that preservation of human adipose stem cell-derived EVs using lyophilization and cryoprotection could be utilized to prevent hypoxic injury and reperfusion in primary human muscle cells (Figure 1). Our in vitro studies for milestone 2 and major task 2 also showed that the small molecule mitochondrial pore inhibitor NIM-811 successfully promoted cell survival and reduced toxicity with ischemia in human myoblasts (Figure 2). The studies utilizing NIM-811 are described in detail in an additional manuscript published in *Scientific Reports* (2021, 11:6152), which also demonstrates NIM-811 is effective at attenuating ischemic muscle damage in vivo (Fig. 3).

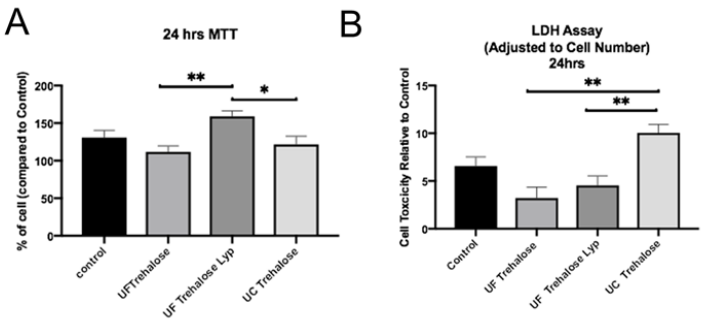

Figure 1. *(A) MTT assay for primary human muscle cell viability (% of MTT positive cells) shows that addition of trehalose improves the pro-viability effects of lyophilized (Lyp) EVs isolated from cultured ADSCs by tangential flow ultrafiltration (UF) compared to EVs isolated by ultracentrifugation (UC) or by ultrafiltration with no lyophilization. *P<0.05, **P<0.01. (B) LDH assay for cellular toxicity in primary human muscle cells (% of MTT positive cells) shows that addition of trehalose reduces toxicity in cells treated lyophilized (Lyp) EVs isolated by tangential flow ultrafiltration (UF) compared to EVs isolated by ultracentrifugation. **P<0.01.*

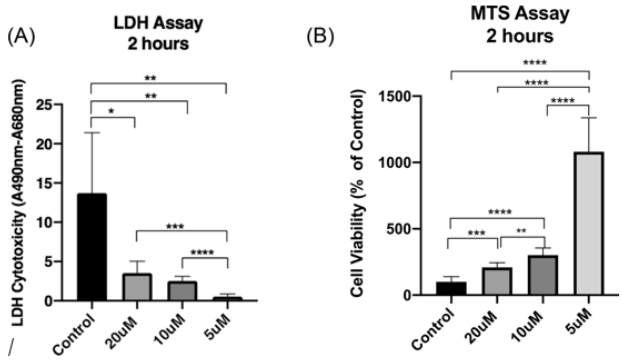

Figure 2*. Effects of NIM-811 treatment on ischemic human myoblasts. Ischemic Human myoblasts were treated with (0–20 μM) NIM-811 for 2 h following the exposure to hypoxia for 6 h. Data shown are for cell viability measured by MTS assay (A) and cell toxicity in different groups as determined by LDH assay (B). Data are expressed as mean ± SD (n = 6). ****P = 0.0001, ***P = 0.0005, **P = 0.0022 and *P = 0.01.*

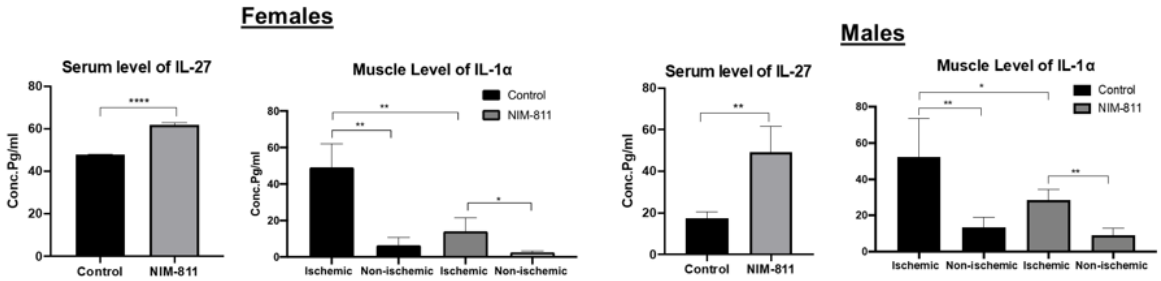

Figure 3. *Effects of NIM-811 treatment on circulating and local cytokines. Mice received vehicle or NIM-811 (10 mg/kg BW) 10 min before reperfusion and 3 h later. Serum IL-27 (anti-inflammatory) level increased significantly in the serum of female and male mice treated with NIM-811 compared to control mice whereas IL-α (pro-inflammatory) was reduced in hindlimb muscle of female and male mice treated with NIM-811 compared to control group. *P < 0.05, **P < 0.01 and ***P < 0.001.*

The more recent paper in *Cells* supporting Milestone 3 shows that while lyophilized EVs do increase perfusion in the rodent model (Fig. 4), the reperfusion appears relatively uncontrolled, resulting in increased local (muscle) and circulating (serum) levels of inflammatory cytokines such as IL-6 (Fig. 5). Proteomic data from this study (Table 1, below) show that a number of muscle structural proteins such as dystrophin, plectin and obscurin are decreased in muscle from mice treated with the EVs. These findings led us to abandon EV treatment in the large animal model, and suggest that pursuing small molecule inhibitors of mitochondrial permeability is a more viable approach.

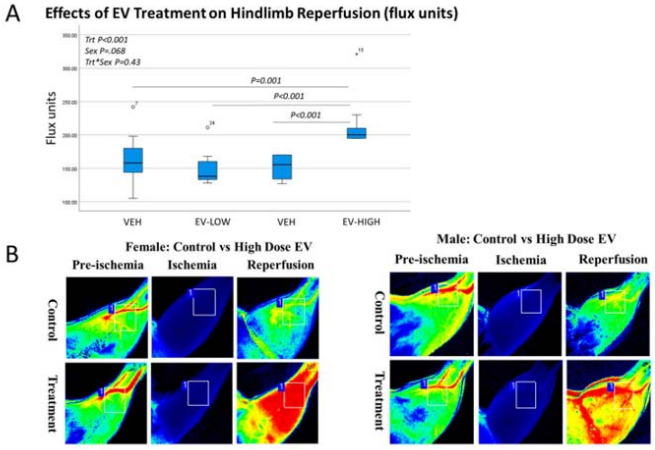

Figure 4. *Effects of EV treatment on hindlimb reperfusion. (A). Box-and-whisker plots showing a significant increase in reperfusion with the high-dose EV treatment. Two-factor ANOVA was performed with treatment and sex as the two factors (n = 5 male, 5 female per group). (B). Laser Doppler images of hindlimbs from male and female mice receiving vehicle (control) or high dose (100 µg) EVs showing increased perfusion in the treated mice. °,* represent outliers in (A).*

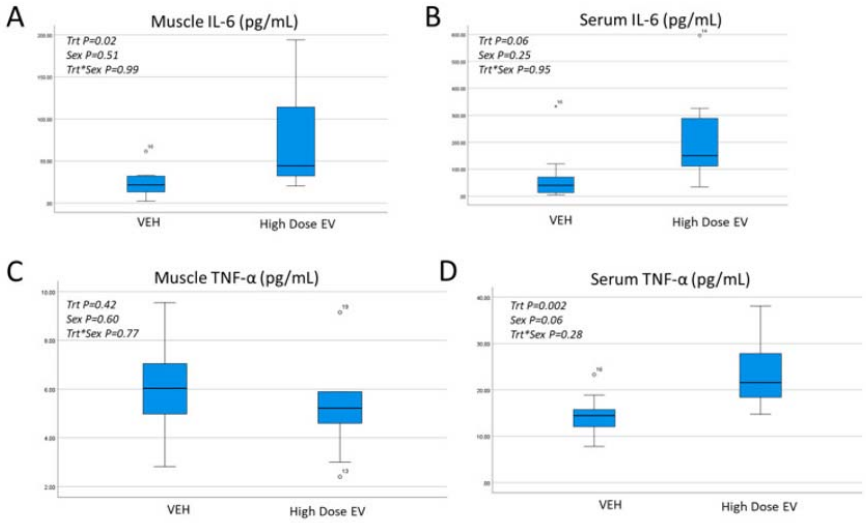

Figure 5*. Effects of EV treatment on local and circulating inflammatory cytokines. Muscle-derived IL-6 (A) is increased significantly with EV treatment, which is associated with a trend toward higher serum IL-6 (B). Muscle-derived TNF-alpha (C) is not significantly increased with EV treatment, but serum TNF-alpha is significantly higher in EV-treated mice (D). Two-factor ANOVA was performed with treatment and sex as the two factors (n = 5 male, 5 female per group). °,* represent outliers.*

Table 1. *Proteins identified from functional enrichment (ToppFun) analysis of downregulated muscle structural proteins (Supplemental file 2) in the EV-treated hindlimb. Fold-change (FC) and P-values are shown for each gene.*

Muscle structural proteins	
Gene symbol	Gene name
Tnni2 (FC=0.69**)	troponin I, fast skeletal muscle
Dmd (FC=0.48**)	dystrophin
Plec (FC=0.44***)	plectin
Obscn (FC=0.42**)	obscurin
Synm (FC=0.43***)	synemin

*P<.05, **P<.01, ***P<.001.

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

The project provided important training opportunities. Dr. Khairat El Baradie worked on the project as a postdoctoral fellow and authored several of the papers resulting from the experiments described in the schedule of work. Two undergraduate students, Andrew Kent and Damon Dunwody, also participated in the project where they performed histological analysis of muscle tissue. Both students also received authorship on papers emanating from their work, which will support their pending applications to medical schools.

How were the results disseminated to communities of interest?

Results were disseminated in the form of published manuscripts and also presentations at national meetings.

1.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 Orlando, FL.
2.Our abstract titled "Targeting the mitochondrial permeability transition pore (mPTP) for repair and recovery of traumatic musculoskeletal injuries" was accepted and published online for the 2021 MHSRS meeting.
3. Our abstract accepted for the 2022 Orthopedic Research Society meeting titled "Skeletal muscle PDGFRα+ fibro-adipogenic progenitor cells are a source of IL-1β in hindlimb ischemia-reperfusion injury" was presented in in Tampa, FL, February, 2022.

4. IMPACT:

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

We explored multiple cryopreservation approaches to optimize our EV preservation strategy. We believe that this technique will significantly impact the field of stem cell biology, as it provides a new and effective method to store a stem cell-derived product for prolonged periods of time in challenging environments.

What was the impact on other disciplines?

Our finding that these preserved vesicles can significantly enhance tissue perfusion may have broader impacts for other disorders where tissue perfusion is compromised, such as heart disease of peripheral arterial disease.

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

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

Nothing to report

Changes that had a significant impact on expenditures

We requested a no-cost extension to complete tissue analysis from the mouse studies.

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

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:

- ## Publications, conference papers, and presentations

Journal publications.

The first 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

The third manuscript 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/

A fifth manuscript titled "**Lyophilized extracellular vesicles from adipose-derived stem cells increase muscle reperfusion but induce a dystrophic phenotype in a mouse model of hindlimb ischemia-reperfusion injury**" was published in *Cells*: https://www.mdpi.com/2073-4409/12/4/557

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**

Our technique for lyophilization and cryopreservation of stem cell-derived EVs was published in *Frontiers in Cell and Developmental Biology* and is freely available through open access.

- **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: Mark Hamrick
Project Role: PI
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 0.6 (quarter 15)
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: 0.3 (quarter 15)
Contribution to Project: Assisted with in vitro experiments using EVs derived from adipose stem cells, supervised all cell culture work.

Name: Yutao Liu
Project Role: Co-I
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 0.15 (quarter 15)
Contribution to Project: Supervised exosome measurements using Zetaview.

Name: Bharati Mendhe
Project Role: Research assistant
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 1.5 (quarter 15)
Contribution to Project: Production and characterization of exosomes.

Name: Ling Ruan
Project Role: Research associate
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 2.1 (quarter 15)
Contribution to Project: Assisted with in vitro experiments, maintain cell cultures, purchasing reagents.

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:

QUAD CHARTS:

9. APPENDICES: