AWARD NUMBER: W81XWH-18-1-0090

TITLE: Exosome Therapy for Stabilization of Extremity Injury

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REPORT DATE: June 2020

TYPE OF REPORT: Annual

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
June 2020	Annual	**1 June 2019 – 31 May 2020**

4. TITLE AND SUBTITLE
Exosome therapy for stabilization of extremity injury.

51 June a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-18-1-0090

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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

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8. PERFORMING ORGANIZATION REPORT NUMBER

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)

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

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

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

15. SUBJECT TERMS

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

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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

Aim 1 will determine the optimal dosage and storage conditions of lyophilized EVs for enhancing cell survival in an in vitro model of muscle ischemia.

Major Task 1: Determine the impact of lyophilization, storage conditions, and storage duration on EV bioactivity in vitro.

Subtask 1: Generate extracellular vesicles from human adipose-derived stem cells using low oxygen tension (hypoxia) culture conditions. (months 1-6)

Subtask 2: Vary storage conditions (e.g., lyophilized or non-lyophilized, frozen or ambient temperature) and storage duration of Evs (months 7-10)

We collected significant data related to subtasks 1 and 2. These data are shown below in Figure 1.

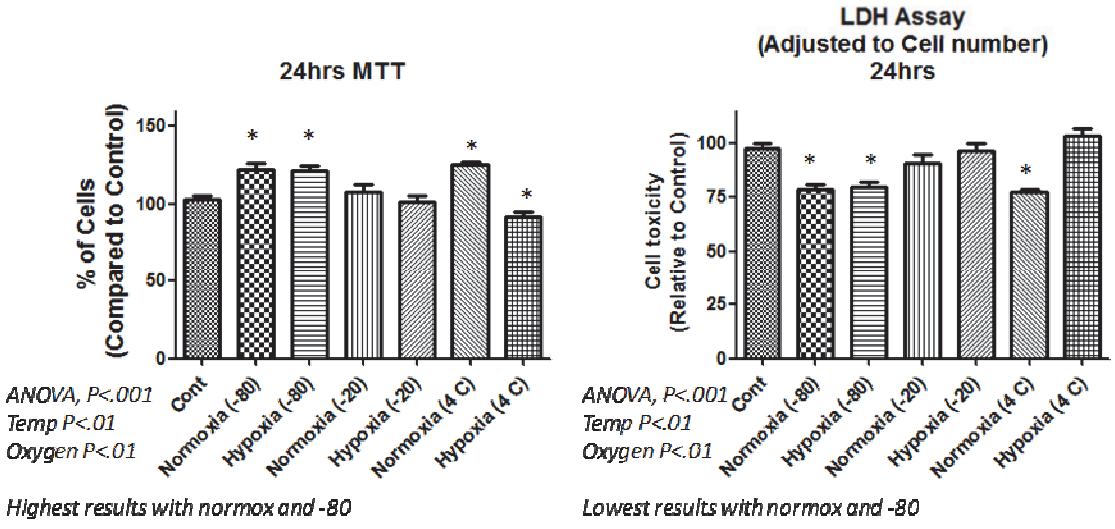


Figure 1A. *Measures of cellular viability (MTT assay) and toxicity (LDH assay) after 24 hours in primary human myoblasts treated with EVs from ADSCS after culture in normoxic or hypoxic conditions, and then stored at different temperatures.*

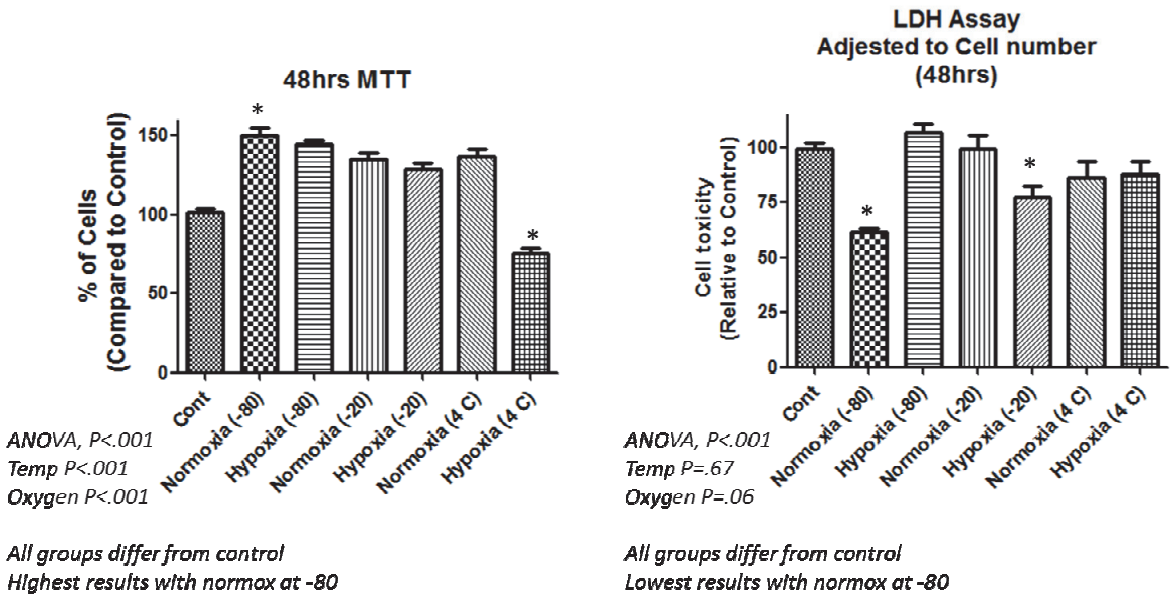


Figure 1B. *Measures of cellular viability (MTT assay) and toxicity (LDH assay)after 48 hours in primary human myoblasts treated with EVs from ADSCS after culture in normoxic or hypoxic conditions, and then stored at different temperatures.*

In brief, we varied both oxygen tension and storage conditions (-80C, -20C, 4C) and evaluated cell survival (MTT assay) and toxicity (LDH assay) in human muscle cells treated with EVs from ADSCs to complete Subtasks 1 and 2. These findings reveal that at both 24 hrs (Fig. 1A) and 48 hrs (Fig. 1B) after treatment (50ug/ml EVs), EVs stored at -80C after culture in normoxic conditions significantly improved cell survival and reduced cell toxicity (*P<.05 compared to control). These findings led us to perform subsequent culture of ADSCs at normoxic conditions, and storage at -80C.

Subtask 3: Treat human primary myoblasts exposed to anoxia with EVs derived from primary human adipose-derived stem cells and determine effects on myotube viability and energy production.

In Subtasks 1 and 2 we varied both oxygen tension and storage conditions (-80C, -20C, 4C) and evaluated cell survival (MTT assay) and toxicity (LDA assay) in human muscle cells treated with EVs from adipose-derived stem cells (ADSCs). These findings revealed that EVs stored at -80C after culture in normoxic conditions significantly improved cell survival and reduced cell toxicity (P<.05 compared to control). In the third quarter of year 1 we worked to optimize EV cryopreservation after EV isolation using tangential flow filtration. These data indicate that addition of trehalose as a cryoprotectant significantly improves the capacity of EVs to promote myoblast survival measured by MTT assay (Fig. 2A) and the ability of EVs to reduce cellular toxicity measured by LDH assay (Fig. 2B).

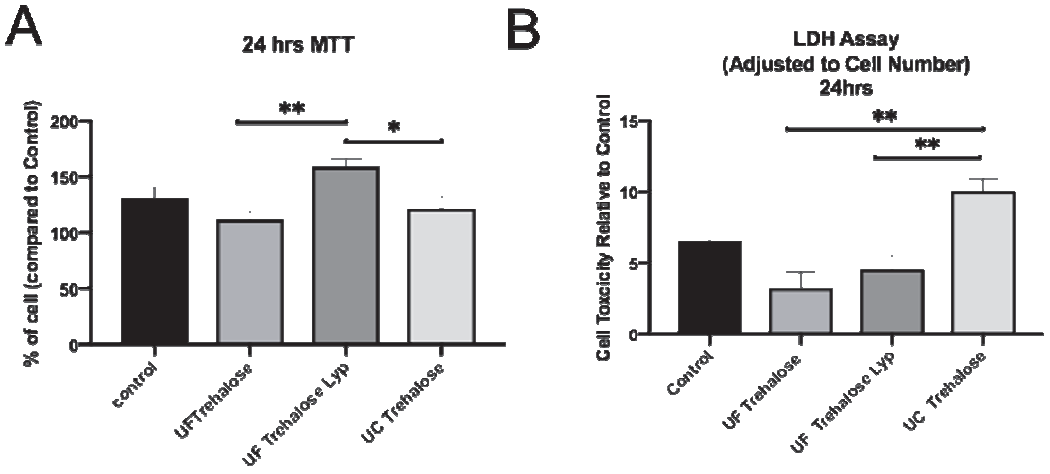


Figure 2. *(A) MTT assay for primary human muscle cell viability (% of MTT positive cells) shows that addition of trehalose improves the pro-viability effects of lyophilized (Lyp) EVs isolated from cultured ADSCs by tangential flow ultrafiltration (UF) compared to EVs isolated by ultracentrifugation (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.*

To further investigate the best condition of lyophilization on exosome number and bioactivity, hADSCs-exosomes were lyophilized in different concentrations of trehalose and polyvinylpyrrolidone (PVP) freeze-drying solutions, rehydrated with the same starting volume of ddH_2O and then the trehalose and PVP4 removed by diafiltration. Nanoparticle tracking analysis (NTA) was performed, and ischemic human myoblasts treated with EVs lyophilized with the different cryoprotectants. Results indicate that the addition of PVP increases EV number (Fig. 3A), and a concentration of 100 mM trehalose plus 2.5% PVP maintains cell viability and decreases cell toxicity (Fig. 3A, B).

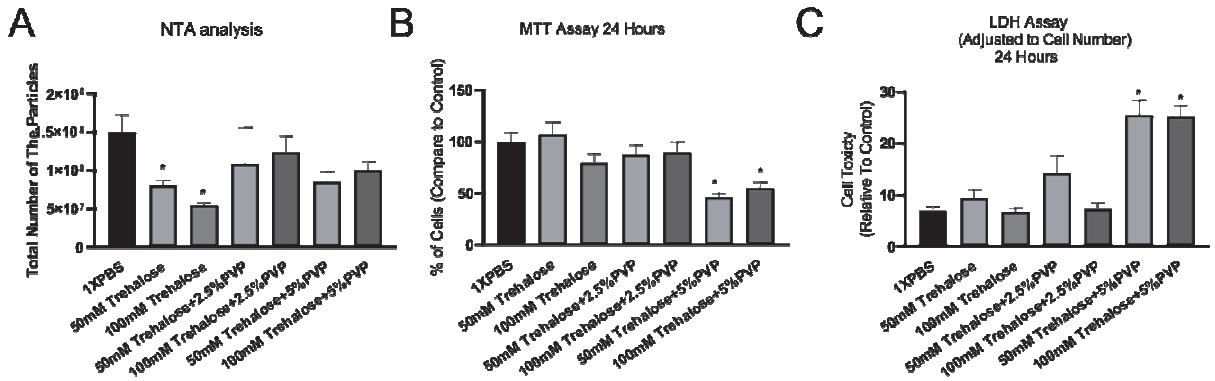


Figure 3*. hADSCs-exosomes were lyophilized in different concentrations of trehalose and polyvinylpyrrolidone (PVP) freeze-drying solutions and then exosome number determined using NTA (A). Ischemic human myoblasts were treated with these EVs and effects on cell viability (B; MTT assay) and toxicity (C; LDH assay) determined. *P<0.05.*

We have completed **Milestone 1** (12 months): 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. Our abstract titled "Optimization of stem cell-derived exosomes for therapeutic application in the prolonged field care environment" has been accepted for the 2019 MHSRS meeting in Orlando, FL. We are therefore on schedule for the timeline proposed in the original Schedule of Work.

The first three quarters of year 2 focused on **Major Task 2: Compare the effects of EV treatment on cell survival with molecules previously determined to attenuate ischemia-reperfusion injury**. The first quarter of year 2 is dedicated to Subtask 1: Obtain stocks of therapeutics (sodium hydrogen sulfide, Etanercept, EPO, NIM-811 C1 esterase inhibitor) for in vitro assays. This subtask as completed, and we have been working on Subtask 2: Treat human primary myoblasts exposed to anoxia with either lyophilized EVs or the therapeutics described above and determine effects on myotube viability and energy production. Hydrogen sulfide is known to promote cell survival under ischemic conditions and so we performed in vitro studies with sodium hydrogen sulfide (NaSH) as well as the hydrogen sulfide donor GYY 4137. The results of these experiments are shown in Fig 4 below. Compared to the control treatment with an exosome-free medium (i.e., phenol red-free DMEM supplemented with 1% exosome-depleted FBS), all tested concentrations of NaHS and GYY4137 were unable to significantly improve the hypoxia-induced ischemic injury to human myoblasts. These findings suggest that NaHS and GYY4137 are ineffective in ameliorating the ischemic injury compared to hADSC-derived exosomes obtained using our methods. On the other hand, the cyclophilin inhibitor and mitochondrial permeability transition inhibitor NIM-811 showed a dose-dependent improvement in cell survival and cell toxicity in ischemic muscle cells (Fig. 5). This is quite impressive and consistent with some previous work showing the effects of this molecule in animal models of TBI. **We have reached Milestone 2, which is to Determine the potential of lyophilized EVs as well as other FDA-approved molecules to promote myotube survival in anoxic conditions.** We have now begun 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.

Unfortunately our activities in the most recent quarter were significantly limited by the Covid-19 outbreak. The requirement for remote work prevented us from moving forward on many of the laboratory-based experiments. Importantly we were able to successfully submit, revise, and have accepted a review paper in *Connective Tissue Research* titled Therapeutic Application of Extracellular Vesicles for Musculoskeletal Repair & Regeneration, officially accepted June 4, 2020.

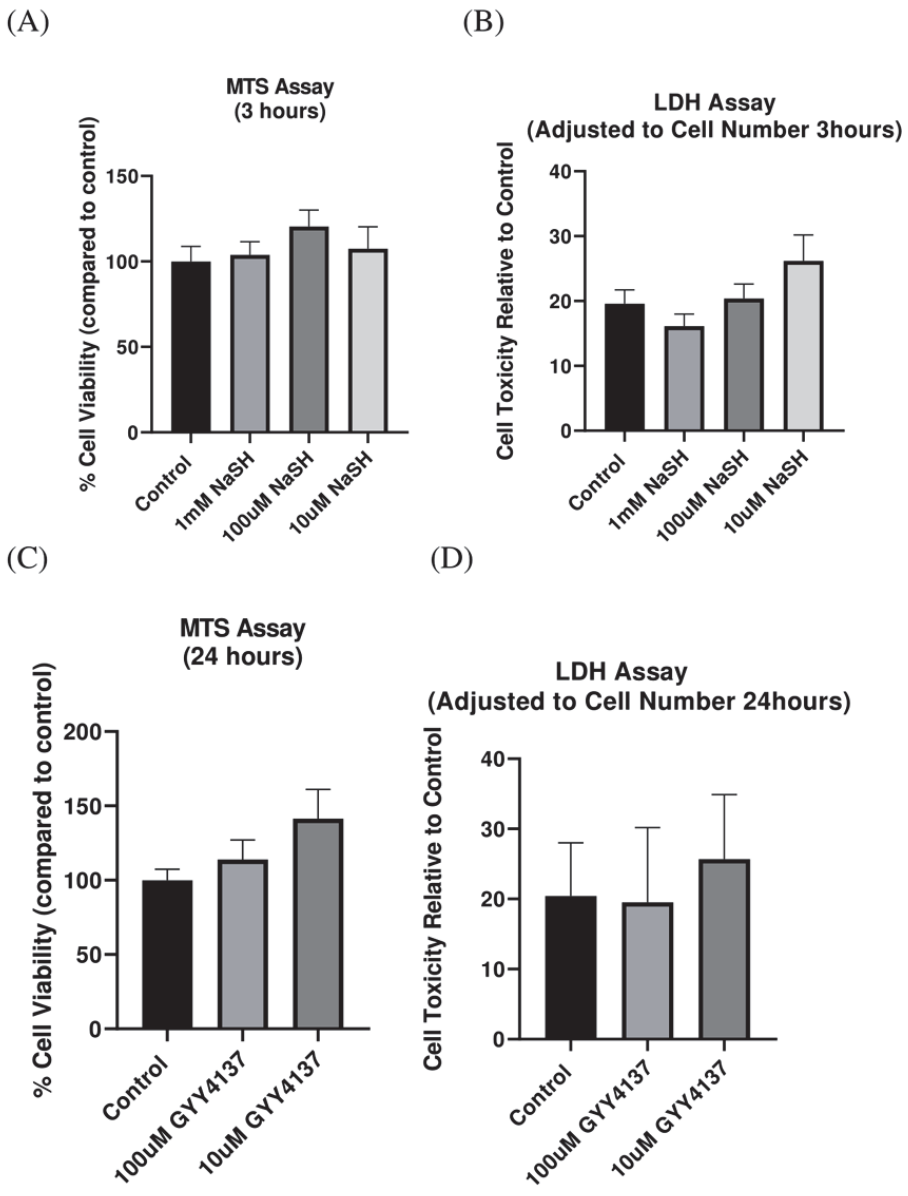
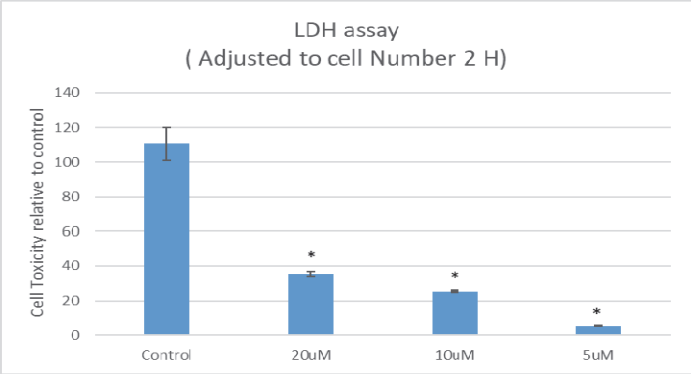


Figure 4. *Effect of sodium hydrogen sulfide (NaHS) and hydrogen sulfide (H2S) donor GYY4137 on ischemic human myoblasts: To test the effect of NaHS, ischemic human myoblasts were treated with 1 mM, 100 μM and 10 μM of NaHS for 20 min before the end of the 6-h hypoxia treatment and then for additional 3 h under the normoxia condition (Fig. A,B). Similar-ly, ischemic human myoblasts were treated with 100 μM and 10 μM of GYY4137 for 24 h (Fig. C,D). The effect of both compounds on cell proliferation and reversal of cell toxicity was determined by MTS (cell survival) and LDH (cell toxicity) assays, respectively. Data are expressed as mean ± SD (n = 6).*



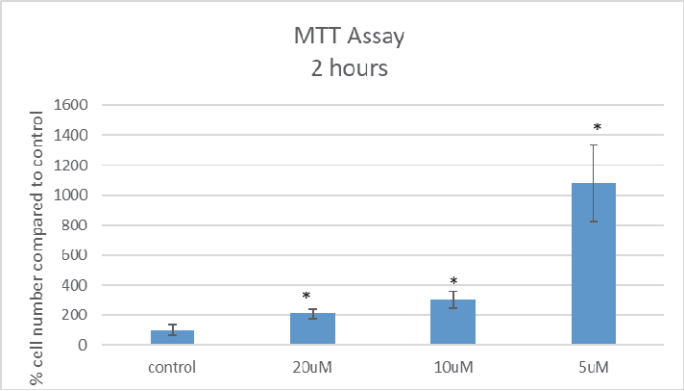


Figure 5. *Effect of NIM-811, a cyclophilin inhibitor and mitochondrial permeability transition inhibitor, on ischemic human myoblasts: To test the effect of NIM-811, ischemic human myoblasts were treated with 20uM, 10 μM and 5 μM of NIM-811 for 20 min before the end of the 6-h hypoxia treatment and then for additional 3 h under the normoxia condition. The effect on on cell proliferation and reversal of cell toxicity was determined by MTT (cell survival) and LDH (cell toxicity) assays, respectively. Data are expressed as mean ± SD (n = 6). *: 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?**
  - ***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?**

We will spend Quarter 1 of year 3 (Quarter 9 cumulative) 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. We have evaluated the effects of NIM-811 in vitro and in vivo as a baseline to compare with the lyophilized EVs. We will in Quarter 1 of year 3 prepare a manuscript on the in vivo NIM-811 studies, referenced above in the MHSRS abstract, for publication. We will request a one-year no-cost extension for the project due to delays related to Covid-19.

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.

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

CHANGES/PROBLEMS: Our laboratory time was limited April-July 2020 due to the Covid-19 outbreak. Our institution normalized lab activities starting July 1, 2020; however, as of the time of this writing, case numbers in Georgia are increasing and we may be directed to telework in the future.

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* (Impact Factor=5.2) on "Exosomes as Therapeutic Systems": https://www.frontiersin.org/articles/10.3389/fcell.2020.00181/full
A manuscript to 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 accepted June 4, 2020 and is now available online:
https://www.tandfonline.com/doi/full/10.1080/03008207.2020.1781102 .
As noted above, we also submitted our work on the NIM-811 molecule to the 2020 MHSRS meeting. We will plan to submit the manuscript resulting from this work in the first quarter of year 3 of the funded project.

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: 12.0
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: 6.0
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.***