Accelerating Translation of Biostasis-Inducing Compounds into Clinical Practice Using Porcine Models

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FINAL REPORT

Date: January 23, 2023

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ACCELERATING TRANSLATION OF BIOSTASIS-INDUCING COMPOUNDS INTO CLINICAL PRACTICE USING PORCINE MODELS

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
01/23/2023	Final Report	October 2021 – September 2023

4. TITLE AND SUBTITLE

Accelerating translation of Biostasis-inducing compounds into clinical practice using porcine models

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER
DS21RES27

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
United States Air Force, 59th Medical Wing (59MDW/ST).
1255 Wilford Hall Loop, Building 4430
Lackland Air Force Base, 78236-9980

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
United States Air Force, 59th Medical Wing
(59MDW/ST).
1255 Wilford Hall Loop, Building 4430
Lackland Air Force Base, 78236-9980

10. SPONSOR/MONITOR'S ACRONYM(S)

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

12. DISTRIBUTION / AVAILABILITY STATEMENT
Distribution A: Approved for public release; distribution is unlimited.

13. SUPPLEMENTARY NOTES

14. ABSTRACT-

In 2006, the military improved body armor to reduce fatalities, but vulnerable areas remain. Injuries from blasts, penetrative, and blunt force can cause severe vascular, neurologic, musculoskeletal, and pulmonary injuries. The body's response to trauma triggers a survival mechanism, which can lead to long-term injury and decrease the chance of survival. Biostasis is a technique that reduces metabolic demands to preserve tissue and organ system viability. A collaborative pilot study with Wyss Institute and Vascular Perfusion Solutions demonstrated successful biostasis in an *ex vivo* porcine model. The goal of this project is to gain military feedback by challenging biostasis compounds in a swine model simulating traumatic extremity vascular injury, which can improve survival by limiting metabolic demand and increasing the time a wounded warfighter can get definitive treatment. We will test the hypothesis that *the biostasis therapeutic, Wyss drug will afford protection against ischemia reperfusion injury at the hindlimb of swine via limiting metabolic demand to enable preservation of neuromuscular health and function.* We propose translating the leading stasis drug into a porcine in vivo hind limb ischemia reperfusion model, which will be conducted concurrently with studies to develop stasis induction protocols and biomarkers. This offers the ability to truly validate the stasis induction protocols and biomarkers, since the ultimate validation of stasis efficacy is enhanced viability and lack of toxicity following tissue and organ reintegration into the host. Additionally, this task will enable validation of stasis biomarkers, which may enable their translation into eventual human use, to include predictions of the likelihood of limb reattachment or organ transplantation success. In this model, the iliac artery and vein will be isolated, and the WCx drug will be infused using a closed-loop system. The limb will remain ischemic for 6 hours without reperfusion, mimicking a military combat situation. The animal will be monitored for 7 days following restoration of native circulation to evaluate the functional outcome of the limb and the whole-body effects of drug washout. This study will also enable analysis of the effects of subtherapeutic doses of WCx on the systemic circulation. It will be the first in vivo survival study in a large mammalian model of WCx-induced biostasis that evaluates the functional outcome of a limb that has undergone biostasis and ischemia without the potential technical complications of limb re-implantation. If the results continue to be promising, this will lead to the evaluation of porcine organ systems and eventually human tissue in future optional work. Despite facing a range of challenges, from delays in contracts and IACUC approvals to equipment acquisition, unforeseen scheduling issues at the facility, and recruitment of a post-doctoral fellow and surgery residents, the project was deemed unfeasible without additional time and funds to maintain the team. It was therefore decided to close the study and assess future funding calls for additional funding for a more easily executable animal model.

15. SUBJECT TERMS- biostasis, ischemia reperfusion injury, combat injury, porcine, hypometabolism, limb preservation

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT:**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			Lt Col Thomas J Percival
U	U	U	UU	23	**19b. TELEPHONE NUMBER** *(include area code)* 210-867-5255

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In the model development, there was a challenge in developing a closed loop perfusion system for the isolated limb. The exposure and achievement of ischemia in the hind limb was

1.0 EXECUTIVE SUMMARY

As the United States engages in conflicts with more sophisticated adversaries, the challenge of mitigating hemorrhage and transporting combat casualties to medical facilities for lifesaving treatment has become increasingly important. Current treatments for extremity vascular injury focus on limiting hemorrhage and replacing blood loss in a forward position. However, in resource-limited environments, immediate blood loss replacement may not always be feasible, resulting in ischemic insult to the extremity through the use of tourniquets. This can lead to direct ischemic injury to muscle and nerves, as well as reperfusion injury after prolonged ischemia, ultimately resulting in chronic pain and loss of function in the extremities. Therefore, there is an urgent need for the development of strategies to mitigate ischemic insult.

One such strategy is biostasis, which involves artificially reducing metabolic demands while leveraging natural body processes to conserve energy, ultimately prolonging tissue and organ system viability. The objective of this project is to evaluate the efficacy of biostasis-inducing therapeutics in a model of extremity vascular injury, with the goal of accelerating the transition to military operational medicine and combat casualty care. The significance of biostasis lies in its potential to limit the metabolic demands of damaged tissue, which can prolong the time available for a wounded soldier to receive definitive treatment.

To achieve this goal, this study aims to gain military end-user feedback on the development of biostasis compounds early in the research and development cycle. This will be accomplished by challenging biostasis compounds in an ischemia reperfusion swine model that simulates traumatic extremity vascular injury. This model was chosen due to the prevalence of extremity vascular injuries in combat settings and to test the hypothesis that biostasis offers a strategy to improve survival by limiting metabolic demand and increasing the time that wounded soldiers must receive definitive treatment.

Despite facing several challenges, including delays in contracts and IACUC approvals, equipment acquisition, unforeseen scheduling issues, and difficulties in recruiting post-doctoral fellows and surgery residents, the project was deemed unfeasible without additional time and funds to maintain the team. It was therefore decided to close the study and assess future funding opportunities for a more readily executable animal model. The outcome of these studies will result in lab prototypes and the advancement of biostasis formulations for military applicability and preservation of life and limb, making it a crucial area of research for improving the care of combat casualties.

2.0 INTRODUCTION

In 2006, the military introduced an improvement in body armor which significantly decreased fatality rate at point of injury. Unfortunately, the face, head, and extremities continue to be vulnerable despite armor improvements; increased injury severity scores, primarily to the extremities and maxillofacial regions, have been reported. Blast, penetrative and blunt force injuries are also accompanied by severe vascular, neurologic, musculoskeletal and pulmonary injuries. Initial trauma triggers a physiologic response to survive. The body begins shunting blood from less essential organs to the vital organs such as the heart and brain. The ensuing hemorrhage from the extremity increases stress on the body with an ensuing massive inflammatory response. Once the hemorrhage is controlled, the body enters shock and experiences significant oxygen deprivation. This deficit is what causes long term injury to the

warfighter and decreases their chance for survival. A retrospective analysis of advanced resuscitative strategies and modern vascular techniques revealed successful limb salvage is achievable with excellent graft patency. **However, in austere settings with delayed evacuation likely, additional measures and novel biotechnological interventions are needed to preserve extremity viability and save the warfighter's life.** Biostasis is a term used to describe methods for artificially reducing metabolic demands while leveraging natural body processes to conserve additional energy, ultimately prolonging tissue and organ system viability. By inducing whole body biostasis, injury and inflammatory responses can be mitigated by decreasing oxygen deficit. This project will utilize direct or systemic introduction of chemical therapeutic agents to damaged tissue, which will reduce the metabolic demands of the body and effectively induce biostasis. *The proposed project builds upon encouraging results returned from the DARPA-sponsored Biostasis program and will extend these efforts to challenge our novel formulation in a more complex, military relevant model.*

In a collaborative pilot study with the Wyss Institute at Harvard University and Vascular Perfusion Solutions (VPS), biostasis was successfully achieved by infusion of Wyss Compound (WC) via perfusion pump in an *ex vivo* porcine model (Figure 1). This treatment slowed the relative metabolic rate by 50% without evidence of necrosis, depleted glucose content, intracellular edema, myofibril disruption and cellular degradation when compared with baseline samples. Further investigation in military relevant *in vivo* simulations to demonstrate its efficacy in patient survival and outcome are required. The goal of this project is to gain military end-user feedback on development of biostasis compounds early in the research and development cycle. This will be accomplished by challenging biostasis compounds in an ischemia reperfusion swine model simulating traumatic extremity vascular injury (Figure 2). This model was chosen since 20% of total vascular injuries occur in the extremities in combat settings and to test the **hypothesis** that biostasis offers a strategy to improve survival by limiting metabolic demand and increasing the time a wounded warfighter could get to definitive treatment.

We will test the hypothesis that *the biostasis therapeutic, Wyss drug will afford protection against ischemia reperfusion injury at the hindlimb of swine via limiting metabolic demand to enable preservation of neuromuscular health and function.* The following aims will address the hypothesis/research question:

Specific Aim 1: Establish biostasis-induction in ischemia reperfusion injury swine model. Induction of biostasis will be evaluated by directly measuring limb metabolic rate during ischemia reperfusion and infusions with biostasis therapeutics.

Specific Aim 2: Evaluate the effects of biostasis therapeutics on hindlimb functional recovery following ischemia. Functional recovery will be determined on days 1, 3, and 7 of reperfusion by nerve conduction velocity, gait, arterial blood flow and electromyography.

Specific Aim 3: Determine the effects of biostasis therapeutics on hindlimb tissue viability following ischemia. Tissue biopsies will be collected on days 1, 3, and 7 of reperfusion. Nerve degeneration and muscle necrosis will be evaluated by histology as an indicator for tissue viability. Furthermore, glucose content, intracellular edema, myofibril disruption and cellular degradation will be compared to pre-ischemic samples.

Specific Aim 4: Elucidate the effects of biostasis therapeutics on ischemia-induced cellular inflammation, metabolic dysfunction, and oxidative stress in swine. Markers of cellular inflammation will be evaluated in serum and muscle tissue pre-ischemia and days 1, 3, and 7 of reperfusion by Luminex assays. Metabolic dysfunction and oxidative stress will be evaluated with well-established spectrophotometric assays.

The Wyss Institute has utilized NemoCAD computational pathway enrichment analysis for rapid biostasis drug discovery. They have leveraged the *Xenopus laevis* model to create an organism-level high throughput screening tool to evaluate positive hits and identify additional biostasis inducers. Through this discovery pipeline, the Wyss Institute has discovered a set of 10 promising biostasis inducing drugs, Wyss Compounds (WC_x, x=iteration), that have successfully induced biostasis in several invertebrate and vertebrate model systems (data unpublished). These 10 biostasis biologics have been extensively screened in at least two animal models to identify the top performing candidate that will be evaluated in the protocol outlined here. The animal models used in these prescreening studies include *Xenopus*, Caco epithelial cells, Gut-on-a-Chip, liver endothelial cells, and/or in a perfusion pump in an *ex vivo* porcine model (Figure 2).

Prior to the animal studies proposed here, 10 biostasis biologics have been extensively screened in a relevant xenopus model that simulates ischemia reperfusion injury (IRI). Currently, these biologicals are being screened in an ex vivo porcine semitendinosus vascularized composite allotransplantation model to identify the biologic that affords the greatest protection against IRI-mediated cell and tissue death (listed in Table 2). In the protocol outlined here, the top performing candidate will be evaluated in a well-established swine model of IRI that simulates traumatic vascular injury 1) to validate the induction of biostasis in swine tissue, and 2) establish a dosing regimen for the selected WC candidate for a full pre-clinical study to begin in Spring 2022.

Prescreening studies suggest the best candidate for these studies is Donepezil, a commonly prescribed medication to improve cognitive function in Alzheimer's patients. Donepezil was shown to induce reversible stasis in *Xenopus laevis* tadpoles within 2 hours of administration. Biostasis was confirmed by lower oxygen consumption rate and activity levels of tadpoles (data unpublished). Donepezil at biostasis inducing concentrations (50 μM) does not affect tadpole development nor survival. Non-cytotoxic concentrations (50 μM) of donepezil modulate mitochondrial respiratory function in both caco-2 intestinal epithelial cells and liver endothelial cells. In both cell lines, ATP/ADP ratio and oxygen consumption is reduced within 20 minutes of administration indicating successful induction of biostasis (data unpublished). Donepezil has been further investigated in a 3D co-culture system (Gut Chip) to evaluate the drug's efficacy. In this model system, oxygen consumption decreases significantly compared to vehicle without significant drug cytotoxicity or loss of gut barrier function (data unpublished). Drug cytotoxicity was defined as the absence of DNA damage and minimal lactate dehydrogenase activity in the culture media.

A simulated hemorrhage via cannulation of the iliac artery and vein will be performed in swine. The WC compound will be infused via closed loop pump system to circulate compound via an isolated limb perfusion strategy. The limb will then be made ischemic for various time points: 1, 3 and 6 hours (n=8). Perfusion will be restored after patch angioplasty and resuscitation with

shed blood. Markers of ischemia will be obtained. The animal will survive for two weeks with functionality of the limb being evaluated daily. Nerve conduction studies, arterial flows in the repair and lab evaluation will be monitored at various time points. Histology will be performed evaluating nerve degeneration and muscle necrosis will be completed. These studies are significant because they will be the first to evaluate a novel biostasis therapeutic compound in an ischemia reperfusion extremity trauma model. *The results of these studies will establish a prototype biostasis-inducing therapeutic in a validated model which will lead to efficacy testing for a variety of other combat-relevant injuries including traumatic brain injury, lung contusion and cardiac contusion.* Continued partnerships with our world-renowned partners will rapidly accelerate development and modernization of therapies that will be applicable in resource-limited environments.

3.0 METHODS, ASSUMPTIONS AND PROCEDURES

Experimental Design and General Procedures:

This study is organized into 3 major tasks:

- Task 1: Model Refinement/Dosing Determination with Donepezil
- Task 2: *In vivo* Ischemia/Reperfusion with Donepezil
- Task 3: Tail-end Funding

3.1 TASK 1: MODEL REFINEMENT/DOSING DETERMINATION:

These studies will utilize a swine limb model of ischemia reperfusion to 1) determine the appropriate dosing regimen for Donepezil to achieve a tissue concentration of 50 μM, and 2) validate biostasis induction for further evaluation in a full pre-clinical study.

TABLE 1: MODEL REFINEMENT/DOSING DETERMINATION

Group/Model (Total n=15)		Treatment	Duration of Drug Exposure & Ischemia	End Point
1	Control, n=3	Sham	Sham	7 days
2	Drug Control, n=3	Vehicle	6h/6h	7 days
3	Exp, n=3	1 mg/kg of Donepezil	6h/6h	7 days
4	Exp, n=3	2 mg/kg of Donepezil	6h/6h	7 days
5	Exp, n=3	3 mg/kg of Donepezil	6h/6h	7 days

3.1.1 Ischemia Reperfusion Injury Swine Model:

A simulated hemorrhage via cannulation of the iliac artery and vein will be performed in swine. Donepezil will be infused via closed loop pump system to circulate compound via an isolated limb perfusion strategy. The limb will be made ischemic and closed loop perfusion of WC will be performed for six hours after cannulation of the artery and vein has been performed. Tissue oxygenation and blood flow are continuously measured in the hindlimb muscles with the OxyFlo and OxyLit probe to validate ischemia insult, level

of biostasis induction and the reinstatement of blood flow following the surgery.

3.1.2 Surgical Preparation:
The animals will be catheterized by ultrasound-guided percutaneous approach as follows: 1) left external jugular vein – resuscitation fluid administration 2) right carotid artery – invasive arterial pressure. If percutaneous insertion fails, cut-downs will be performed for vascular access. Animals will be instrumented for the following: invasive arterial pressure, rectal temperature, $ETCO_2$, PaO_2, and ECG.

3.1.3 External Iliac Artery Exposure:
A low midline incision will be made with a retroperitoneal dissection to expose a 5-6 cm segment of the external iliac artery. Side branches of the external iliac will be ligated but not divided. The proximal and distal segments of the external iliac artery and vein will be isolated and controlled with vessel loops. The artery and vein will be cannulated with a Cordis or catheter equivalent for infusion and recirculation of the infusion compound.

3.1.4 Ischemia:
During the 6-hour ischemic period the external iliac artery and vein will circulate donepezil in a closed loop system. WC Drug Concentrations in Affected Hindlimb Muscles: 0, 30, 60, 90, 120, 180, 240, 300, and 360 min. (Potentially vs. other side). Biopsies of other tissues to evaluate systemic leakage? Kidney and liver to evaluate toxicity? Tissue Oxygenation via Oxylite in Affected Hindlimb Muscles to determine level of ischemia in surrounding tissue: every 15-30 min. Tissue blood flow with OxyFlo in Affected Hindlimb Muscles to validate ischemia in surrounding tissue: every 15-30 min. Evaluate Tissue Health via Histology: 0 and 360 min. Serum/plasma Collection: Every 15-30 min to evaluate systemic inflammation and drug toxicity and overall state of pig

TABLE 2. ISCHEMIA REPERFUSION INJURY CRITERIA
1. Blood flow to ligated major vessels is absent as determined by the OxyFlo sensor.
2. Tissue oxygenation in adjacent muscles of the affected hindlimb is reduced compared to non-affected hindlimb muscles as determined by the OxyLit sensor.
3. Blood flow is restored in the affected hindlimb as determined by the OxyFlo sensor.
4. Tissue oxygenation in adjacent muscles of the affected hindlimb increases following restoration of blood flow as determined by the OxyLit sensor.

3.1.5 Surgical Repair:
After completion of the ischemic period, the artery is flushed with heparin and balloon thrombectomy performed. The artery will then be repaired with a patch angioplasty and vein will primarily be repaired. The wound will be

closed in layers. Surgical repair of the artery will be performed with a patch angioplasty, and the animal will survive for one week. Throughout this one-week period, gait will be assessed following 24-, 72-, and 168-hours reperfusion (post-ischemia) to validate loss of neuromuscular function in vehicle control group by Tarlov score.

3.1.6 Survival Study for Model Development:

3.1.6.1 Behavioral/Functional:

3.1.6.1.1 Daily gait using Tarlov
3.1.6.1.2 Electrophysiological measurements: 1, 3 and 7 days
3.1.6.1.3 Tissue blood flow with OxyFlo: 1, 3 and 7 days
3.1.6.1.4 Tissue Oxygenation with OxyLite: 1, 3 and 7 days

3.1.6.2 Muscle Biopsies: 1, 3 and 7 days

3.1.6.2.1 Histology
3.1.6.2.2 Muscle fiber contractility

3.1.7 Dosing:

To determine the dosing regimen for a full pre-clinical study, swine will be organized into five groups ranging from low to high concentrations of Donepezil (listed in Table 1). The three testing concentrations of the selected Donepezil drug used in this study will be based on preliminary studies performed by the Wyss Institute. To evaluate the effects of biostasis therapeutics on hindlimb viability and functional recovery following ischemia reperfusion injury as described above. In addition, drug levels will also be evaluated in blood throughout the drug infusion period (0, 30, 60, 90, 120, 180, 240, and 300 min) to measure potential drug loss into the circulation. Blood samples will be snap frozen and sent to the Wyss Institute to measure drug levels. This is important to establish as this will indicate any areas of improvement for model development. Tissue biopsies will be collected throughout the infusion and wash-out period to measure drug uptake. The tissue concentration that closely resembles the EC_{50}, determined from studies in tadpoles, tissue culture and human organ chips at the Wyss Institute, will be used in Task 1.2. The appropriate dosing regimen for all 5 compounds with known EC_{50}s, can be elucidated based on the dosing study of WC_1.

3.1.8 Safety Evaluation:

To determine the safety of the Donepezil drug, blood, and necropsies (iliac artery, liver, kidney, bilateral anterior tibialis, and peroneal nerves) will be collected at the time of euthanasia to evaluate 1) tissue viability using histology, and 2) biomarkers of inflammation and cytotoxicity. For the evaluation of tissue viability, tissues will be stored in formalin and sent to CIRS Histology Core for H&E staining and analysis. For the evaluation of biomarkers of inflammation and cytotoxicity, tissues will be snap frozen and sent to the Wyss institute on dry ice for processing and analyzing.

3.1.9 Biosamples

Animal is euthanized and the iliac artery, liver, kidney, bilateral anterior tibialis and peroneal nerves are collected for histopathology and metabolic assays on day 7. Roughly 100 mg of tissue will be collected for each analysis. Blood samples are required to be drawn at 0, 30, 60, 90, 120, 180, 240, 300, and 360 min throughout the ischemia period and 24 and 72 of reperfusion. Blood (~2 mLs) is drawn from the external or internal jugular artery.

3.1.10 Data Analysis:

The focus of this study is performing the pilot study. No power analysis is planned, as this is a pilot study collecting an initial baseline data. The number of animals was decided to maximize data collection and meet study goals within the study resource and time while minimizing animal use.

3.2 TASK 2: *IN VIVO* ISCHEMIA/REPERFUSION OF MOST SAFE AND EFFICACIOUS WC CONCENTRATION

In vivo closed loop perfusion of swine hind limb with perfusion of WCx with subsequent ischemic period and evaluation of limb function for a one-week survival period – Total 26 swine.

Evaluation of biostasis capability in model of ischemia/reperfusion. Task 2 will show the effect of biostasis in a limb model of ischemia/reperfusion and allow for evaluation of tissue viability one-week observation period. This will also allow for evaluation of the systemic effects of potential drug loss into the systemic circulation as well as validation of stasis biomarkers with a clear functional outcome.

TABLE 3: Task 2

Group/Model (Total n=26)		**Treatment**	**Duration of Drug Exposure & Ischemia**	**End Point**
1	Control, n=6	Sham	Sham	7 days
2	Drug Control, n=10	Vehicle	6h/6h	7 days
3	Experimental, n=10	Donepezil	6h/6h	7 days

Description: The external iliac artery and vein of the swine will be isolated. The limb will be made ischemic and closed loop perfusion of WC will be performed for six hours after cannulation of the artery and vein has been performed. The best performing dose determined in previous tasks of this project will be used in this study. Surgical repair of the artery will be performed, and the animal will survive for one week. Daily assessment of limb function will be performed.

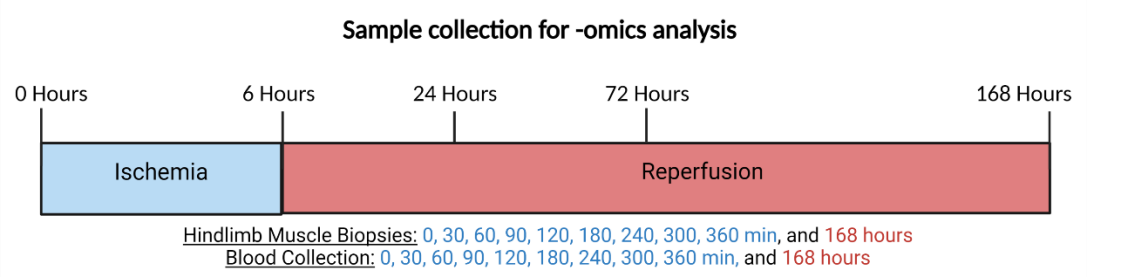
Specific Aim 1: Establish biostasis-induction in ischemia reperfusion injury swine model. Induction of biostasis will be evaluated by directly measuring limb metabolic rate during ischemia reperfusion and infusions with biostasis therapeutics.

Specific Aim 2: Evaluate the effects of biostasis therapeutics on hindlimb functional recovery following ischemia. Functional recovery will be determined on days 1, 3, and 7 of reperfusion by nerve conduction velocity, gait, arterial blood flow and electromyography.

Tarlov assessments will be performed 24, 72, and 168 hours of reperfusion (post-ischemia). Blood samples will be collected to perform standard ABGs and measure inflammatory markers at 24, 72, and 168 hours of reperfusion (post-ischemia).

Electrophysiological measurements will be performed on the left sciatic nerve of isoflurane anesthetized animals on a heating pad and recorded with a Sierra Summit to measure compound muscle action potential (CMAP) and nerve conduction velocity (NCV). To measure CMAP, the nerve will be simulated by a single 0.5 ms duration. A needle electrode is placed intramuscularly at the sciatic notch (in the left gluteal region) to stimulate the sciatic nerve. The recording electrode is placed in the central region of the left gastrocnemius. The current (in mA) will be increased to achieve the maximum CMAP reading to be recorded as the maximum CMAP. To measure NCV, a single pulse of 0.5 ms duration is used to stimulate the sciatic nerve first placed in the left sciatic notch (proximal site) and secondly in the gastrocnemius (distal site). The recording electrode is placed in the plantar of the foot. NCV (m/s) is then calculated as the distance between stimulation sites (proximal – distal, m), divided by the difference between the latency of the proximal site and distal site (s).

Specific Aim 3: Determine the effects of biostasis therapeutics on hindlimb tissue viability following ischemia. Tissue biopsies will be collected on days 1, 3, and 7 of reperfusion. Nerve degeneration and muscle necrosis will be evaluated by histology as an indicator for tissue viability. Furthermore, glucose content, intracellular edema, myofibril disruption and cellular degradation will be compared to pre-ischemic samples.



Specific Aim 4: Elucidate the effects of biostasis therapeutics on ischemia-induced cellular inflammation, metabolic dysfunction, and oxidative stress in swine. Markers of cellular inflammation will be evaluated in serum and muscle tissue pre-ischemia and days 1, 3, and 7 of reperfusion by Luminex assays. Metabolic

dysfunction and oxidative stress will be evaluated with well-established spectrophotometric assays (listed in Table 4).

TABLE 4. ASSAYS TO ASSESS METABOLIC DYSFUNCTION, OXIDATIVE STRESS, AND INJURY

ASSAY	OBJECTIVE/PURPOSE
Luminex Assay	Assess inflammatory state of affected tissues.
Citrate Synthase Assay	Overall Mitochondrial Activity
Proteasome Activity Assay	Proteasome activity is altered following induction of biostasis in several animal models
Calpain Activity Assay	Skeletal muscle remodeling.
Myeloperoxidase (MPO) Activity Assay	Involved in ischemia reperfusion injury.
Superoxide Dismutase (SOD) Activity	Oxidative stress state. Altered following ischemia reperfusion injury.
Chemical Calcium Assay	Loss of calcium homeostasis following ischemia reperfusion injury.
Mitochondrial DNA Copy Number	For mtDNA Copy number analysis. Mitochondrial dynamics
Glycogen Assay	Assess overall metabolic state.
Lactate Assay	Assess overall metabolic state.
SERCA (Sarco(endo)plasmic reticulum calcium ATPase)	Responsible for transporting calcium (Ca(2+)) from the cytosol into the lumen of the sarcoplasmic reticulum (SR) following muscular contraction. The Ca(2+) sequestering activity of SERCA facilitates muscular relaxation in both cardiac and skeletal muscle.
Single Fiber Contractility	Test functionality following injury. https://www.jove.com/t/52695

Biosamples Animal is euthanized and the iliac artery, liver, kidney, bilateral anterior tibialis and peroneal nerves are collected for histopathology and metabolic assays on day 7. Roughly 100 mg of tissue will be collected for each analysis. Blood samples are required to be drawn at 0, 30, 60, 90, 120, 180, 240, 300, and 360 min throughout the ischemia period and 24 and 72 of reperfusion. Blood (~2 mLs) is drawn from the external or internal jugular artery.

Data Analysis Continuous outcome variables will be assessed for normality by the Shapiro-Wilks test. Normally distributed continuous outcomes will be presented as mean and standard deviation, and one-way analysis of variance (ANOVA) testing with a Tukey's post hoc test will be performed to determine significant differences among the groups. Otherwise, non-normally distributed outcomes will be presented as median and interquartile range (IQR) and will be analyzed using Kruskal-Wallis test (a non-parametric method) with Dwass-Steel-Chritchlow-Fligner (DSCF) pairwise comparisons. To test the group and time effects, a generalized linear model (GLM)

will be performed. Significance will be set to $p < 0.05$. Statistical analyses will be performed using SAS version 9.4 (Statistical Analysis Software, Cary, NC).

3.3 TASK 3: TAIL END FUNDING

Tail Funding Request for the Continuation of Studies: This add-on request will enable further exploration of novel biostasis agents and the generation of predictive models to accelerate lead candidate development.

3.3.1 Determine the mechanistic features of biostasis drug with evaluation via the omics pathways.

Blood and tissues samples (sampling outlined below) in the current study and newly proposed study will be collected prior to, during, and after drug administration to generate RNA-seq, proteomics, and metabolomics data to assess the role of stasis-inducing pathways of previously discovered biostasis pathway. Furthermore, we will build upon this data with phosphoproteomic studies to assess intracellular signaling pathways and networks mediated by reversible phosphorylation in response to stasis drugs. These studies in conjugation with transcriptomics and metabolomics will help to uncover new cell signaling pathways and stasis drug targets. The increased tissue sampling frequency will allow us to develop a predictive model to help identify time required to induce biostasis as well as its duration along with providing a pathway to determine the mechanistic features of the drug with evaluation via the omics pathways. The goal of this task is to identify trajectories of stasis and viability metrics to determine the duration of tissue preservation obtainable as well as optimal dosing strategies and pinpoint associated mechanisms.

3.3.2 Validate efficacy of the top 2 performing biostasis drugs against simultaneous ischemia and hemorrhagic shock in porcine to simulate a more clinically relevant model of limb-threatening vascular injury.

Hemorrhagic shock reduces the ischemic threshold to 3 hours where recovery is absent without intervention. In this study, once ischemia is established with the method described above, hemorrhage will be induced by removing 35% of total blood. Blood flow will be restored at the ischemia interval determined in the model refinement study (Task 2). The efficacy of the selected biostasis agents will be evaluated in the same fashion as the current study (described in Research Timeline and Research Plan 1.) and will include -omics analysis to develop a predictive mechanistic model of biostasis pathways. Additionally, this task will enable validation of stasis biomarkers, which may enable their translation into eventual human use, to include predictions of the likelihood of limb reattachment or organ transplantation success.

4.0 MAJOR EVENTS/MILESTONES/SUCCESS

In preparation for the execution of this project,

- Kick Off Meeting – April 2022
- IRB/IACUC Approval – April 2022
- Project manager hired – April 2022
- Project research technicians hired – May 2022
- Project post-doctoral fellow hired – N/A
- All experimental procedures completed – N/A
- Data Analysis – N/A
- Poster presentation – provide location and date: N/A
- Manuscript submitted to – name of journal and date: N/A
- Dissemination of Results – N/A

5.0 RISK ASSESSMENT

6.1 Risk Analysis:

5.1.1 FY22Q1:

Risk: Slight delays in getting IACUC protocol submitted for approval. This has delayed our start date of model refinement studies tentatively to April with the anticipation of having personnel hired to support studies.
Mitigation Plan: Team will work closely with the attending vet (AV) to increase the likelihood of approval.
Update: This delay should not hinder completion of studies.

5.1.2 FY22Q2:

Risk #1: IACUC was circulated for signatures, this has delayed initiation of model development.
Mitigation Plan #1: N/A
Update #1: IACUC was routed and officially approved on 4/20/22

Risk #2: There have been delays in receiving equipment and room assignment for studies.
Mitigation Plan #2: In the interim, the team will make use of a laboratory space at CIRS that is already established and used by another research group for training purposes. Additionally, the team has developed standard operating procedures (SOPs) in preparation for the official start of the study.
Update #2: Despite scheduling conflicts, the team successfully initiated the training for some of the bench studies.

5.1.3 FY22Q3:

Risk: Delays in IACUC approval and hiring technical team have led to significant delays in kicking off the model refinement study.
Mitigation Plan: The animal surgeries have been authorized to be conducted through OY2, as per the agreement with Mr. Barnicott at CIRS. Extending into OY2 will guarantee the completion of the surgical portion of the studies. It is

important to note that this study has received supplementary funding to facilitate the completion of the proposed biological assays in FY23.
Update: CIRS costing for entire study was finalized. Animal-related costs are projected to cost a total of $72K. The remaining $72K will be deobligated to ODCs for OY2. CIRS staffing for FY23 will need to be paid during OY2 (estimated costs: $40K). Biomedical Associate and Technician were identified and have been on-boarded. We did not receive any ORISE post-doc applicants. OY1 ORISE funds will be deobligated and moved to OY2 ODCs. Funds set aside for OY2 ORISE will be used toward an additional OY2 1.0 FTE Biomedical Associate.

5.1.4 FY22Q4:

Risk: The surgical instruments and protocol-specific supplies that were ordered earlier in the year have not yet been received, which has resulted in a delay in commencing the model refinement phase of the study.
Mitigation Plan: Utilize CIRS training animals for PI to train surgical residents so they can perform surgeries in lieu of PI.
Update: Until all the necessary supplies have been received, any scheduling discussions at the CIRS facility will not be considered. During the discussion, it was communicated that the facility can no longer support four swine surgeries per day, but only up to two per day. This is different from the initial agreement reached between the PI and CIRS Facility. Consequently, the model refinement project overview and timeline had to be revised to account for the changes in facility support (Appendix A). This has also resulted in a modification to the full pre-clinical approach and timeline (Appendix B).

5.1.5 FY23Q1:

Risk: Owing to the availability of the CIRS schedule and the revision to be only being able to support a single swine surgery per operating day, it is not possible to complete the surgical portion of the biostasis swine study before the end of the PoP. Consequently, this study has been discontinued, and the tail end funding (FY23-24) has been suspended.
Mitigation Plan: A mitigation plan was formulated for the biostasis swine study, which involves the use of an alternative animal model along with a comprehensive research strategy plan (including experimental design) scheduled for September 2022, as well as a project timeline in October 2022 (refer to Appendix C). The technical team has been trained and is ready to execute these studies.
Update: Considering uncertainty regarding the acceptance of the alternative plan by the sponsor and pending IACUC approval, it was ultimately decided to discontinue the project entirely. The concern was that it would not be possible to complete the project by the September 2024 deadline due to potential delays caused by sponsor and IACUC approval.

5.2 Technical Challenges

In the model development, there was a challenge in developing a closed loop perfusion system for the isolated limb. The exposure and achievement of ischemia in the hind limb was performed without significant difficulty. Once the arterial and venous systems were cannulated, to create a closed loop system, there were challenges with maintaining consistent perfusion of the due to the lack of volume in the limb. There was also difficulty maintaining cannulation of the vessel due to the arterial and venous anatomy.

6.0 TRANSITION PLAN

6.1 Military Relevance:

As the US engages in conflicts with more sophisticated adversaries, the time to mitigate hemorrhage and transport injured individuals to medical facilities for lifesaving treatment is expected to increase. In such austere military settings, the traditional approach to preserving extremities has been limited to controlling hemorrhage and replacing blood loss. However, in resource-limited environments, immediate blood loss replacement may not always be feasible, resulting in an increased ischemic insult to the extremity through tourniquet use. To address these challenges, this project proposes a novel approach to reducing limb ischemia by inducing biostasis early on, thereby limiting metabolic processes. The goal is to evaluate the potential of biostasis compounds for extremity preservation, with the aim of reducing limb loss and improving patient outcomes in high-risk military settings. The novelty of this approach lies in the fact that current treatments for extremity vascular injury primarily focus on blood flow restoration. However, the induction of biostasis offers a new avenue for managing casualty care, as it has the potential to stabilize patients and reduce the need for immediate blood loss replacement. This, in turn, may increase manpower assets in high volume patient situations, allowing medics to attend to others until patient transfer occurs. Moreover, the evaluation of biostasis compounds and their potential implementation on a systemic scale could further enhance patient outcomes. Biostasis has the potential to stabilize patients, allowing medics to attend to multiple casualties, thereby increasing manpower in critical situations. Ultimately, the development and evaluation of biostasis compounds can lead to the reduction of limb loss and improved survival rates in austere military settings.

6.2 Transition Strategy:

It is anticipated that at the end of these studies, at least one biostasis compound will be identified and worthy of advanced development. Although regulatory approval processes are lengthy, this pioneering research must be conducted in partnership with early, mid, and late developers to ensure that there is a clear path to product transition; therapeutic batch scaling up and conceptualization and more complex systemic testing is expected to occur and be completed within 3 years of this project closure. Progress reports will be completed regularly to keep services apprised of breakthroughs and risks associated with the project.

Successful completion of these experiments will serve as a launchpad for further exploration of biostasis compounds as feasible strategies for preservation of limb and life. This pioneering research must be conducted in partnership with early, mid, and late developers to ensure that there is a clear path to product transition; therapeutic batch scaling up and conceptualization and more complex systemic testing is expected to occur and be completed within 3 years of this project closure. Progress reports will be completed regularly to keep services apprised of breakthroughs and risks associated with the project. Furthermore, we will work with our industry partners to obtain additional data to pursue regulatory approval for such therapeutics by the US Food and Drug Administration. Additional proposals will be submitted to opportunities such as Congressional Derived Medical Research Program calls and Joint Program Committee 6 (Combat Casualty Care) to leverage other resources for continuation of biostasis prototype development. TRL Level III. TRL/KRL Start and Completion Date: 25-60 months from completion.

7.0 RESULTS

The project team faced several challenges which prevented them from executing the project the end of the period of performance following the expiration of FY22 funds. These challenges included delays in contracts and Institutional Animal Care and Use Committee (IACUC) approvals, as well as difficulties in post-doctoral fellow recruitment and acquiring necessary equipment. In addition, unforeseen scheduling issues at the facility, which could have been related to other research projects or staffing shortages, may have further complicated matters. As a result, the study was closed, and future funding calls will be assessed for additional funding for a more feasible animal model. Despite the challenges, Science & Technology has acquired surgical instrumentation that would enable the project to be carried out if funding were secured. However, at present, no scientific results are available for this project.

8.0 CONCLUSION/DISCUSSION

The challenges we encountered during the execution of this project have provided us with valuable insights into our internal processes, allowing us to enhance our strategies and minimize missed deadlines and contract delays in the future. Nevertheless, certain obstacles that arose during the project were unexpected and beyond our control. This study represents an exceptional opportunity to close crucial gaps in our understanding of the current standard-of-care resuscitation practices for combat casualties, particularly in the context of traumatic vascular injury to the extremity and limb salvage. Given the increasing prevalence of traumatic injuries in the military, the ability to save limbs and prevent amputations is of utmost importance. This study aims to identify the most effective resuscitation techniques and provide evidence-based recommendations for improving limb salvage rates and overall patient outcomes. Considering the investments in this study, we plan to evaluate future funding calls and seek additional funding to ensure its successful completion and achievement of its subsequent objectives. By advancing the knowledge of standard-of-care resuscitation practices for combat casualties, we can improve the care provided to our service members and ultimately save more lives.

9.0 DELIVERABLES

9.1 Publications: N/A

9.2 Presentations: N/A

10.0 COST

Total funding for this study: $2,109,850.00

11.0 REFERENCES

1. S Cuzzocrea, DP Riley, AP Caputi, D Salvemini, Antioxidant Therapy: A New Pharmacological Approach in Shock, Inflammation, and Ischemia/Reperfusion Injury Pharmacological Reviews. March 1, 2001. Vol 53(1) 135-159
2. Nimesh S.A. Patel, Edward J. Sharples, Salvatorre Cuzzocrea, Prabal K. Chattergee, Domenico Britti, Muhammad M. Yaqoob, and Christoph Thiermmeman. Pretreatment with EPO reduces the injury and dysfunction caused by ischemia/reperfusion in the mouse kidney in vivo Kidney International, Vol. 66 (2004), pp. 983–989
3. Shimoda M, Sawada T, Iwasaki Y, Mori S, Kijima H, Okada T, Kubota K.Erythropoietin strongly protects the liver from ischemia-reperfusion injury in a pig model. Hepatogastroenterology. 2009 Mar-Apr;56(90):470-5.
4. Deyan Mihov, Nikolay Bogdanov, Beat Grenacher, Max Gassmann, Gregor Zünd, Anna Bogdanova, Reza Tavakoli. Erythropoietin protects from reperfusion-induced myocardial injury by enhancing coronary endothelial nitric oxide production. *Eur J Cardiothorac Surg* 2009; 35:839-846.
5. Dejan Kupnik • Štefec Grmec. Erythropoietin in post-resuscitation neurological recovery: is there light at the end of the tunnel? Signa Vitae 2007; 2(1): 11 - 14
6. Ates E, Yalcin AU, Tilmaz S, Koken T, Tokyol C.Protective effect of erythropoietin on renal ischemia and reperfusion injury. ANZ J Surg. 2005 Dec;75(12):1100-5.
7. Bakan V, Ciralik H, Tolun FI, Atli Y, Mil A, Oztürk S. Protective effect of erythropoietin on torsion/detorsion injury in rat model.J Pediatr Surg. 2009 Oct;44(10):1988-94.
8. Faikah Gueler, Joon-Keun Park, Song Rong, Torsten Kirsch, Carsten Lindschau, Wen Zheng, Marlies Elger, Anette Fiebeler, Danilo Fliser, Friedrich C. Luft, and Hermann Haller Statins Attenuate Ischemia-Reperfusion Injury by Inducing Heme Oxygenase-1 in Infiltrating Macrophages. Am J Pathol. 2007 April; 170(4): 1192–1199.
9. Sabine Steffens and François Mach. Drug Insight: immunomodulatory effects of statins—potential benefits for renal patients? Nature Clinical Practice Nephrology (2006) 2, 378-387
10. Semenas, Egidijus & Nozari, Ala & Wiklund, Lars. (2010). Sex differences in cardiac injury after severe haemorrhage and ventricular fibrillation in pigs. Resuscitation. 81. 1718-22. 10.1016/j.resuscitation.2010.08.010.

12.0 FIGURES AND TABLES:

13.0 LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMS

ABGs	Arterial Blood Gases
ADP	Adenosine Diphosphate
ASI	ABSS Solutions Inc.
ATP	Adenosine Triphosphate
Caco-2	Human epithelial colorectal adenocarcinoma cell line
CMAP	Compound Muscle Action Potential
CIRS	Clinical Investigator Research Support
DARPA	Defense Advanced Research Projects Agency
DNA	Deoxyribonucleic Acid
ECG	Electrocardiogram
ETCO2	End-Tidal Carbon Dioxide
FDA	U.S. Food and Drug Administration
FTE	Full-Time Equivalent
FY	Fiscal Year
GLM	Generalized Linear Model
H&E	Hematoxylin and Eosin staining
IACUC	Institutional Animal Care and Use Committee
IRB	Institutional Review Board
IRI	Ischemia Reperfusion Injury
KRL	Knowledge Readiness Level
µM	Micromolar (unit of concentration)
NCV	Nerve Conduction Velocity
n	Number of subjects or samples in a group
ODCs	Other Direct Costs
PaO_2	Partial Pressure of Oxygen
PoP	Period of Performance
RNA-seq	RNA Sequencing
SOPs	Standard Operating Procedures
TRL	Technology Readiness
VPS	Vascular Perfusion Solutions
WC	Wyss Compounds