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TITLE: Supercooled Ex-Vivo Porcine VCA Preservation to Extend the Timeline Between Procurement and Transplantation and Enable Tolerance Induction to Eliminate Immunotherapy Needs and Risks

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Supercooled Ex-Vivo Porcine VCA Preservation to Extend the Timeline Between Procurement and Transplantation and Enable Tolerance Induction to Eliminate Immunotherapy Needs and Risks

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

Massachusetts General Hospital
55 Fruit St Boston MA 02114

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

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Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The project aims to develop a novel technology to preserve vascular composite allografts for extended periods. This project uses a porcine model.

In the second year the focus was on identifying ideal perfusion parameters and initiating transplant studies.

15. SUBJECT TERMS
Organ Preservation, VCA transplantation, limb transplantation, supercooled storage

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a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	16	19b. TELEPHONE NUMBER *(include area code)*

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10. INTRODUCTION: *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

Enabling prolonged preservation of vascularized composite allografts (VCA) is critical to enable their use in a practical manner clinically. Machine perfusion technologies have enabled dynamic organ storage for many organs, in stark contrast to the current gold standard of static cold storage. Supercooling technology, which builds on machine perfusion, has been shown to further extend preservation, allowing the increase of viable preservation time to 27 hours for human livers, 3 times the clinical average. This project aims to translate these exciting results in livers to VCA, also leveraging prior studies in rats.

11. KEYWORDS: *Provide a brief list of keywords (limit to 20 words).*

VCA, preservation, supercooling, cryopreservation, transplantation, machine perfusion, Ischemia Reperfusion Injury

12. ACCOMPLISHMENTS: *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?
List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

1. *Milestone # 1 ACURO approval obtained*. **100% complete** (February 2020)
2. *Milestone #2 Complete evaluation of Machine perfusion on VCA viability.* **80% Complete**
3. *Milestone #3 Develop a method to extend preservation duration for porcine limbs.* **50% Complete**
4. *Milestone #4 Develop a method to enable using mixed chimerism for VCA transplantation.* **10% Complete**

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

We started the project by scaling up from our prior experience in rats to a large animal limb ex vivo perfusion system.

1) Ex vivo subnormothermic machine perfusion (SNMP): Development of an Optimized protocol for 24-hr preservation of swine hindlimbs

12 swine hind limbs were procured from Yorkshire pigs during terminal procedures in the Knight surgery operative room, with about 20minutes warm ischemia during recovery. They were then subjected to different perfusion protocols. Perfusion parameters in each protocol are displayed in Figure 1.

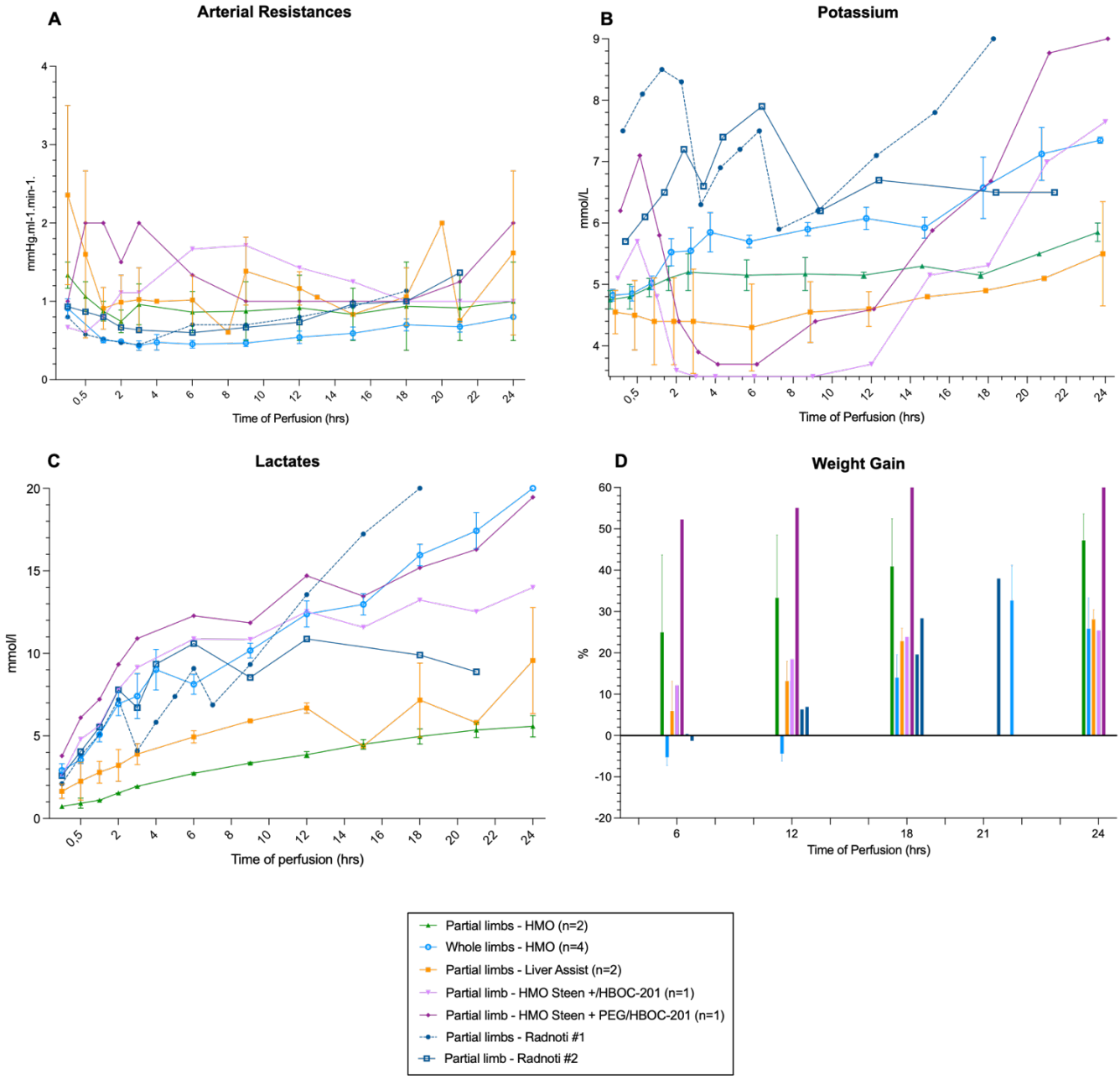

Figure 1. Perfusion parameters observed while optimizing the subnormothermic perfusion protocol to swine hindlimbs.

Two porcine partial limbs were first perfused with a system of our design including a glass oxygenator (Radnoti LLC, Covina, CA), same as used for the perfusion of rat limbs. One (Partial limb - Radnoti #1) had a recirculating volume of 500ml of Steen+ for an initial limb weight of 476g. The massive release of potassium and lactate required us to perform two media exchanges, each with 500ml of

perfusate after 2.5 and 6 hrs of perfusion. Excessive potassium and lactate concentrations of >9mmol/L and >20mmol/L respectively after 18 hours (Fig. 1 B-C) ultimately led to discontinuation of the perfusion. For the next iteration, we therefore increased the perfusate volume to 1L for a 458g limb. Potassium and lactate concentrations were still high. To prevent further increases, we added 500ml of perfusate at 2.5hr (total circulating volume 1.5L), followed by two exchanges of 1L at 6 and 12 hrs and one of 500ml at 18 hrs. The perfusion was stopped at 21 hours due to non-viable parameters and significant edema.

Since this system did not meet the metabolic needs of a porcine limb at 21°C, we made changes: for the next experiment the addition of an oxygen carrier (HBOC-201, Hemopure®) was tested along with a hollow fiber membrane oxygenator. Simultaneous perfusion of 2 limbs was performed, with the limbs weighed 632 and 629g. Two perfusion media were tested, the first one with Steen+ as before with HBOC-201, the second one with modified concentrations of 12% BSA (vs. 15%) and 2% PEG (vs. 0.5%). For both limbs, medias exchanges (350ml of perfusate) were performed at 6, 12, and 18hrs. In both perfusions, initial potassium concentrations were very low (Fig. 1 B), but lactate, resistances, and weight gain still indicated a limb in distress. The 12% BSA solution was worse in terms of weight gain.

Thus, we made a further change in the limb perfusion system, replacing the Radnoti oxygenator with a Medtronics® Hollow fiber Membrane Oxygenator (HMO). The first 2 perfusions with this system, performed with 2L of Steen+ without need for media exchange, produced very encouraging results, with stable resistances, low potassium and lactate concentrations throughout the experiment. However, edema was still significant at >30%.

2) Study of the impact of the surgical model on the perfusion protocol

To identify the source of edema, we next performed 4 whole limb perfusions to determine whether the edema was related to the surgical design that suppresses distal limb microcirculation. The weight of the entire limbs was much greater and averaged 1200g for the first experiment. Both limbs were procured from the same pig and perfused with our custom-designed Machine Perfusion (MP) system (Figure 2), starting with a perfusate volume of 2L. A single 1L exchange was performed after 5-hr SNMP. In this experiment, final weight gain was 18 and 33% with low resistances, but potassium and lactate concentrations were high and well above the acceptable ranges (K+ 7.5mmol/L; Lactate > 20mmol/L). Hence, for the next bilateral limb perfusion we perforemed more perfusate exchanges to counter the toxic metabolites accumulation. Using 2L of perfusate in the circuit, we did a 50% perfusate exchange at 2hrs, and 25% at 6, 12 and 18 hours. Edema was low during the first 12 hours (Figure 1D), but potassium and lactate release were still significant (Figure 1B-C). As a result, we concluded that with this model, the amount of perfusate that would have been required to maintain good parameters was too large for our system and not cost-effective.

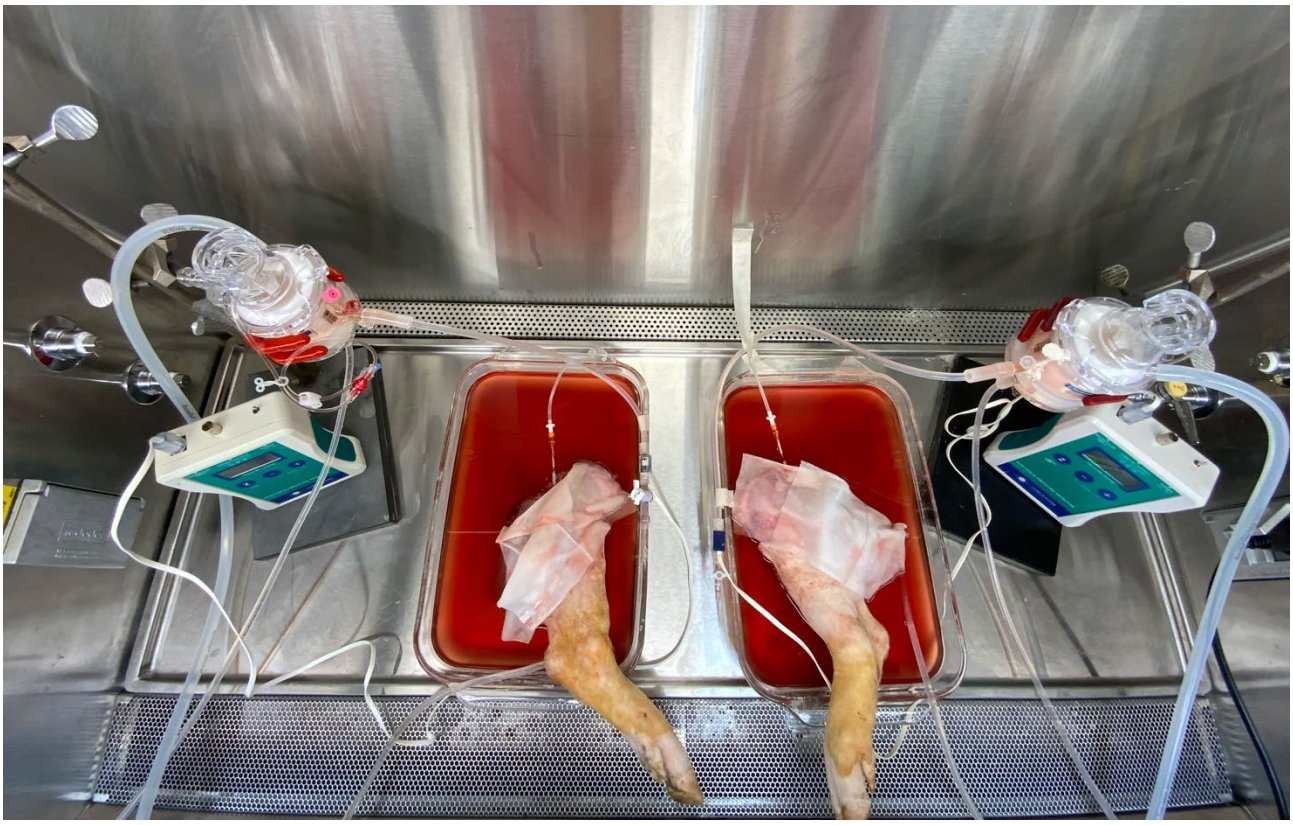

Figure 2. 24-hr SNMP of whole limbs harvested from Yorkshire pigs. *2 liters of Steen+ recirculating in the circuit.*

3) Role of the pulsatile flow in ex vivo perfusion of porcine partial limbs

We then studied the impact of pulsatily as a source of endothelial injury, and hence swelling, (in the Liver Assist® device – Fig. 3A) versus continuous flow (in our custom-built MP, Fig. 3B) on VCA perfusion preservation. Two partial hindlimbs procured from 20-30kg Yorkshire pigs from the same donor and placed on the liver assist or the custom device. We used our HMO oxygenated Steen+ perfusion solution. The weight of recovered limbs in each group was comparable. In each group, the limbs were placed on perfusion after approximately 16 minutes of cold ischemia (transportation to the perfusion system). Perfusion parameters are shown in Figure 4.

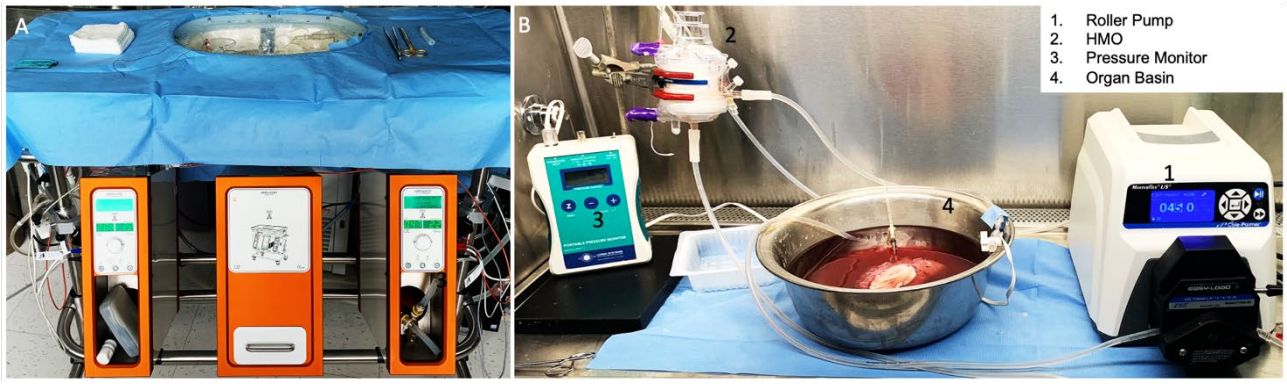

Figure 3. Comparison of limbs from same donor with pulsatile vs. continuous perfusion. *Left: pulsatile flow at 60bpm with Organ Assist perfusion device. Right: Continuous perfusion with Custom Built Machine Perfusion device featuring a Hollow fiber Membrane Oxygenator.*

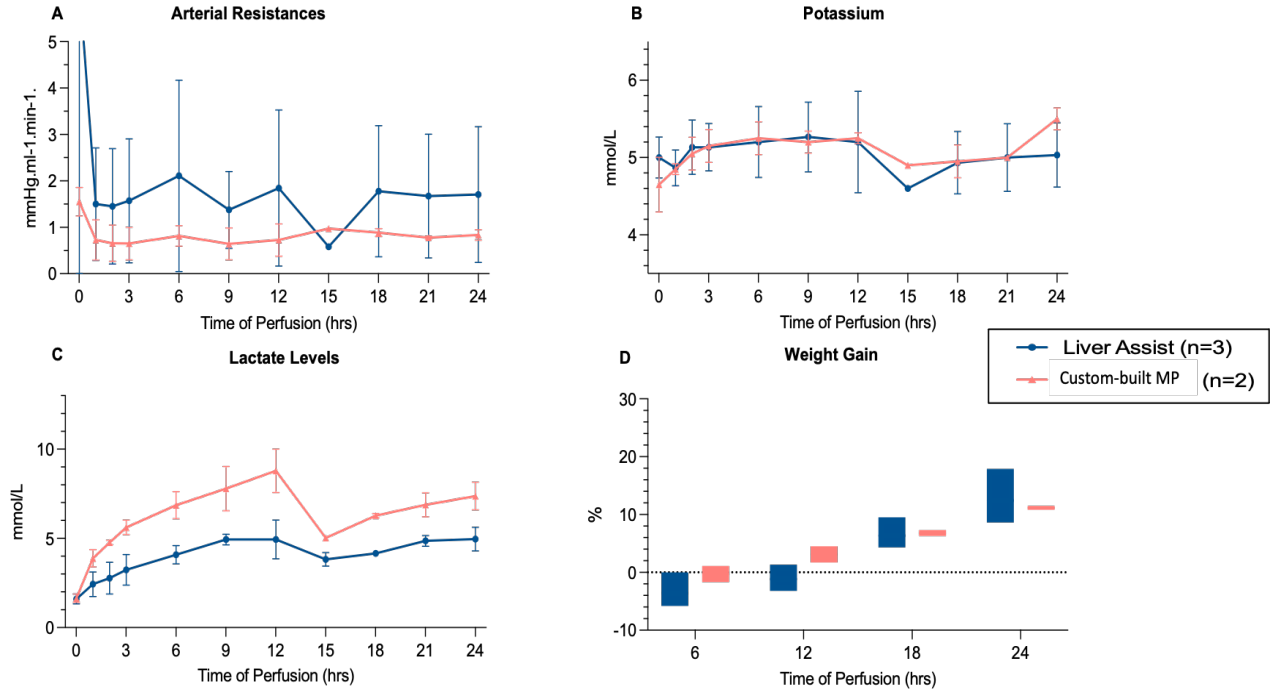

Figure 4. Optimized Continuous (Custom MP) vs. Pulsatile (Liver Assist) perfusion parameters. *These graphs represent the last three experiments where the limbs were perfused simultaneously, one on the custom-built MP, and the contralateral on Liver Assist. Only two replicates are reported for the MP, since one perfusion had to be stopped after 12 hours due to a tissue embolism in the system.*

With this final optimization of our system, protocol and media, we were able to achieve edema limited to about 10% in both systems after 24 hours of perfusion. Edema was not different in either protocol ($p=0.71$). Resistances were also more stable in our custom system. Potassium concentrations were the same in both systems ($p > 0.05$ at each timepoint), lactates slightly higher in the custom-buit MP at 3 hour time point ($p = 0.008$) but remaining comparable at all other measurements ($p > 0.05$).

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

If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Two post-doctoral research fellows were trained. Training topics included surgical techniques of partial hind limb harvest in a swine model (by attending plastic surgeon Dr Lellouch and Vice Chair of MGH IACUC Mark Randolph), machine perfusion, as well as scientific writing, experimental design, and various data analysis techniques.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Pulsatile vs Continuous Flow in Swine Hindlimb Preservation using Subnormothermic Machine Perfusion
P. Tawa, M. Goutard, R.J. de Vries, A. G. Lellouch, G. Romano, V. Pozzo, S. Maggipinto, L. Lantieri, M. A. Randolph, C. L. Cetrulo, Jr., K. Uygun.
Submitted to the American Society for Reconstructive Transplantation Meeting – November 2021

A Simplified Perfusion Protocol for 24-hr VCA Ex Vivo Preservation in a Swine Limb Transplantation Model
M. Goutard, P. Tawa, R.J. de Vries, A. G. Lellouch, G. Romano, V. Pozzo, C. Pendexter, S. Maggipinto, L. Lantieri, S. N. Tessier, M. A. Randolph, C. L. Cetrulo, Jr., K. Uygun.
Submitted to th American Society of Reconstructive Microsurgery Meeting – January 2022

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

The immediate next step will be to peform VCA transplants after 24-hr ex vivo perfusion on our custom-built system. Controls will be 24-hr cold storage control transplants and fresh control transplants. Preliminary data will be gathered for the supercooling of swine limbs simultaneously with our ongoing study on rat limb supercooling.

4. **IMPACT:** D*escribe 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?
If there is nothing significant to report during this reporting period, state "Nothing to Report." Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

The key accomplishment is the development of a novel swine limb machine perfusion protocol for VCA 24-hr preservation without major edema. This is a 4x increase in viable preservation of limbs at the large animal scale based on literature.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

As an interdisciplinary project, the results are expected to have impact on the fields of plastic surgery, transplantation, biopreservation and medical systems engineering.

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Licensing of patents previously developed in project W81XWH-17-1-0680, precursor to this project, are in discussion. We also expect new IP may result from this work, or alternatively the data will supporting prior patent applications.

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to report.

5. **CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

Changes in approach and reasons for change
Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Covid19 crisis led to major delays in large animal work. Accordingly we requested a change in project SOW to reduce transplant experiments and focus on the perfusion milestones, which was approved in May 2021. No significant changes has been necessary after that point.

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

As noted in the SOW change request which was approved, There is some risk that the supercooling protocol will not be ready in time for the pig limb transplants. If that is the case, we will perform extended preservation with perfusion. There is strong literature evidence that perfusion itself reduces immunogenicity https://pubmed.ncbi.nlm.nih.gov/30518863/

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing additional to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Not applicable.

Significant changes in use or care of vertebrate animals

Nothing to report.

Significant changes in use of biohazards and/or select agents

Nothing to report.

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

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Two publications being prepared for submission based on the meeting abstracts noted above in the dissemination section. A third small article on method development is under consideration

  Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report.

  Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Nothing to report yet.

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

- Nothing to report.

Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

A novel protocol for swine limb ex vivo perfusion preservation was developed.

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to report.

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:
  - *data or databases;*
  - *physical collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

Nothing to report.

7. 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: Curtis Cetrulo, MD
Project Role: Co-Principal Investigator
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 1
Contribution to Project: Dr. Cetrulo is responsible for overall design and direction of proposed studies, and interpretation of results.

Name: Mark Randolph, MASc
Project Role: Co-Investigator
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 1
Contribution to Project: Dr. Randolph is responsible of the animal surgeries and the interpretation of results

Name: Alec Andrews
Project Role: Research Technologist
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 5
Contribution to Project: Mr. Andrews assists the PI and study staff in all aspects of study coordination.

Name: Alexandre Lellouch, MD
Project Role: Research Fellow
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 3
Contribution to Project: Dr. Lellouch is leading the surgeries and participates in the interpretation of results.

Name: Hayshem Lancia
Project Role: Graduate Assistant
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 12
Contribution to Project: Mr. Lancia is assisting with limb recovery surgeries

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

See attached Other support documentation. No effects on the effort in this project.

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:

Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

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

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

See attached quad chart.

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

No additional document to report.