AWARD NUMBER: W81XWH-17-1-0680

TITLE: Development of a Supercooled Limb Preservation Protocol

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REPORT DATE: October 2018

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
October 2018	Annual	1 Sep 2017 - 31 Aug 2018

4. TITLE AND SUBTITLE

Development of a Supercooled Limb Preservation Protocol

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-17-1-0680

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

The project aims to develop a novel technology to preserve vascular composite allografts for extended periods. This project uses a rat limb transplant model and aims to preserve grafts for 3days or more.

In this first year, we developed a machine perfusion protocol for rat limbs. Several aspects had to be optimized, including media, additives, oxygen carriers among others to identify a protocol that does not lead to ischemia, exhibits sustained low vascular resistance, and energy levels. We further started evaluation of the perfused grafts by transplantation with success in the first tests. We also started optimization of the supercooled graft preservation, which will be the main goal in year 2.

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

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

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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

Static cold storage (SCS) of allografts with University of Wisconsin (UW) solution at 4 degrees C is the current clinical standard in transplantation, but is limited to few hours for vascular composite allografts (VCAs). In our prior studies, our group develop a subzero nonfreezing (SZNF) preservation approach to extend storage of rat livers up to 3days. In this project, we aim to develop a similar SZNF preservation approach for rat limbs and demonstrate viability after 3 days or longer storage with transplantation.

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

Organ Preservation, VCA transplantation, limb transplantation, supercooled storage

3. **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. (Months 1-5) **100% complete**
2. Milestone #2 Complete evaluation of Machine perfusion on VCA viability. (Months 5-24) **75% complete**
3. Milestone #3 Establish a SZNF protocol for VCA tissues (Endpoint: Tissue Energy Charge). (Months 12-15). **30% Complete.**
4. Milestone #4 Identify limits of SZNF for rat limbs ((Endpoint: >80% transplant survival). (Months 12-36) **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 with building a ex vivo machine perfusion systems for rodent limbs. Figure 1 below displays the system and its components.

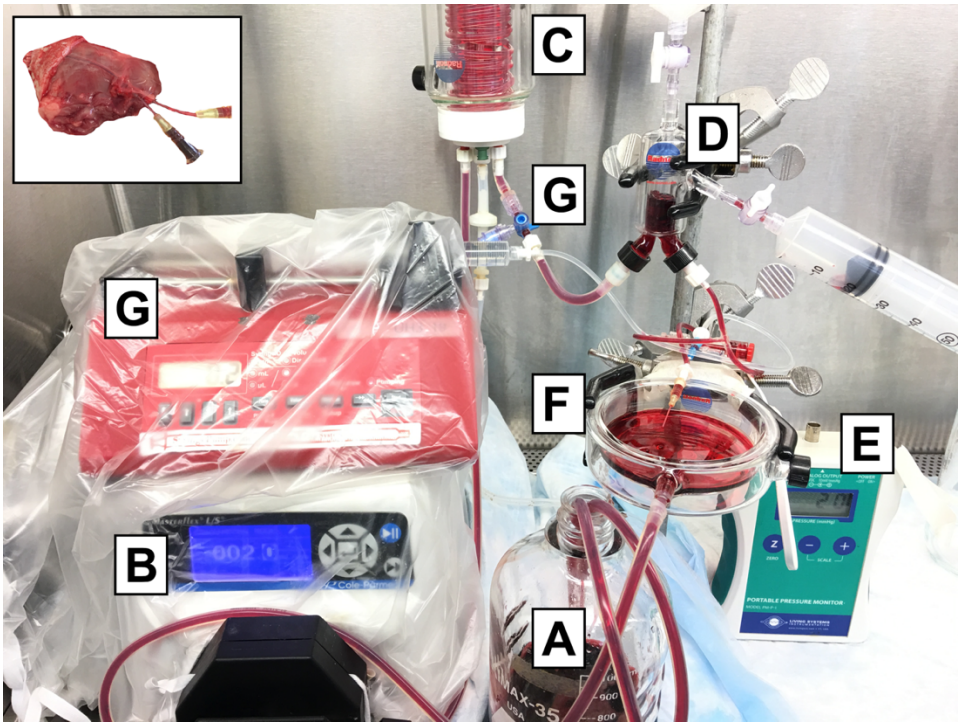

Figure 1. *Ex vivo* subnormothermic machine perfusion set up. The circuit consists of perfusion solution (A) that is pumped via a roller pump (B) to the oxygenator (C), that is oxygenated with a carbogen mixture (5% CO_2 and 95% oxygen). The solution then goes through the bubble trap (D) to prevent air bubbles going into the limb. The pressure is measured (E) at the level of the limb that is laying the basin (F). Inflow samples are measure at the inflow valve (G) with outflow samples are measured directly from the venous outflow canula (as shown in upper left panel).

Next, we tested several perfusates for optimum perfusion of the limbs. Please note that the process itself was iterative, and several steps of optimization and comparison are skipped for sake of brevity. Table 1 below shows the testing of two key additives, PEG, an oncotic agent used to minimize edema observed in prior iterations, and HBOC (hemopure), ax oxygen carrier which proved very beneficial likely by reducing hypoxia in the tissues as indicated by reduced lactate levels. Figure 2 below displays the results of perfusing rat hindlimbs with these perfusates. Use of base media with only albumin added as an oncotic agent led to edema, high vascular resistance, and sustained ischemia. Addition of BSA helped reduce these significantly, but the addition of HBOC was the critical step that eliminated edema and produce the best results of other indicators tested.

Table 1. Overview of machine perfusion solutions tested.

	Group 1 BSA n=4	**Goup 2 BSA + PEG n=4**	**Group 3 HBOC-201 n=4**
Solution base			
- PromoCell muscle media (mL)	500	500	375
- HBOC-201 (mL)	-	-	125
Differentiating additives			
- Bovine serum albumin (BSA) (g)	10	10	10

- Polyethylene glycol (PEG) (g)	-	15	15
- Prostaglandin (μL/min)	-	-	0.2
Additional supplements			
- Penicillin-Streptomycin (mL)	2	2	2
- L-glutamine (mL)	5	5	5
- Insulin (μL)	100	100	100
- Heparin (mL)	1	1	1
- Hydrocortisone (μL)	100	100	100
- Dexamethasone (μg)	8	8	8

BSA = bovine serum albumin, PEG = polyethylene glycol and HBOC-201 = hemoglobin based oxygen carrier-201.

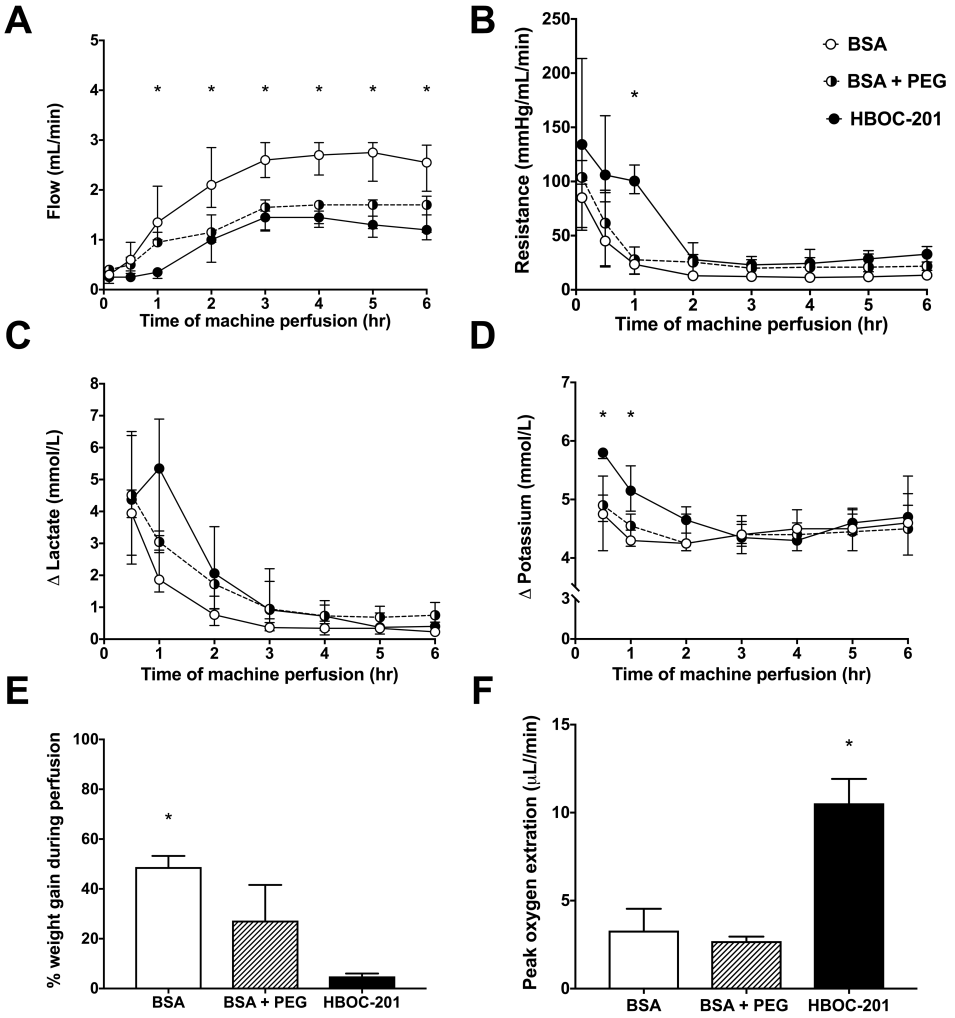

Figure 2. Overview of perfusion parameters. In all groups, arterial flow increased while vascular resistance decreased over the course of perfusion (Panel A&B). Lactate levels peaked during the first hour of perfusion and decreased thereafter (Panel C). Potassium levels peaked during the first hour of perfusion and stabilized thereafter (Panel D). Weight gain was calculated the difference compared to baseline (Panel E). Oxygen extraction was significantly higher in the HBOC-201 group (Panel F). Abbreviations

used: Abbreviations used; BSA = bovine serum albumin, PEG = polyethylene glycol and HBOC-201 = hemoglobin based oxygen carrier-201.
To evaluate the viability of the grafts perfused with the cocktails tested, we assessed the tissue energy charge [defined as Energy Charge = *(ATP + 1/2ADP)/(ATP + ADP + AMP)]*. As shown below, all perfusion groups led to increased energy charge compared to static cold storage, and levels statistically same as in vivo. While not statistically different from other perfusates, the HBOC group was the highest.

We are now in the process of testing the HBOC based perfusion protocol with transplantation. Figure 4 below displays the results from the first few transplants, where perfused grafts survive for one week post transplant similar to fresh controls, indicating viability.

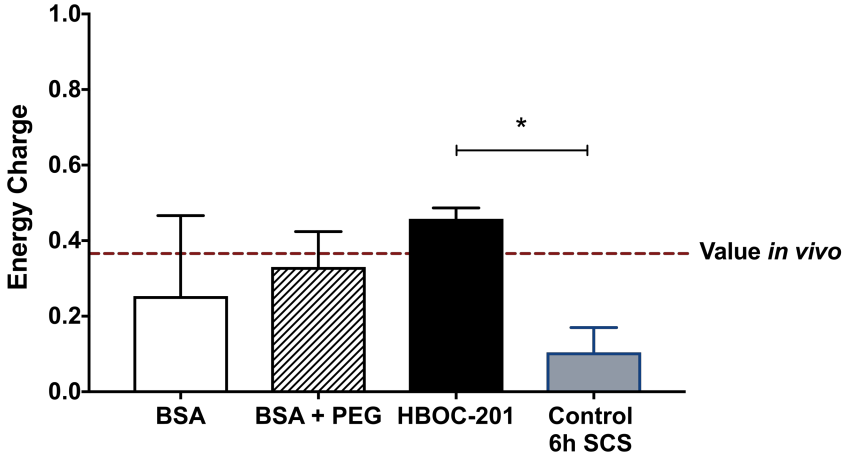

Figure 3. Energy charge. Energy charge ratios of SCS control limbs were significantly lower compared to HBOC-201 perfused limbs, but not BSA and BSA + PEG limbs (P = 0.002) respectively. The red dotted line indicates median energy charge levels *in vivo*. Abbreviations used: Abbreviations used; BSA = bovine serum albumin, PEG = polyethylene glycol and HBOC-201 = hemoglobin based oxygen carrier-201.

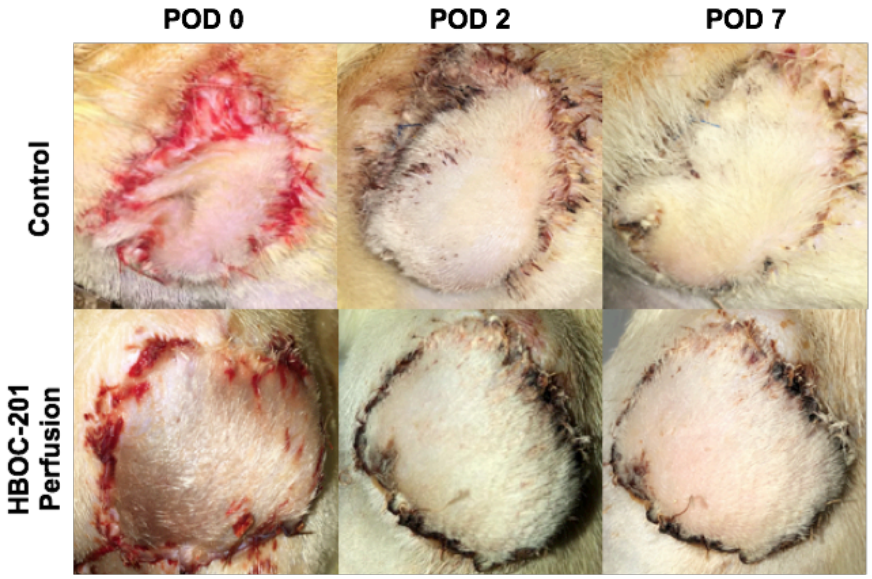

Figure 4. Heterotopic hind limb transplant grafts on post-operative days 0, 2 and 7. Post-operative follow up of heterotopic hind limb transplant grafts. Abbreviations used; HBOC-201 = hemoglobin based oxygen carrier-201, POD = post-operative day.

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.

Fellows and technicians have received microsurgery training from Dr. Randolph including operating under magnification. Fellows did participate in writing of meeting abstracts, progress reports, manuscript drafts, as well as preparation and presentation of oral and poster talks at meetings.

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.

We presented the results of this research in three meetings relevant to the field. The first manuscript describing the results of perfusion protocol development is in process of being submitted (expect submission in November 2018). We also published a review article. See *Products* below for detailed list.

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 plan for year 2 includes testing of the limb perfusion protocol by transplantation. Depending on the graft survival, the need for further optimization of the protocol will be evaluated.

The second component for year is the development of the subzero preservation protocol. The first step is optimize the supercooling protocol, which is already in process. We will perform initial screening of the protocol based on tissue energy charge, and then test extending the duration ex vivo. Year 3 will focus on testing and further optimizing this protocol with transplants.

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 rational development of a limb perfusion protocol. As detailed above, the process involved starting with the liver perfusion protocol, then iteratively addressing its shortcomings and adopting to rat limbs. To our knowledge this is the first study in literature that follows such a rigorous development process, and is expected to be a key technology development in the field of VCA preservation. We are also evaluating filing new IP based on this development.

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.*

We are currently evaluating filing new IP based on the results. While discussions with industry have not taken place yet, we plan to initiate discussions after filing.

What was the impact on society beyond science and technology?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

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. We expect to have such impact after the first set of publications, and especially once the technology is scaled up to large animal or human VCA tissues.

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.

Nothing to Report.

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.

There was a slight (1.3 months) delay in obtaining ACURO approval in the prior quarterly report. We have mostly caught up with this, thanks to advance planning and ordering of supplies necessary for perfusion.

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.

Lab supplies for preservation appear to cost less than anticipated; however we will need a new surgical microscope to perform the transplants. A separate application for budget change request will be filed.

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).*

Burlage LC, Tessier SN, Etra JW, Uygun K, Brandacher G. Advances in machine perfusion, organ preservation, and cryobiology: potential impact on vascularized composite allotransplantation. Curr Opin Organ Transplant. 2018;23(5):561-7. DoD support acknowledged.

  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.*

1. Lellouch AG, Karimian N, Ng ZY, Mert S, Geerts S, Uygun K, Cetrulo CL. Ex-vivo Subnormothermic Oxygenated Machine Perfusion of Swine Forelimbs Enables Prolonged Graft Preservation Prior to Transplantation. Plastic and Reconstructive Surgery-Global Open. 2016 Sep 1;4(9S):55-6.
2. Burlage L, Lellouch A, Tessier S, Pendexter C, Cronin S, Schol M, Randolph M, Porte R, Lantieri L, Cetrulo C, Uygun K. Ex-Vivo Subnormothermic Oxygenated Machine Perfusion of Rodent Hindlimb: Feasibility Study to Extend Preservation Time of Vascularized Composite Allograft. American Journal of Transplantation 2018 Jun 1 (Vol. 18, pp. 763-763).
3. Lellouch SAG, Tessier SN, Cronin SE, Schol IM, Pendexter CA, Randolph MA, Lantieri L, Uygun K, Cetrulo C. Optimization of ex-vivo Subnormothermic Oxygenated Machine Perfusion in Vascularized Composite Allograft on Rat to Prolong Preservation Duration. Journal of Burn Care & Research. 2018;39(S1):S44-S.

- **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.

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

A novel protocol for limb machine perfusion 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, however an IP disclosure is being prepared.

- **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".

Example:

Name:	*Mary Smith*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	*1234567*
Nearest person month worked:	*5*
Contribution to Project:	*Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support:	*The Ford Foundation (Complete only if the funding support is provided from other than this award.)*

Name:	*Korkut Uygun*
Project Role:	*PI*
Nearest person month worked:	*1*
Contribution to Project:	*Co-led the project*

Name: *Curt Cetrulo*
Project Role: *Co-PI*
Nearest person month worked: *1*
Contribution to Project: *Co-led the project*

Name: *Mark Randolph*
Project Role: *Investigator*
Nearest person month worked: *1*
Contribution to Project: *Assisted in IACUC protocol development, experimental design, planning for animal studies, training of personnel in small animal surgery, anesthesia, limb transplantation.*

Name: *Alexandre Lellouch*
Project Role: *Fellow*
Nearest person month worked: *4*
Contribution to Project: *Led the graft recoveries and transplantations*

Name: *Shannon Tessier*
Project Role: *Fellow/Investigator*
Nearest person month worked: *1*
Contribution to Project: *Led development of the new perfusion cocktail*

Name: *Safak Mert*
Project Role: *Research Fellow*
Nearest person month worked: *1*
Contribution to Project: *Assisted in tissue viability assessment and biological assays*

Name: *Sonal Nagpal*
Project Role: *Technologist*
Nearest person month worked: *2*
Contribution to Project: *Assisted in development of the new perfusion cocktail & perfusion studies*

Name: *Peony Banik*
Project Role: *Research Technician*
Nearest person month worked: *7*
Contribution to Project: *Assisted in perfusion studies*

Name: *Sonal Nagpal*
Project Role: *Research Technician*
Nearest person month worked: *2*
Contribution to Project: *Assisted in perfusion studies*

Name: *Gaelle Gabrielle Anna Saviane*

Project Role: *Research Technician*
Nearest person month worked: *2*
Contribution to Project: *Assisted in limb transplants and assessment*

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 documents to report.