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TITLE: Subnormothermic Ex Situ Limb Perfusion Using Red Blood Cells and Plasma: Critical Component Analysis

PRINCIPAL INVESTIGATOR: Kagan Ozer, MD

CONTRACTING ORGANIZATION: University of Michigan
Arbor, MI 48109-1274

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6. AUTHOR(S)
KAGAN OZER, MD

E-Mail: kagan.ozer@uc.edu

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University of Michigan
3003 S. State St., Ann Arbor, MI 48109-1274

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13. SUPPLEMENTARY NOTES

14. ABSTRACT: Introduction: The long-term objective of this application is to keep vascularized composite tissues viable at or beyond 48 hours using ex-vivo perfusion systems. In this project, we used rat hind limb allograft transplantation model along with a miniaturized version of our custom-made ex-vivo perfusion system and tested red blood cell based perfusate solutions. **Methods:** Under SA1, we tested the efficacy of three plasma components in maintaining muscle force generation on limb allografts after 8 hours of SNT ex-vivo perfusion/transplantation. These components were separated based on their molecular weight (full, high molecular weight, and low molecular weight plasma) and mixed with red blood cells to perfuse limbs, which were then transplanted to another recipient orthotopically to observe limb survival as well as the long-term outcomes of muscle and nerve regeneration 3 months following transplantation. Outcome measures included analysis of the EMG data, muscle and nerve histology, and proteomic analysis of the perfusate. **Results:** Under SA1, recipients in the group perfused with RBC and HMW plasma proteins had severe reperfusion injury and did not survive beyond 3 days. In other groups, the addition of whole plasma did not appear to improve amplitude and latency on gastrocnemius and EDL muscles compared to immediately transplanted limbs. Groups perfused with RBC only and RBC with LMW plasma demonstrated excellent amplitude and latencies on all muscle groups with no significant difference compared to immediately transplanted limbs ($p>0.05$). The EDL muscle in these groups also generated similar tetanic and twitch forces compared to the immediate transplantation group ($p>0.05$). Under SA2, we used the best perfusate combination established under SA1 and perfused limbs following various ischemic intervals after the limb amputation and demonstrated protective effects of ex-situ perfusion up to 4 hours following ischemia compared to static cold storage. **Conclusions:** Ex-situ perfusion system at 20-22°C using red blood cells and low molecular weight plasma is superior to static cold storage in both transplantation and replantation setting.

15. SUBJECT TERMS
NONE LISTED

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1. INTRODUCTION:

The long-term objective of this application is to keep vascularized composite tissues viable at or beyond 48 hours using ex-vivo perfusion systems. To achieve this goal, we use ex-vivo perfusion systems to deliver oxygen and other nutrients to amputated vascularized composite tissue parts to maintain their survival and function. In this application, we propose to use various components of the human blood under subnormothermic (21-24□C) temperatures using established methods of pulsatile ex-vivo perfusion systems. There is no doubt that full blood is the best perfusate perfected after billions of years of evolution. Red blood cells are essential to carry the oxygen, and the plasma contains many proteins and growth hormones essential for endothelial stability. Both red blood cells and plasma are vital for oxygenation, energy production, and survival of tissues. The critical components of the plasma needed for survival of the tissue are not identified previously. Identification of these critical components will have the potential for indefinite survival of tissues, not only for VCAs, but also for other solid organs. Proposed research has a strong potential to be quickly translated into clinical practice as the machine perfusion systems and plasma separation technologies are already in place.

2. KEYWORDS:

composite tissue allograft, ex-situ, ex-vivo, in-situ, perfusion, reperfusion, ischemia, , machine perfusion, near-normothermic, sub-normothermic,

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Specific Aim1: To define the critical molecular weight proteins in plasma that maintains muscle force generation on limb allografts after 8 hours on subnormothermic ex-vivo perfusion and transplantation.
Specific Aim 2: To determine the effects of critical ischemia time on muscle force generation of limb allografts after 8 hours of subnormorthermic ex-vivo perfusion using red blood cells and plasma.
Both of these specific aims were completed within the proposed timetable. For SA1, all 3 subtasks were completed within 9 months from its start. For SA2, all 3 subtasks were completed within 18 months from its start. Both tasks were completed 100%.

What was accomplished under these goals?

Red blood cells based perfusates, synthetic oxygen carriers, and ex-situ perfusion at various temperatures (normothermic, near-normothermic, sub-normothermic, and hypothermic) have all been studied in solid organ transplantation models. Limb allografts due to their unique energy requirements, represent a challenge in terms of preservation as principles established in other organ system are not applicable to limb allografts. Briefly in this current study, we tested viability and functional recovery of limb allografts under various ex-situ perfusion temperatures and compositions. We used rat hind limb allograft transplantation model and a custom-made ex-situ perfusion system. Amputated limbs were perfused using a red blood cell based perfusate with added plasma proteins (full, low, and high molecular weight). Following 8 hours of ex-situ perfusion, limbs were transplanted to the recipient. All surviving recipients' muscle strength were tested in 3 months at the end of expected neuromuscular recovery to determine the overall functional outcome. In addition, we also assessed their muscle and nerve structure using histology. We performed 76 transplantations in 19 groups and collected over 100 data points on each transplant recipient including perfusate chemistry, survival, electrodiagnostic evaluation, muscle and nerve histology and muscle contractile force. Our findings can be summarized under the following headline.

1. Ideal perfusion temperature for limb allografts is sub-normothermic temperatures around 20 to 24 °C.
2. Static Cold Storage is detrimental to muscle architecture and nerve regeneration beyond 3 hours in this model.
3. At Sub-normothermic temperatures, the right balance between perfusate viscosity and effective microcirculation is achieved when red blood cell (RBC) was used alone as the perfusate or when they are mixed with low-molecular weight plasma.
4. Freshly obtained plasma through paracorporeal circulation did not result in significant changes in muscle force generation.
5. Plasma filtration system used in the study resulted in significantly improved survival, muscle and force generation compared to a system relying on plasma exchange.

There were some technical limitations of the study. First, current plasma filtration systems available to use in the laboratory setting separates protein molecules based on their molecular weight. Depending on the pore size of the filter, 2 groups of small and large size can be separated. This makes it challenging to identify protein molecules that play a role in endothelial stability. A more granular separation method is required for better results. Second, rat hind limb allograft transplantation model is the only small animal model allowing assessment of the neuromuscular regeneration. However, rats are difficult to keep under anesthesia for prolonged perfusion testing such as 24 hours. Despite these difficulties, our study findings will have a fundamental impact of limb preservation at the clinical setting.

Currently, any amputated part in the field is preserved using methods of static cold storage. This is detrimental to microcirculation as it has been demonstrated by us and others. In this study, we found that the dynamic machine circulation of an amputated part using RBCs at 20-24°C results in significantly better survival and function. This is the most ground braking finding with potential to change the current practice paradigm but will require manufacturing appropriate portable machines for limb perfusion.

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

s.

In general, rat hind limb allograft transplantation requires top level microsurgical skill with ability to anastomose vessels that are as small as 500 microns. Two fellows involved in these experiments have improved their surgical skills and currently matched in top ranking plastic and reconstructive surgery residency programs.

How were the results disseminated to communities of interest?

Results have been presented in two national meetings and will be submitted to two peer reviewed journals before the end of the year.

Viability and Functional Outcomes of Limb Allografts Following Sub-normothermic Plasma and Red Blood Cell Based Perfusion. Amir Dehdashtian MD, MPH; Erin Guy, BS; Anna E. Rieggers, BS; Keith Kozma; Paul S Cederna, MD; Stephen WP Kemp, PhD; Kagan Ozer, MD. American Society for Surgery of the Hand (ASSH) 77th Annual Meeting October 1st, 2022.

The Effects of Subnormothermic Plasma and Red Blood Cell Based Perfusion on the Function and Viability of Limb Allografts Amir Dehdashtian, Erin Guy, Anna Rieggers, Keith Kozma, Paul S Cederna, Stephen WP Kemp, Kagan Ozer. Plastic Surgery Research Council (PSRC), 65th Annual Meeting, June 8-12, 2022.

What do you plan to do during the next reporting period to accomplish the goals?

Nothing to report.

4. **IMPACT:**

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

From the practical standpoint, this study provides the basis for a new preservation method amputated parts following trauma. Traditionally, amputated hand and upper extremity parts are preserved using cold storage methods. These methods have limited longevity particularly if the part contains large sums of muscle as seen in amputations proximal to the wrist joint. In the light of the current study, amputated parts, once cannulated, can be connected to ex-situ perfusion device and can be perfused at room temperature. This will not only remove waste products from the circulation, but will also provide with the oxygen and other nutrients for longer time periods. In addition to this short-term gain, we anticipate developing a preservation solution, not just including red blood cells but also parts of the plasma. From that perspective, current study provides potential to generate longer survival times in mid and long-terms.

What was the impact on other disciplines?

Principles established in this study are directly applicable to transplantation of the vascularized composite tissue allografts including hand, face, abdominal wall, uterus, and penis. In addition, hand surgeons involved in replantation of amputated extremity parts will be able to perform emergent surgeries on an elective setting.

What was the impact on technology transfer?

Out study demonstrated the possibility to keep amputated limb parts viable for longer periods of time under sub-normothermic conditions. For these findings to be impactful in clinical practice, a portable ex-situ perfusion system should be manufactured for surgeons to use. At this point, we have not made any efforts to initiate a start-up company, also did not reach out to an existing one for manufacturing.

What was the impact on society beyond science and technology?

Nothing to report.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to report.

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

The most significant delay throughout the grant period was COVID. Like other institutions, we had to shot down for almost 12 months.

Changes that had a significant impact on expenditures

Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

Nothing to report

Significant changes in use or care of vertebrate animals

Nothing to report

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. PRODUCTS:

- **Publications, conference papers, and presentations**

- **Journal publications.**

Three manuscripts are currently in preparation and will be submitted before the end of the year.

Books or other non-periodical, one-time publications.

n/a

Other publications, conference papers and presentations.

Viability and Functional Outcomes of Limb Allografts Following Sub-normothermic Plasma and Red Blood Cell Based Perfusion. Amir Dehdashtian MD, MPH; Erin Guy, BS; Anna E. Rieggers, BS; Keith Kozma; Paul S Cederna, MD; Stephen WP Kemp, PhD; Kagan Ozer, MD. American Society for Surgery of the Hand (ASSH) 77th Annual Meeting October 1st, 2022.

The Effects of Subnormothermic Plasma and Red Blood Cell Based Perfusion on the Function and Viability of Limb Allografts Amir Dehdashtian, Erin Guy, Anna Rieggers, Keith Kozma, Paul S Cederna, Stephen WP Kemp, Kagan Ozer. Plastic Surgery Research Council (PSRC), 65th Annual Meeting, June 8-12, 2022.

- **Website(s) or other Internet site(s)**

n/a

- **Technologies or techniques**

n/a

- **Inventions, patent applications, and/or licenses**

Nothing to report

- **Other Products**

n/a

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: Kagan Ozer
Project Role: PI
Researcher Identifier (e.g. ORCID ID): 0000-0001-7397-3696
Nearest person month worked: Worked throughout the project
Contribution to Project: Oversaw all aspect of project execution, trained the postgrad student to perform experiments, analyzed preliminary results.

Name: Amir Dehdastian
Project Role: research fellow
Nearest person month worked: Worked throughout the project
Contribution to the project: Performed all microsurgical procedures, analyzed data, help perform EG testing and histology.

Name: Erin Guy
Project Role: research tech
Nearest person month worked: Worked throughout the project
Contribution to the project: Performed all microsurgical procedures, analyzed data, help perform EG testing and histology.

Name: Steve Kemp
Project Role: Co-PI
Researcher Identifier (e.g. ORCID ID) : 0000-0002-5608-3584
Nearest person month worked: Worked throughout the project.
Contribution to Project: Oversaw and analyzed EMG and nerve histology data,

Name: Jana Moon
Project Role: Research tech
Nearest person month worked: Worked throughout the project.
Contribution to Project: Performed EMG testing, helped to process nerve samples

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

None

What other organizations were involved as partners?

None.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

9. APPENDICES:

Please see detailed statistical analysis