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24-Hour Rat Hind Limb Preservation Using a 3D-Printed Subnormothermic Portable Machine Perfusion Device

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Abstract

Background: Currently, severed limbs after combat or traumatic injuries are preserved with cold ischemic storage. However, this method maintains limb viability for no more than 12 hours. In this study, a new device, referred to as the Universal Limb Stasis System for Extended Storage (ULiSSES™), was used to maintain rodent skeletal muscle viability for 24 hours, after 4 hours of ambient temperature ischemia.

Methods: Hind-limbs from 5 Sprague Dawley rats were recovered and allowed to lie on a counter for approximately 4 hours at room temperature (19-24ºC) to simulate delayed limb recovery. Limbs were then perfused for 24 hours using room temperature Krebs Henseleit solution. Arterial and venous pressure, flow, PaO_2, PvO_2, perfusate pH, and temperature were recorded hourly.

Results: Ambient ischemia time was 3.4 ± 0.5 hours. Perfusion pressure was 8.2 ± 2.0 mmHg with a mean flow to the limbs of 9.5 ± 5.0 ml/min. The pH and temperature of the KH perfusate were stable throughout preservation at 7.38 ± 0.05 and 23.7 ± 0.5ºC, respectively. Oxygen consumption reached a plateau of 0.28 ± 0.04 ml O_2/min/100 g by 17 hours with vascular resistance hovering around 1.0 ± 0.2 mmHg/ml/min initially, then declining by about 50% after 18 hours. Mean limb weight gain was 37.7 ± 31.8%.

Conclusions: ULiSSES™ appears to open the potential for stabilization and preservation of avulsed limbs for 24 hours or longer, leading to the feasibility of cost-effective transport from any recovery site to any re-plantation site with minimal tissue deterioration.

Keywords: Ex-vivo perfusion • Limb preservation • Machine perfusion • Limb preservation • Limb replantation • Perfusion • Machine perfusion technology • Portable devices • Military medicineh-homeomorphism • h-totally continuous functions • h-contra-continuous functions

Abbreviations: ABS: acrylonitrile butadiene styrene • ºC: degree Celsius • MP: machine perfusion • mmHg: millimeters of mercury • PaO_2: partial arterial pressure of Oxygen • PvO_2: partial venous pressure of Oxygen • ml: milliliter • min: minute • ULiSSES™: Universal Limb Stasis System for Extended Storage • g: grams • L: liter • Ga: gauge •KHB: Krebs-Henseleit Buffer

Introduction

Combat and mass casualty situations invariably produce a significant amount of soft tissue damage that requires application of a tourniquet, sometimes associated with extremity avulsion. Unfortunately, skeletal muscle will not remain viable for more than 6 hours at ambient temperatures following cessation of blood flow due to the onset of ischemia-induced necrosis. Tissue degradation ultimately leads to loss of cell homeostasis, releasing a multitude of factors that prime the tissue for free radical production, leading to irreversible ischemic and reperfusion injuries [1,2]. Thus, rehabilitation or replantation of large limbs that have experienced prolonged ischemia may result in a number of serious complications [2-6].

In mass casualty situations and particularly in the combat setting, many limbs may simultaneously present for evacuation. Current preservation methods may not provide sufficient time for collecting the extremities, stabilizing the patients, and transport to a medical facility with the resources needed for limb replantation. In most cases, especially at remote locations, extremities may need to be preserved for several hours or even days. The current method for preserving severed limbs is based on cold ischemic storage and is most often used because of its simplicity, low cost, and relative effectiveness. However, this method preserves limb viability for no more than 12 hours [7,8].

Ex-vivo machine perfusion (MP) for organ preservation has been effective at maintaining tissue viability for extended periods in hearts [9-11] kidneys [12-13] lungs [14], livers [15-17], and other vascularized tissue, such as limbs [18-20], in both normothermic and hypothermic environments. While the optimal perfusate for limb preservation is still debated, some studies report positive preservation and transplantation results using autologous blood[20] and others report low edema and optimal viability with acellular perfusates [21].

Machine perfusion devices reported in the literature tend to be complex, with common structural components: electric pumps, oxygenators, heat exchangers, filters, and an oxygen supply. However, MP devices that have been reported in the literature are not portable and not easily translatable for use in combat casualty settings.

Bunegin et al previously described an oxygenated MP system using acellular perfusates for the hypothermic preservation of canine hearts for up to 12 hours [10-21], and rodent kidneys for up to 24 hours[13]. These studies showed that stroke work by the heart after 12 hours and glomerular filtration rate in kidneys after 24 hours of hypothermic oxygenated perfusion were not statistically different from freshly recovered control organs.

In this proof of concept study, a limb-oxygenating MP device that evolved from our organ preservation work was used to provide limb preservation at sub-normothermic temperatures for 24 hours in a simulated limb avulsion combat setting, using a rodent model. The device, referred to as the Universal Limb Stasis System for Extended Storage (ULiSSES™), was used to maintain rodent skeletal muscle viability following 4 hours of ambient temperature ischemia. Muscle viability was inferred by measuring oxygen consumption in the experimental rat limbs during the perfusion period and

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comparing it to a reference value based on previously published measures of healthy, freshly recovered rodent hind limbs at similar temperatures.

Materials and Methods

Limb recovery

All limbs used in this study were recovered from animals that were euthanized in accordance with humane standards and compliant with the "Guide for the Care and Use of Laboratory Animals". The protocol was approved by an Institutional Animal Care and Use Committee (IACUC) at The University of Texas Health Science Center in San Antonio, Texas.

Hind-limbs from 5 Sprague Dawley rats weighing approximately 200 to 300 g were recovered 15 to 20 minutes following euthanasia. After making a circumferential incision at the upper to mid-thigh level, sharp dissection was used to separate the hind limb muscle groups from the pelvic girdle. The location of the femoral artery and vein were noted, followed by exposure of the femur. The femur was cut with a bone saw and the severed limb was allowed to lie on a counter for approximately 4 hours at room temperature (19-24°C) to simulate delayed limb recovery - similar to what might be expected during combat operations. After this resting period, limbs were weighed, followed by cannulation of the arterial and venous stumps with 22 Ga catheters. The arterial cannula was flushed with room temperature, heparinized (3,000 units/L) Krebs-Henseleit (KH) solution until the venous outflow was clear. The limb was then connected to the ULiSSES™ MP device using the 22 Gacatheters with the arterial connector attached to the ULiSSES™ head unit.

ULiSSES™ preservation device description

All parts of the ULiSSES™ MP device were 3D printed in acrylonitrile butadiene styrene (ABS) by an Airwolf 3D HD2X printer. The ULiSSES™ device consists of two parts (Figure 1): a fluid-filled container in which the limb is stored, and an oxygenation pumping head to which the limb is attached. Within the head, oxygen permeable capillaries are integrated with a pumping membrane designed to achieve three functions simultaneously. The first is to drive perfusate though the attached limb, the second, to prevent retrograde flow though the oxygen permeable capillaries, and the third, to remove carbon dioxide and oxygenate the perfusate. A small portable oxygen tank (size M9, with a capacity of 255 L) was attached to a battery-driven micro-solenoid operating at 60 pulses/minute via a miniature pressure regulator set to achieve a peak driving pressure pulse of 20 mmHg.

Limb perfusion

The limb was attached to the outflow port of the head unit via the femoral artery catheter and both the head unit and the limb were lowered onto the container, previously filled with 1 L of KH preservation solution at room temperature (24°C). Joining the two parts of the device forms a limb storage compartment (Figure 2). The micro-solenoid was set to operate at 60 pulses/min with a peak driving pressure of 20 mmHg. Pressure pulses applied to the pumping membrane pushed oxygenated KH perfusate through the limb. The perfusate exited via the femoral vein cannula into the organ/limb storage compartment. Pressure in the organ/limb storage chamber increased to reflect the pulse volume exiting the venous cannula. Fluid in the storage compartment was forced by the pressure pulse through the oxygenator where CO_2 was removed, and O_2 added, to begin the next cycle.

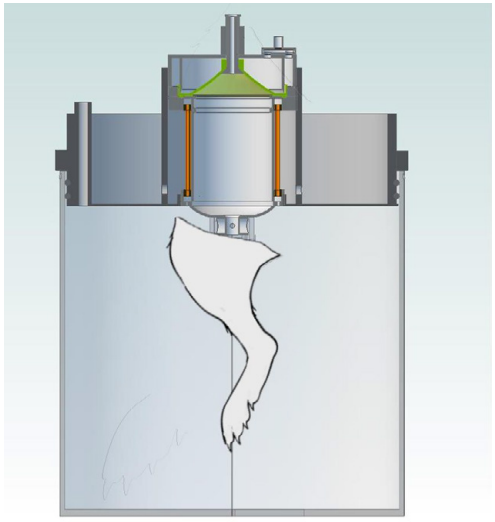

Figure 1. Schematic of the ULiSSES™ device and set up for rodent limb perfusion.

Figure 2. Rodent hind limb submerged in room temperature KH solution during oxygenated perfusion preservation.

Exterior sampling ports were attached to the arterial and venous cannulas for perfusate sampling. Fiber optic oxygen probes (NeoFox, Ocean Insight, Largo, FL, USA) were inserted into these arterial inflow and venous outflow ports to measure arterial and venous pO_2, Arterial and venous pressure were measured continuously (Statham Pressure Transducers, Waltham Mass.) and recorded hourly. Flow was calculated as the product of the storage chamber pulse pressure and the compliance of the system to yield a pulse volume, which was then multiplied by the pulse rate to obtain flow in ml/min. Pressure, pH, temperature and pO_2 were recorded hourly during the 24-hour perfusion period using Data Acquisition software (WinDaq, Dataq, Akron Ohio). Oxygen consumption was calculated as the difference between the arterial and venous pO_2 measurements, multiplied by the oxygen solubility in mlO2/ml perfusate/mmHg, and the measured flow. Vascular resistance was calculated as the quotient of the perfusion pressure and flow, and reported as mmHg/ml/min. Following the perfusion period, limbs were removed from the device and weighed to assess the development of edema.

Data are presented as the mean ± standard deviation. Pre- and post-limb weights were compared using a paired student's t test. Time course data were subjected to regression analysis with calculation of a correlation coefficient using Curve Expert Professional 2.6.0 software (Copyright © 2017 Daniel G. Hyams).

Results

Rodent hind limbs experienced 3.4 ± 0.5 hours of room temperature ischemia before being flushed and attached to the ULiSSES™ device for perfusion preservation. The formulation of the KH perfusion solution is shown in (Table 1). Pulsatile perfusion of the rat hind limbs with oxygenated, KH solution was initiated at a rate of 60 pulses/min and a peak arterial pressure of 22.5 ± 1.9 mmHg. The temperature of the perfusate remained within a tight envelope: 23.7 ± 0.5°C over the entire 24-hour perfusion period. Mean perfusion pressure was 8.2 ± 2.0 mmHg, with a mean flow to the limbs of 9.5 ± 5.0 ml/min. The pH of the perfusate was stable throughout preservation

at 7.38 ± 0.05 (Table 2). The pO_2 of the perfusate delivered to the femoral artery rose steadily throughout the perfusion period, reaching a mean of 522 ± 120 mmHg. Venous outflow rose similarly to 332 ± 83 mmHg. The average A-VO_2 difference was 219 ± 44 mmHg over the perfusion period (Figure 3). Oxygen consumption was low initially, rising to a mean plateau of 0.28 ± 0.04 ml O_2/min/100g by 17 hours (Figure 4). Mean vascular resistance in the limbs hovered about 1.0 ± 0.2 mmHg/ml/min initially, declining rapidly after 18 hours of perfusion by about 50% (Figure 5). Initial limb weight was 23.0 ± 2.9 g. Mean limb weight post perfusion increased to 31.3 ± 5.7 g representing a 37.7 ± 31.8 % increase ($p<0.05$) (Figure 6). Previously published data yielded a reference value for the mean oxygen uptake for oxygenated, hypothermically machine perfused rat hind limbs at 25 °C of 0.35 ± 0.08 ml/min/100 g. Oxygen uptake by limbs immediately following the ischemic period was 0.09 ± 0.07 ml/min/100g (Figure 7).

Table 1. Krebs-Henseleit composition.

NaCl	118.0 mM
KCl	4.7 mM
$MgSO_4$	1.2 mM
$CaCl_2$	1.25 mM
$KH2PO_4$	1.2 mM
$NaHCO_3$	25.0 mM
Glucose	11.0 mM
Osmolarity	280 mO/L
pH	7.4

Table 2. Perfusion parameters during 24-hour subnormothermic preservation.

Perfusion Parameters During 24-hour Subnormothermic Preservation	
Pulse rate	60 pulses min^{-1}
Perfusion flow	9.5 ± 5.1 ml min^{-1}
Perfusion Pressure	8.2 ± 2.0 mmHg
Temperature	23.7 ± 0.5 deg C
Perfusate pH	7.38 ± 0.05

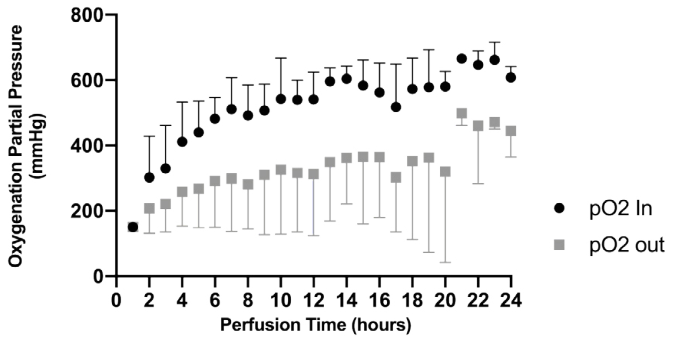

Figure 3. Perfusate oxygen partial pressure; arterial O_2 inflow and venous O_2 outflow.

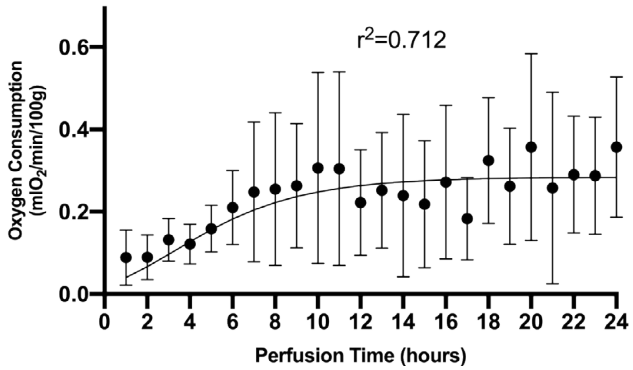

Figure 4. Oxygen uptake by rodent hind limbs during perfusion preservation at 24°C following 3.4 hours of ambient temperature ischemia.

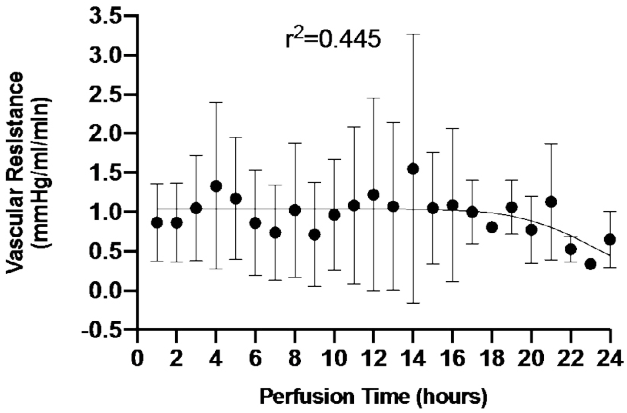

Figure 5. Vascular resistance during perfusion preservation at 24°C.

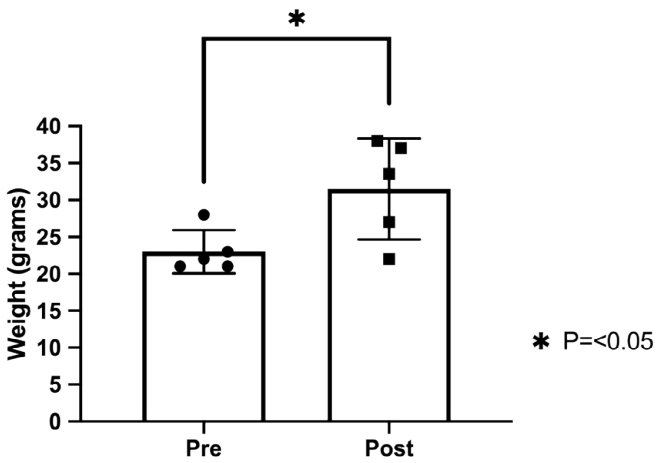

Figure 6. Hind limb weight gain resulting from perfusion preservation.

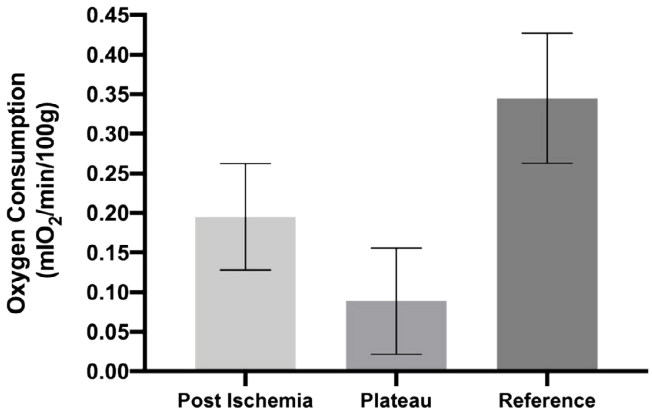

Figure 7. Reference, post ischemia and plateau oxygen uptake.

Discussions

Kaltenborn et al. recently proposed the hypothesis that a portable, clinically applicable, ex-vivo, limb perfusion device (EVLP) could address the lack of replantation possibilities associated with military operations with high rates of amputation [22]. A hypothetical implementation of this proposal might include a tactical evacuation of the patient and limb transport (with static cold storage) to a forward field hospital where the patient could be stabilized, and the limb connected to an EVLP device. Aeromedical evacuation to an advanced care facility could then proceed without the time constraints imposed by limb ischemia. There, the initial focus would be improving the patient's health status to a level that would support reattachment of the limb, allowing a microsurgical team to replant the limb under optimal conditions [22].

Kaltenborn suggested that three critical characteristics of a "clinically applicable" device would be ease of use in stressful situations, such as those posed in forward combat areas; resistance to the effects of climate; and compact enough to facilitate transport, especially by air.

Given the complexity and size of machine perfusion devices currently used in EVLP experimentation, it is unlikely that these satisfy Kaltenborn's definition of "clinically applicable".

The ULiSSES™ device, on the other hand, does appear to conform to Kaltenborn's definition of "clinically applicable" in that it is a compact, lightweight, and simple to operate. The device utilizes a combination of fluidics, and mechano-elastic principles for the hypothermic, oxygenated, perfusion and preservation of limbs and other vascularized tissue. Oxygen, perfusate, and fluidics logic combine to perfuse, preserve, and potentially resuscitate tissue at a rate that mimics a natural heartbeat. Capillary fibers with a large surface area, permeable to O_2/CO_2, provide gas exchange and efficient oxygenation of the perfusate. These innovations result in a dramatic reduction in size, weight and cost, leading to a portable, single-use device that has, thus far, successfully maintained rodent limb viability for at least 24 hours.

Bunegin et al described a predictive, linear relationship between oxygen consumption by organs during oxygenated hypothermic machine perfusion and post preservation function on a Langendorff at normothermia. This prediction is supported by data showing that oxygen uptake during perfusion preservation appears to mirror post perfusion preservation function [23].

During prolonged ambient temperature ischemia, skeletal tissue rapidly deteriorates - transitioning from a reversible ischemic phase to an irreversible phase and becoming completely unrecoverable after about 6 hours. By some estimates, after 4 hours of ambient temperature ischemia, less than 24 percent of the muscle remains viable.

Previous investigations of oxygen uptake by healthy, freshly-recovered rat hind limbs at 25°C during oxygenated machine perfusion yielded a reference, mean oxygen consumption, of 0.35 ml/min/100 g [24-34]. In this study, after 3.4 hours of ambient temperature ischemia, oxygen consumption by the rodent hind limbs was reduced to 26% of the reference O_2 uptake, consistent with the estimates of tissue viability by Blaisdell et al.

The ULiSSES™ technology appears to have resuscitated, at least to some extent, profoundly ischemic skeletal muscle, slowly improving its oxygen uptake almost three-fold over 15 hours of oxygenated perfusion. After 24 hours of oxygenated perfusion, oxygen uptake in the limbs reached 80% of the reference uptake, suggesting the possibility of recovered function. Most important, this was achieved at a very low perfusion pressure, potentially reducing shear stress on the vascular endothelium, and diminishing reperfusion injury to the muscle.

Unfortunately, the limbs exhibited a substantial edema following perfusion. This finding is not surprising, because the isosmotic perfusate did not contain oncotic agents to help mitigate fluid accumulation.

The ULiSSES™ device opens the potential for stabilization and preservation of the avulsed limbs for 24 hours, and beyond. Limbs that have been ischemic for several hours could potentially be resuscitated, increasing the potential for successful reattachment. Ultimately, preservation may be possible for a length of time that allows the patient and surgical facilities to better prepare for replantation. Additionally, it will make the re-attachment of separated extremities feasible and cost-effective, enabling transport from any recovery site to any replantation site, with minimal tissue deterioration. This, of course, assumes that ULiSSES™ technology is scalable to human limbs.

The current configuration is capable of supporting a human hand at ambient temperatures based on the measured oxygen delivery and the tissue's calculated oxygen requirement (Figure 8). A recent configuration update to the ULiSSES™ oxygenator resulted in raising perfusate oxygen saturation to greater than 90% (700 mmHg) at 4°C. Similar oxygen delivery and demand calculations suggest that the updated oxygenator, combined with

Figure 8. ULiSSES™ prototype configured for avulsed human hand perfusion preservation.

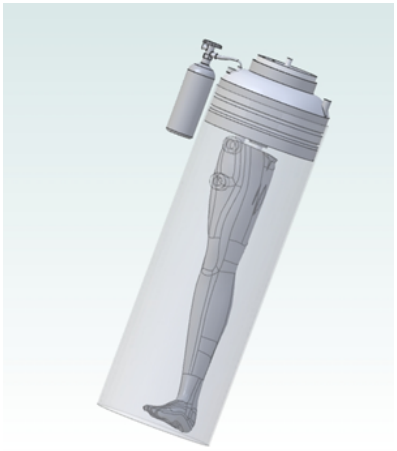

Figure 9. Proposed ULiSSES™ configuration for avulsed whole lower extremity perfusion preservation.

a slightly larger limb canister, would be sufficient to support the metabolic requirements of the amount of muscle tissue present in an adult leg at 4°C (Figure 9).

The long-term impact of extended preservation and potential resuscitation of skeletal muscle, as provide by the ULiSSES™ device, include better limb function and shortened post-reattachment recovery. Replantation of an avulsed limb has the potential to alleviate many of the physical, psychological, emotional, and societal issues that complicate an amputee's life. Moreover, the lifetime healthcare costs associated with limb loss may be significantly reduced.

Authorship Page

Participated in research design: RJV and LB

Participated in the writing of the paper: RJV, JM, LB

Participated in the performance of the research: RJV, JM, JG, LB

Contributed new reagents or analytic tools: RJV, JM, JG, LB

Participated in data analysis: RJV and LB

Critical manuscript review and approval for submission: all authors contributed equally

Disclosure

LB is the inventor of the ULiSSES™ device and owns shares of the company commercializing the device.

RJV, JM, and JG have no conflict of interest to declare.

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