Ex Situ Perfusion of Human Limb Allografts for 24 Hours

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Background. Vascularized composite allografts, particularly hand and forearm, have limited ischemic tolerance after procurement. In bilateral hand transplantations, this demands a 2-team approach and expedited transfer of the allograft, limiting the recovery to a small geographic area. Ex situ perfusion may be an alternative allograft preservation method to extend allograft survival time. This is a short report of 5 human limbs maintained for 24 hours with ex situ perfusion. Methods. Upper limbs were procured from brain-dead organ donors. Following recovery, the brachial artery was cannulated and flushed with 10 000 U of heparin. The limb was then attached to a custom-made, near-normothermic (30-33°C) ex situ perfusion system composed of a pump, reservoir, and oxygenator. Perfusate was plasma-based with a hemoglobin concentration of 4 to 6 g/dL. Results. Average warm ischemia time was 76 minutes. Perfusion was maintained at an average systolic pressure of 93 ± 2 mm Hg, flow 310 ± 20 mL/min, and vascular resistance 153 ± 16 mm Hg/L per minute. Average oxygen consumption was 1.1 ± 0.2 mL/kg per minute. Neuroromuscular electrical stimulation continually displayed contraction until the end of perfusion, and histology showed no myocyte injury. Conclusions. Human limb allografts appeared viable after 24 hours of near-normothermic ex situ perfusion. Although these results are early and need validation with transplantation, this technology has promise for extending allograft storage times.

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Vascularized composite allografts (VCA) have become a reality in the last 18 years. The first hand transplant was performed in 1998,¹ and since that time, over 100 hand transplantations have been performed.² VCAs, particularly hand allografts, are subject to reperfusion injury if warm ischemia time is prolonged. Among the various tissue types in VCAs, muscle appears the most susceptible to irreversible ischemic injury, which occurs as early as 4 hours.³ Once the blood flow is reestablished, immediate increases in reactive oxygen species and intracellular calcium result in mitochondrial damage, eventually leading to apoptotic or necrotic cell death within the muscle.⁴ This ultimately triggers the immune approval of article version to be published. B.N. participated in project design, critical revision of the article, and approval of article version to be published. R.E.P. participated in project design, critical revision of the article, and approval of article version to be published. P.L. participated in project design, critical revision of the article, and approval of article version to be published. S.M.R. participated in project design, critical revision of the article, and approval of article version to be published. C.L.M. participated in data analysis, critical revision of the article, and approval of article version to be published. A.R.-P. participated in project design, data acquisition, data analysis, data interpretation, critical revision of the article, and approval of article version to be published. R.H.B. participated in project design, data interpretation, critical revision of the article, and approval of article version to be published. J.C.M. participated in project design, data interpretation, critical revision of the article, and approval of article version to be published. K.O. participated in project design, data acquisition, data analysis, data interpretation, drafted the article, and approval of article version to be published.

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system and affects both the early and long-term graft function. To minimize the ischemic injury, procured hand allografts are currently flushed with preservative solution and kept at 4°C until transplantation. Lowering the temperature reduces the cellular energy demand and curbs the detrimental effects of anaerobic respiration. However, at 4°C, the overall energy expenditure only drops 10%, which is often inadequate to prevent reperfusion injury.

To optimize the use of critically ischemic organs, dynamic preservation methods, in the form of continuous ex situ perfusion have been proposed. These methods promote aerobic cellular respiration that is thought to improve cellular ion and signal homeostasis. For limb allografts, ex situ perfusion systems have only been tested on porcine limbs, resulting in promising outcomes for up to 24 hours. In this study, we report the outcome of human limb allografts maintained on ex situ perfusion for 24 hours.

MATERIALS AND METHODS

The institutional review board, in cooperation with the local organ procurement agency, Gift of Life Michigan, approved this study (HUM00097424). Five human limb allografts underwent near-normothermic ex situ perfusion.

Limbs

Upper limbs were procured from brain-dead adult donors. Three donors were men and 2 donors were women. Ages ranged from 37 to 69 years. Body mass index was between 22.5 and 43.9 kg/m². Two donors had essential hypertension and hyperlipidemia, 1 donor had diabetes, and otherwise, there was no known history of cardiovascular or peripheral vascular disease.

Procurement

An upper arm tourniquet was inflated to 250 ± 25 mm Hg. A fish mouth incision was made at the level of the elbow joint. The brachial artery and vein were dissected and divided. The median, radial and ulnar nerves were identified and transected. The forearm muscles were detached from the lateral and medial epicondyles of the humerus, and the limb was disarticulated at the elbow. The stump was then closed and tourniquet released. The donors were then prepared for thoracic and/or abdominal organ procurement.

Back table instrumentation of the limb included insertion of a short cannula (2.4 or 3.2 mm diameter barbed connector; Quosina, Ronkonkoma, NY) into the brachial artery, opening of the brachial and cephalic veins to allow for venous drainage, and flushing the limb with 10 000 units of sodium heparin in 250 mL of 0.9% normal saline, without any other preservative solution. The limb was then transported, without cooling, to our research laboratory for ex situ perfusion. No cooling of the limb was performed in order to minimize injury caused by cold ischemia time (CIT).

Ex Situ Perfusion System

The ex situ perfusion system was an open venous drainage circuit consisting of a roller pump (Shiley Roller Pump; Stockert Instruments, Munich, Germany or M pump; MC3, Ann Arbor, MI), reservoir with oxygenator (Baby Capiox-Rx; Terumo, Tokyo, Japan), and a custom-made limb chamber designed to facilitate perfusate return to the reservoir (Figure 1; Video 1, SDC, http://links.lww.com/TP/B358). Circuit flows were controlled through the pump, with target flow set to 6% to 8% of the donor’s estimated cardiac output. Perfusion pressure was transduced at the arterial cannula. Systolic pressure was maintained less than 110 mm Hg; circuit flow was adjusted, when necessary, to stay below this limit. Temperature of the system was maintained at 30°C to 33°C. The sweep gas was a combination of oxygen (40-60% by concentration), carbon dioxide (5-10%), with the balance nitrogen.

The perfusate was plasma-based with packed red blood cells added to achieve a hemoglobin concentration of 4 to 6 g/dL. All blood products were blood type matched with the donor and obtained from the University of Michigan Health Systems’ blood bank. Additives to the perfusate included concentrated albumin, sodium bicarbonate, tromethamine, calcium chloride, and sodium heparin. Dextrose was added as needed to maintain perfusate glucose greater than 100 mg/dL, whereas regular insulin was administered if the glucose concentration was greater than 300 mg/dL. Antibiotics were added to cover skin flora. Total perfusate volume in the circuit was 250 to 300 mL. Partial perfusate exchange was performed every 3 to 5 hours. Methylprednisolone...
(200 mg) was given upon start of perfusion and with each perfusate exchange.

**Perfusion and Monitoring**

Each limb was attached to the circuit with a perfusion goal of 24 hours. Circuit pressure, flow, and perfusate temperature were monitored continuously. Vascular resistance was calculated by dividing mean circuit pressure by circuit flow. Activated clotting times were checked to verify anticoagulation of the circuit. Plasma-free hemoglobin was measured every 6 hours by multiple wavelength spectrophotometry (Ultraspec 500 Pro; Amersham Biosciences, Pittsburgh, PA) to monitor for circuit hemolysis.

Perfusate was collected prelimb and postlimb to evaluate pH; partial pressure of carbon dioxide and oxygen; oxygen saturation; bicarbonate, glucose, lactate, sodium, potassium, and hemoglobin concentrations every hour. Oxygen consumption was calculated using a modified Fick equation using circuit flow and the prelimb and postlimb oxygen contents. Perfusate myoglobin, a marker of myocyte injury, was measured every 6 hours using a latex enhanced immunoturbimetric assay (Integra 800; Roche Diagnostics, Indianapolis, IN).

The limb was weighed before perfusion and at the end of the experiment. Skin temperature at the palm was measured hourly (Thermo Tech TT1022 Noncontact Infrared Digital Thermometer; Max Tool, Ontario, CA). Continuous pulse oximetry was performed (Nellcor N-595 Pulse Oximeter; Nellcor, Pleasanton, CA). Neuromuscular electrical stimulation (Neruosign 100 Never Monitor; Neurosign Surgical, Whitland, United Kingdom) of the median and ulnar nerves was performed every 2 hours.

**Histology and Single Muscle Fiber Contractility**

For limbs 3 through 5, biopsy samples from the muscle bellies of flexor carpi radialis were taken at 0, 12, and 24 hours of perfusion. Samples were formalin fixed, cross-sectioned, and stained via hematoxylin-eosin to evaluate structural injury. After staining, all muscle sections were digitally captured using a bright field microscope and mean fiber cross-sectional area was calculated using ImageJ software (v.1.45) by outlining the circumference of each fiber within representative samples.

Muscle biopsies were also taken to assess muscle function and the maximum isometric contractile force of permeabilized single muscle fibers, following previously published techniques. Briefly, biopsies were placed in a relaxing solution containing Ca\(^{2+}\) chelating agents in a pH-buffered solution and disected into 3 × 0.75 mm fiber bundles. Bundles were permeabilized in dissecting solution for 30 minutes, incubated in storage solution for 16 hours, and then stored at −80°C. On the day of testing, the bundles were thawed individual fibers were secured at one end to a servomotor (Aurora Scientific, Aurora, ON) and at the other end to a force transducer (Aurora Scientific). Using a laser diffraction measurement system, fiber length was adjusted to a sarcomere length of 2.7 μm. Assuming an elliptical cross-section, the average fiber cross-sectional area was estimated by measuring the fiber diameter at 5 locations along the fiber midsection from high-magnification images at 2 different views (top and side). Maximum fiber isometric force (Fo) was elicited by immersing the fiber in a solution containing a high concentration of Ca\(^{2+}\) and adenosine triphosphate. Specific force of fibers was calculated by dividing forces by fiber cross-sectional area. Five fibers were tested from each biopsy sample.

Differences in fiber cross-sectional area and contractile force between the 0- and 24-hour biopsy groups were analyzed using paired t tests (IBM SPSS, v21.0. Armonk, NY). Values are reported as means ± standard deviations, and P values less than 0.05 were considered to be significant.

**RESULTS**

Average procurement time was 17 ± 5 minutes, and all had less than 20 mL of blood loss. Average total ischemic time was 76 minutes (range, 40 to 100 minutes).

Hemodynamic parameters of perfusion for each limb are shown in Figure 2. Systolic pressure was maintained at an average of 93 ± 2 mm Hg and vascular resistance 153 ± 16 mm Hg/L per minute. The circuit flow was an average 310 ± 20 mL/min, which was 6% to 10% of the donor’s estimated cardiac output. Temperature of the perfusion system was maintained at an average of 32.0 ± 0.2°C. Activated clotting times were all greater than 1000 seconds. Plasma-free hemoglobin was elevated at the start of perfusion and mildly increased with perfusion time (Figure 2).

Changes in perfusate pH, partial pressure of carbon dioxide and oxygen, electrolyte concentration, and glucose concentration for each limb are shown in Figure 2. Average oxygen consumption was 1.1 ± 0.2 mL/kg per minute. Perfusion myoglobin was elevated at the start of perfusion (43 μg/mL) and increased with perfusion time (92 μg/mL at 24 hours).

Average percentage weight change of the limbs was -0.4%, with a range of (-7% to +7%). Pulse oximetry was 100% for all limbs throughout the perfusion. Average palm temperatures were 31.3 ± 0.6°C. Neuromuscular electrical stimulation revealed M4 (contraction against gravity) muscle contraction throughout the experiment on all 5 limbs (Video 2, SDC, http://links.lww.com/TP/B359).

Histology of the flexor carpi radialis biopsies at 0, 12, and 24 hours did not show a difference in fascicular architecture and shape. There were no changes in mean fiber cross-sectional area between start and end of perfusion (265 ± 34 vs 280 ± 67 μm², P = 0.819). None of the samples at 24 hours showed necrosis, degeneration, fatty, or inflammatory cell infiltration. (Figure 3) Additionally, single muscle fiber contractility testing revealed no changes in either the maximum fiber isometric force (Fo: 0.63 ± 0.46 vs. 0.64 ± 0.26 mN, P = 0.952) or the specific force of fibers (129 ± 26 vs. 151 ± 28 kPa, P = 0.557) between start and end of perfusion.

**DISCUSSION**

Five human limb allografts appeared viable after 24 hours of ex situ perfusion, as demonstrated by ongoing oxygen consumption, stable vascular resistance, intact response to nerve stimulation, and biopsy results. This represents a clinically significant expansion of preservation time compared to current practice for VCAs.

Ex situ perfusion of donor organs is gaining increased attention by transplant professionals. Success has been realized in renal transplantation, where hypothermic (4-6°C) ex situ perfusion is known to reduce delayed graft function compared...
to cold storage. In pulmonary and cardiac transplantation, normothermic (37°C) ex situ perfusion allows for reconditioning of potential allografts, including those from donors after circulatory death, which has resulted in an increased number of transplants. For VCA transplantation, our results suggest ex situ perfusion could increase storage time, which in turn may allow additional time to coordinate between operative centers, and allow for a single operative team for limb preparation and attachment. Furthermore, the extended time has the potential to transform hand and limb transplantation from an emergent surgery to an elective.

In addition to these logistical advantages, near normothermic perfusion may prevent the damage caused by cold storage. In experimental limb allograft models, the degree of muscle damage correlates with CIT. Irreversible damage to myocytes occur between 3 and 6 hours of CIT. van der Heijden et al. studied skeletal muscle contraction and histology using various preservative solutions at 4°C. They demonstrated that after 16 hours of storage, at least 25% of muscle fibers were necrotic and unresponsive to electrical stimulation. Later, Hautz et al. flushed rat hind limb isografts and stored them at 4°C for 2, 10, or 30 hours. They demonstrated that 2 hours of CIT led to mild inflammation on day 10, whereas those with CIT of 10 and 30 hours had extensive inflammation and tissue damage. The primary area of injury was muscle, along with significant perineural infiltration.

FIGURE 2. Individual perfusion parameters (systolic pressure, circuit flow, and vascular resistance), perfusate composition (pH; gas partial pressures; electrolyte, lactate, and glucose concentration), oxygen consumption, serum myoglobin levels, and plasma free hemoglobin over perfusion time.
Histomorphometric alterations seen in these experimental studies, however, do not seem to correlate with the clinical experience. Landin et al. reviewed 28 hand and forearm transplant patients and did not observe a correlation between the functional outcome and CIT.

This contrast between experimental and clinical findings may stem from a variety of factors. First, experimental studies are often performed on rat hind limb allografts in which functional assessment of the limb is not practical. Second, experimental limb allografts contain disproportionally large amount of muscle compared to human hand allografts, and consequently, the damage is more noticeable. Third, on a standard hand transplant patient, the intrinsic muscles of the hand are the most susceptible tissue to ischemic injury, and these muscles have minimal contributions to those functions measured by the Disabilities of the Arm, Shoulder and Hand outcome instrument. Nevertheless, there seems to be at least 1 report describing a correlation between perioperative ischemic injury and fibrotic muscle degeneration or decreased allograft function. In the current study model, we are unable to evaluate for evidence of ischemic-reperfusion injury in the presence of an immune system. Therefore, no conclusions can be drawn regarding the long-term function of the tested allografts. However, the muscle contracted against gravity as seen in Video 2, SDC, http://links.lww.com/TP/B359; this requires a structurally intact nerve, neuromuscular endplate, and contraction of 80% of all myocytes within the compartment tested. This strong response seems to suggest well-preserved allograft function at the end of 24 hours of perfusion.

The perfusion system was pressure-limited and flow-regulated: we controlled flow through the circuit, but limited the flow based on preset pressure targets (systolic blood pressure less than 110 mm Hg). The pressure limitations were set to minimize pressure-induced endothelial injury, which was not observed in our histologic analysis. We targeted system flow to be 6% to 8% of estimated donor cardiac output, which was based on physiologic estimates of healthy upper extremity blood flow. This level of flow helped to ensure adequate oxygen delivery, as it is known that prevention of ischemia may potentially reduce the incidence of acute rejection following VCA transplantation. Wang et al. tested a 20°C ex situ perfusion strategy incorporating hyperoxygenated University of Wisconsin solution on gracilis fasciomyocutaneous allografts to evaluate the onset of acute rejection. Muscle flaps were perfused for 5 hours before transplantation and demonstrated a significantly delayed acute rejection even in the absence of systemic immunosuppression. These results...

FIGURE 3. Representative muscle histology (H&E) and quantitative fiber cross-sectional area and contractile force measurements of the flexor carpi radialis at 0 hours, 12 hours, and 24 hours of perfusion. Muscle fibers showed polygonal structure representing normal adult muscle. Single muscle fiber contractility testing confirmed normal contraction and force generation at the end of 24 hours as seen in specific force of fibers. H&E, hematoxylin-eosin.
are in concert with other studies on cardiac allografts demonstrating a decrease in acute rejection when ischemia-reperfusion injury is abrogated.22–27

Temperature of the system was maintained at near-normothermia (30–33°C) rather than hypothermic (4–6°C) to minimize peripheral vasoconstriction and shunting, and possibly to improve posttransplantation function. In experimental studies, normothermic or near-normothermic preservation of porcine liver and kidney allografts seemed to have better function compared to cold stored grafts.28 Although no comparative studies are available on human limb allografts, previous studies using near-normothermic perfusion of swine limbs appears to have provided satisfactory short-term outcome following transplantation.13–15 Muller et al15 tested the feasibility to use machine perfusion at 32°C using whole blood on swine forelimb allografts and demonstrated no significant tissue injury in replant limbs after 7 days. Ozer et al13 used a similar temperature (27–32°C) but was able to extend perfusion time up to 24 hours without signs of reperfusion injury.

Perfusate for this system was blood-based because it offered the oxygen carrying capacity of red blood cells, free radical scavengers, buffers, and oncotic properties. Perfusate at a lower-than-physiologic hemoglobin concentration (4–6 g/dL) also seemed to minimize vascular congestion. An acellular perfusate could have been used14; this has the advantage of not containing any immune cells, not requiring a cross-match before administration, and being readily available. However, for this initial study, we defaulted to blood-based to maximize oxygen delivery and minimize possible ischemia. Perfusate exchange was performed at regular intervals with the intention to normalize electrolyte concentrations and minimize metabolite accumulation. Electrolytes stayed near a physiologic range for the 24 hours of perfusion; however, fluctuations were observed and there was minimal correction achieved with perfusate exchanges. These fluctuations may represent the limb’s stress response to the procurement and reperfusion process along with the lack of hemostatic organs such as kidneys and lungs. The addition of a hemofilter to the circuit will be necessary for tighter electrolyte control. Lactate concentration slowly increased throughout the experiment. Plasma lactate levels are known to increase with severe hypoxemia, but it also can be increased by nonhypoxic causes such as delayed clearance in the setting of renal or hepatic failure, or during hypermetabolic states where glucose uptake by a cell exceeds the rate of metabolism by mitochondria.29 Increases in lactate have been observed during the ex situ lung perfusion, and good outcomes after transplantation were achieved despite elevated lactate concentrations.30 In our study, the increased lactate concentration did not seem to affect the muscle or the neurovascular endplate function, and thus the increasing lactate concentration likely represented a lack of clearance from the system. Perfusate myoglobin measured at the beginning of the experiment was higher than normal, perhaps due to surgical trauma or prolonged immobilization as in all our patients were in intensive unit care before procurement. In the absence of renal clearance, the myoglobin concentration doubled by the end of perfusion at 24 hours. This level of increase is substantially less than what would be observed an acute compartment syndrome or ischemic myopathy.

Limitations of this short report include the small sample size, as only 5 limbs have been perfused. Additional limbs will be necessary to see if similar results at 24 hours are consistently maintained. Also, there were no limbs that underwent cold storage to serve as a comparative control group. Third, our custom-made system is not readily transportable. The optimal ex situ perfusion system would travel to the site of procurement to reduce ischemia time. Although limb procurement was performed under sterile conditions, perfusion system was not set to be sterile. Further use of the system at the clinical setting will require rigorous microbiologic testing to prevent infection. Additional histological studies to identify tissue damage may help to understand the change in lactate concentration and electrolyte fluctuations. Finally, although our results showed at least M4 muscle contraction after 24 hours of perfusion, and single muscle fiber contractility testing is normal; no conclusions can be drawn about the performance after transplantation, nor the long-term survival of the extremity. The lack of proinflammatory mediators during the perfusion period could potentially mask preservation injury that may manifest only after reperfusion.

In conclusion, ex situ perfusion may be a promising modality of preservation and storage for vascularized composite tissue allografts. It offers the benefits of extended storage time and real-time functional testing. Future research is necessary to refine the perfusion system and determine the impact this method has on reducing ischemia-reperfusion injury.

REFERENCES


