The Effect of *Ex Situ* Perfusion in a Swine Limb Vascularized Composite Tissue Allograft on Survival up to 24 Hours

Kagan Ozer, MD,* Alvaro Rojas-Pena, MD,† Christopher L. Mendias, PhD,* Benjamin S. Bryner, MD,† Cory Toomasian, BS,‡ Robert H. Bartlett, MD†

**Purpose** To test the potential for the *ex situ* limb perfusion system to prolong limb allograft survival up to 24 hours.

**Methods** We used 20 swine for the study. In group 1 (control), 4 limbs were perfused with heparin solution and preserved at 4°C for 6 hours. In group 2, 4 limbs were perfused with autologous blood at 27°C to 32°C for 24 hours. In both groups, limbs were transplanted orthotopically to recipients and monitored for 12 hours. In addition to perfusion parameters, we recorded perfusate gases and electrolytes (pH, pCO₂, pO₂, O₂ saturation, Na, K, Cl, Ca, HCO₃, glucose, and lactate) and obtained functional electrostimulation hourly throughout the experiment. Histology samples were obtained for TUNEL staining and single-muscle fiber contractility testing.

**Results** In both groups, hemodynamic variables of circulation remained stable throughout the experiment. Neuromuscular electrical stimulation remained intact until the end of reperfusion in group 2 vs no response in group 1. In group 2, a gradual increase in lactate levels during pump perfusion returned to normal after transplantation. Compared with the contralateral limb in group 2, single-muscle fiber contractility testing showed no significant difference at the end of the experiment.

**Conclusions** We demonstrated extended limb survival up to 24 hours using normothermic pulsatile perfusion and autologous blood.

**Clinical relevance** Successful prolongation of limb survival using *ex situ* perfusion methods provides with more time for revascularization of an extremity. (*J Hand Surg Am.* 2016;41(1):3–12. Copyright © 2016 by the American Society for Surgery of the Hand. All rights reserved.)

**Key words** *Ex situ*, perfusion, reperfusion, swine, vascularized composite tissue allograft.

Upper extremity loss proximal to the wrist is a major disability. In case of major upper extremity amputations, replantation needs to be performed within 4 to 6 hours to prevent permanent muscle damage. Delay in transportation, lack of an available microsurgery team, or concomitant life-threatening injuries results in limb loss. Vascularized composite tissue allograft (VCA) transplantation is also subject to time constraints. Once a donor is available, preoperative preparation, transfer of the extremity part, and revascularization need to be completed within a few hours to succeed.

After traumatic or elective amputations (as in VCA transplantation), the extremity is preserved in cold storage solutions at 4°C. Although this reduces the metabolism and lactate production in the short term, beyond a certain critical point it leads to extensive damage to the cell membrane, resulting in cold
ischemia-reperfusion injury and accelerated graft loss. Such damage is well documented in solid organs and hand allografts.5–12

To optimize the timing of surgery and minimize cold ischemia-reperfusion injury, normothermic ex situ perfusion systems have been used successfully in solid organ transplantations.13–20 Overall, experience with VCA is limited. Various authors have prolonged limb allograft survival successfully up to 12 hours in preclinical studies.21–23 These were statistically significant compared with native ischemic tolerance of a limb (4 hours) but more time is needed to make a clinically meaningful difference. In this study, we studied the potential to achieve such a goal up to 24 hours.

MATERIALS AND METHODS
Our university committee on the use and care of animals approved this study, and its guidelines and policies for the humane use of animals in research along with those from the United States Department of Agriculture and the Association for Assessment and Accreditation of Laboratory Animal Care were followed in this study.

Experimental groups
We disarticulated 4 forelimbs in each of 2 groups at the level of the elbow, perfused them with 10,000 U heparin on 100 mL of saline solution, and weighted them. In group 1 (control), limbs were stored at 4°C for 6 hours before transplantation. In group 2, limbs were immediately connected and perfused for 24 hours according to the protocol described subsequently. At the end of the cold storage in group 1 and perfusion in group 2, all limbs were transplanted to another animal orthotopically and monitored for an additional 12 hours.

Animal model
We used an established swine limb replantation/transplantation model.23 After induction of anesthesia, mechanical ventilation was set to keep PCO2 at 35 to 45 mm Hg and peak inspiratory pressures less than 20 cm H2O. The right carotid artery and right internal jugular vein were used to monitor mean arterial blood pressure, heart rate, central venous pressure, and fluid administration (80–120 mL/h) and to collect blood samples.

Surgical technique
Preparation of the forelimb on donor: All donor forelimbs were disarticulated at the elbow to minimize blood loss.23 After amputation, we cannulated the brachial artery and marked the median and ulnar nerves for monitoring.

Preparation of the recipient and transplantation: Before the end of cold storage or perfusion, each recipient swine was anesthetized and the ipsilateral forelimb was amputated for orthotopic transplantation using the same technique.

Limbs in both groups were weighted before transplantation. We then used 2 Steinmann pins to fix the olecranon to the humerus. All origins of the flexor and extensor tendons on the humerus were approximated using 2-0 suture. This took the tension off the vascular bundle. Under microscopic magnification, the brachial artery and vein and the cephalic and basilic veins were repaired using end-to-end technique and 8-0 nylon suture. Finally, skin was approximated. We used the median nerve to monitor neuromuscular stimulation until the end of the experiment. Before opening the vascular clamps, each recipient was administered 200 μg/kg heparin subcutaneously.

Ex situ perfusion protocol
The perfusion apparatus consisted of a perfusion pump (Waters Medical Systems, Minneapolis, MN) and an oxygenator (Baby CAPIOX RX, Terumo Cardiovascular Systems Co, Ann Arbor, MI). The oxygenator had 0.5-m2 surface area connected to a 70% O2–5% CO2 gas tank. The gas mixture contained 5% CO2 and 95% O2. Pulsatile perfusion pressure was limited to 60 to 80 mm Hg. During perfusion, the limb was maintained in an antiseptic diluted sodium hypochlorite solution dressing (Fig. 1).

Preparation of the perfusate: We first centrifuged donor blood and removed white blood cell and platelet fractions. Packed red blood cells and plasma were mixed with dextran in a ratio of 1:2 to reach a colloid osmotic pressure of 20 to 30 mm Hg. During the experiment, perfusate was replaced at a constant rate (80 mL/h) to remove toxic metabolites from the system in the absence of liver and kidney functions. We also measured perfusate glucose levels every 2 hours and substituted the perfusate either with glucose (1 mL dextrose 50%) (if the level was < 4.5 mmol/L) or with insulin (2 U) (if glucose was > 14 mmol/L). The perfusate was maintained at 27° to 32°C throughout the experiment.

Monitoring
The following variables were measured throughout the experiment:
• From the limb on pump perfusion: pressure, flow, resistance, temperature, O2 delivery and consumption, acid-base status, and blood gases.
From the perfusate and the recipient (every 2 h): hemoglobin, lactate, K\(^+\), Na\(^+\), Cl\(^-\), HCO\(_3\)\(^-\), pH, pCO\(_2\), pO\(_2\), and glucose.

Neuromuscular electrical stimulation every hour using a nerve stimulator.

Limb weight: After the amputation, before the transplantation, and at the end of reperfusion (at 12 hours).

**Histology**

*Single-muscle fiber contractility testing:* Muscle biopsies (10 × 5 mm) were placed in a relaxing solution containing Ca\(^{2+}\) chelating agents in a pH-buffered solution. We then placed muscle bundles in skinning solution to further the permeability of the cell membranes for 30 minutes and stored them at −80°C until the day of testing. On the day of testing, we thawed the specimens and placed them in a chamber containing relaxing solution. The bundles were secured at one end to a servomotor (Aurora Scientific, Aurora, Ontario, Canada) and at the other end to a force transducer (Aurora Scientific) using 2 ties of 10-0 monofilament nylon suture at each end. Fiber length was adjusted to obtain a sarcomere length of 2.7 μm using a laser diffraction system. We then calculated the fiber cross-sectional area at 5 different points.
positions. Maximum fiber isometric force was elicited by immersing the fiber in a solution containing a high concentration of Ca\(^{2+}\) and adenosine triphosphate (ATP). The specific force of fibers was calculated by dividing the fiber isometric force by the fiber cross-sectional area. Fibers were categorized as fast or slow by examining their force response to rapid, constant-velocity shortening, and 10 fast fibers were tested from each biopsy sample (Fig. 2).

Terminal deoxynucleotidyl transferase deoxyuridine-triphosphatase nick end labeling staining
First, we placed muscle biopsies in TissueTek OCT (Sakura Finetek Europe, Alphen aan den Rijn, The Netherlands) and snap-froze them in liquid nitrogen. We then put 10-μm sections on a cryostat and labeled them with a Click-It terminal deoxynucleotidyl transferase deoxyuridine-triphosphatase nick end labeling (TUNEL) assay (Life Technologies, Grand Island, NY), which marked apoptotic...
nuclei with the green fluorophore AF488. Sections were also treated with the blue fluorescent nuclear dye Hoechst 33342 to identify total nuclei and the red fluorescent extracellular matrix marker WGA-lectin-AF555 to identify intramyocellular versus extramyocellular nuclei.

Statistical analysis
Data are presented as means ± SD. We tested differences between the control and perfusion groups using the Student t test. Using the SD generated by the primary outcome variable (single-muscle fiber contractility testing), a power of 80% required a minimum of 4 animals to be tested in each group when statistical significance was set to .05.

RESULTS
Limbs on pump perfusion in group 2 showed increased peripheral vascular resistance within the first hour but returned to lower values after the second hour and vascular resistance remained low until the end of the perfusion at 24 hours (Fig. 3, left
column). This occurred without a significant increase in flow or mean arterial pressures. Recipients in both groups remained hemodynamically stable throughout the experiment. Mean arterial pressure, hematocrit (not shown in Fig. 3), and heart rate did not show a statistically significant difference between groups ($P > .05$) (Fig. 3, right column).

In group 1, the brachial and radial arteries had blood flow throughout the experiment. Capillary refill on the skin remained more than 4 seconds (compared with 2 seconds in group 2). Skin temperature did not return to normal despite warming. Lactate and potassium levels did not increase after revascularization.

In group 2, among metabolic parameters of perfusion, lactate increased steadily until the end of perfusion at 24 hours (Fig. 4, left column). Potassium, pH, and other parameters remained stable, likely owing to periodic perfusion exchange. After transplantation, lactate in group 2 returned to normal levels. Lactate, potassium, pH, and other parameters did not show a statistically significant difference between groups after reperfusion ($P > .05$) (Fig. 4, right column).

Single-muscle fiber contractility testing values showed a statistically significant drop between 18 and 24 hours while limbs were on the pump in group 2 ($P < .05$). This returned to normal at 30 and 36 hours during the reperfusion phase (6 and 12 h after transplantation) ($P > .05$). In contrast, the control group showed a steady decline throughout the experiment (Fig. 5, right column). Muscle samples obtained for TUNEL staining showed a limited number of apoptotic cell nuclei between group 2 and control samples, which suggested a normal cell cycle after 12 hours of reperfusion (Fig. 6).

**DISCUSSION**

Using an *ex situ* perfusion system, we demonstrated the possibility of extending limb allograft survival up to 24 hours as evidenced by metabolic, functional, and histology parameters of limb perfusion. Since hand transplantation became a reality, the standard of care in preserving the procured VCA has been to use static cold storage methods. Although this approach successfully reduces the initial ATP consumption and metabolism of the muscle cell, it eventually leads to anaerobic metabolism and complete depletion of ATP stores. Studies on cardiac allografts in particular demonstrated that the primary
The site of cold injury is the cell membrane of endothelium and myocytes. The magnitude of these injuries seems to correlate with the duration of cold ischemia. Cardiac allografts subjected to 6 hours of cold ischemia had 25% greater graft loss than those subjected to 2 hours. At the cellular level, cold ischemic injury is characterized by coagulative myonecrosis (CMN), which coexists with or mimics acute cellular rejection but also heals with a scar that is indistinguishable from ischemic cell death or necrosis. In hand allografts, Landin et al reported perioperative ischemic injury in a patient with bilateral hand transplantation. They observed the development of a slowly progressive wrist contracture over 7 months on the left side, which was subjected to 3.5 hours of cold ischemia (7 hours total ischemia) before revascularization. The authors thought that this did not result from a localized compartment syndrome or allograft rejection because they found extensive CMN on histology. On both cardiac and hand allografts, CMN develops in days to weeks. In the current study design, we terminated our experiments 12 hours after reperfusion. This was not enough to detect CMN. Instead, we used TUNEL staining to distinguish apoptotic cell death from ischemic necrosis. With single-muscle fiber contractility testing, we further confirmed the presence of healthy myocyte units after 24 hours on pump perfusion.

Normothermic perfusion methods were introduced to circumvent problems associated with cold storage and have been successfully applied in solid organ transplantations. In experimental studies,
FIGURE 6: Samples in the left column were obtained from normal muscle without ischemia. Samples on the right were taken from animals after 24 hours of pump perfusion followed by 12 hours of reperfusion. Apoptotic nuclei, which are the sign of pending tissue necrosis, would appear in green fluorescent dye. A comparison of muscle samples (top row) shows no difference between the 2 sites. WGA, wheat germ agglutinin.
cardiac allografts kept in completely beating status with normothermic blood perfusion displayed excellent myocardial ultrastructure on electron microscopy images. In clinical trials, cardiac allografts have been reported to have fewer primary graft failures and acute rejection when preserved on normothermic ex vivo perfusion compared with cold preservation using histidine–tryptophan–ketoglutarate solution. Overall experience with the use of normothermic ex situ perfusion in VCAs is only preclinical at this point. Two authors have shown successful prolongation of allograft survival on swine models. Constantinescu et al used a modern perfusion technique, autologous blood at 32°C, and reported survival up to 12 hours on swine forelimbs. Ozer et al showed similar results using pulsatile perfusion and diluted blood as the oxygen carrier at 27°C to 32°C. They reported minor increases in limb weight with ongoing contraction after 12 hours on the pump and 12 hours of reperfusion. In the current study, we perfused limbs for 24 hours and observed continuous neuromuscular electrical stimulation and normal force generation on single-fiber contractility testing. Limb weight increased 20% at the end of pump perfusion and then returned to 15% after 12 hours of reperfusion with excellent contraction on all muscles tested. At this point, using the technique described in this article, 24 hours seems to be reliable for ex situ perfusion.

Limitations of the current study include a limited observation period after transplantation. As a result, we do not know the effect of the reperfusion injury beyond 12 hours in either group. No conclusions can be drawn regarding the long-term function of the transplanted extremity, because this would depend on the effect of ex situ perfusion on nerve regeneration in the presence of long-term immunosuppressive therapy. Despite the presence of statistically significant differences between the control and experimental groups in single-muscle fiber contractility testing, it is unknown whether this translates into a difference in the function of the extremity. These limitations can be addressed in another experiment with long-term follow-up using chronic immunosuppression, possibly leading to clinical application.

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REFERENCES


