Ex Situ Limb Perfusion System to Extend Vascularized Composite Tissue Allograft Survival in Swine

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Background. Organ perfusion systems have successfully been applied in solid organ transplantations. Their use in limb transplantation and revascularization has not been widely investigated. In this study, we tested the potential for ex situ perfusion system to prolong limb allograft viability in a swine forelimb amputation/replantation model. Methods. Fourteen swine were used. In group 1 (n = 4), we perfused 4 amputated limbs for 12 hours using warm (27°C–32°C) autologous blood. Group 2 (n = 3) served as a cold preservation control group, preserving limbs for 6 hours at 4°C. All limbs were transplanted into healthy swine (n = 7) and observed for another 12 hours. Hemodynamic variables of circulation, as well as perfusate gases and electrolytes (pH, pCO2, pO2, O2 saturation, Na+, K+, Cr, Ca2+, HCO3−, glucose, lactate) were measured. Muscle samples were used to measure single-muscle fiber contractility. Results. In the control group, no microcirculation was observed after 6 hours of cold storage. In the pump perfusion group, all limbs displayed a gradual increase in lactate levels (P < 0.05) during ex situ perfusion that returned to normal after transplantation and reperfusion (P = 0.05). The pH and potassium remained stable throughout the experiment. Single-muscle fiber contractility testing showed near normal contractility at the end of the reperfusion period (P > 0.05). Limb weight did not increase significantly between the end of pump perfusion and reperfusion (P > 0.05). Conclusions. We demonstrated the potential to preserve limb allograft using ex vivo circulation. This approach promises to extend the narrow time frame for revascularization of procured extremities in limb transplantation.

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Materials and Methods

The University Committee on Use and Care of Animals approved this study. The University Committee on Use and Care of Animals, United States Department of Agriculture and Association for Assessment and Accreditation of Laboratory Animal Care guidelines and policies for humane use of animals in research were followed in this study.

Experimental groups: In group 1, four swine forelimbs were amputated, connected to ex situ perfusion for 11 hours and transplanted to another healthy swine. In group 2, 3 swine microsurgery team and the warm ischemia time. Currently, these issues are addressed by immediate transfer of the patient to specialized centers and perfusing the amputated limb with clear cold solution and preserving it at cold temperature until the re plantation can be done. In amputations proximal to the wrist joint, re plantation is considered futile if the warm ischemia time exceeds 6 hours.

Tissue preservation in extremities before revascularization is a limiting factor in the area of limb transplantation and re plantation. Given the time restraints imposed by tissue viability, immediate transfer of an amputated or harvested limb is essential for success. With recent advances in organ preservation, a prolonged period of tissue survival is possible in solid organ transplantations with excellent function.1–14 Up to 5 days of survival is achieved in experimental kidney perfusion systems.6,7,13 Using the experience accumulated in previous studies, we investigated the feasibility of keeping swine limbs viable on an ex situ limb perfusion system in this study.

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forelimbs were amputated, preserved at 4°C for 6 hours, and transplanted to another healthy swine. Recipients in both groups were observed 12 additional hours during the reperfusion.

**Animal Model**

Swine, 40 ± 5 kg, were sedated with an intramuscular mix of 5 mg/kg tiletamine HCl and zolepam HCl (Telazol Wyeth Holdings Corporation, Carolina, Puerto Rico) and 3 mg/kg xylazine (TranquiVed Vedco Inc, St. Joseph, MO). Animals were intubated and ventilated with 100% O₂ and 1% to 3% isofluorane (Hospira, Inc., Lake Forest, IL). Initial mechanical ventilator settings were adjusted 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 catheterized to monitor mean arterial blood pressure and heart rate, and to collect blood samples. A central line was placed in the internal jugular vein to monitor central venous pressure and maintenance fluid administration (80–120 mL/hr). Prophylactic antibiotic (cefazolin, 1 g) was administered throughout the procedure every 6 hours.

**Surgical Technique**

Preparation of the donor forelimb: The animal was placed supine on the operating table. Both forelimbs were shaved first and prepared using betadine solution for skin cleansing. Two longitudinal incisions were placed on the medial and lateral aspects of the limb, approximately 12 cm each. Both of these incisions were connected to a circumferential incision around the antecubital fossa. First, the incision on the lateral aspect of the limb was explored. Two skin flaps (anterior and posterior) were elevated to expose the lateral aspect of the flexor and extensor compartments. The triceps tendon was detached from the olecranon and marked using 0 silk. On the medial side, 2 skin flaps were elevated anteriorly and posteriorly. Care was taken to protect the cephalic vein during the dissection. The biceps tendon was identified at the antecubital fossa and detached along with the brachialis muscle.

This was followed by elevation of flexor/pronator mass and extensor compartment muscles off the medial and lateral epicondyles, respectively. The median, ulnar and radial nerves were then identified and transected. An electrode was placed on the median nerve for later use in neuromuscular electrical stimulation. Finally, the brachial artery and accompanying veins were transected 5 cm proximal to the antecubital fossa, and the elbow was disarticulated. The brachial artery was cannulated, and limbs were flushed with 10000 units of heparin in 100 mL of saline without any other preservative solution. In the control group, procured forelimb was taken to a cold storage room. In the experimental group, the limb was attached to the ex situ limb perfusion system.

Preparation of the recipient and transplantation: Recipient was anesthesized 2 hours before the end of preservation. Amputation was performed on the ipsilateral forelimb at the same level. At the end of its preservation, the limb was brought to the operating field and fixed to the recipient’s ulnohumeral joint using 2 cross Steinman pins. This was followed by re-approximation of biceps, triceps, forearm flexor, and extensor muscles. The ends of the neurovascular bundle at the antecubital fossa were approximated, and the artery was anastomosed using 8.0 ethilon suture under magnification along with its 2 accompanying veins. In addition, the cephalic vein and/or basilic veins were also anastomosed when available. Before opening the clamps for revascularization of the limb, recipient was injected with 200 μm/kg heparin subcutaneously. Skin was approximated using 2.0 silk.

**Ex-situ Perfusion Protocol**

Throughout this study, we used pressure-limited pulsatile perfusion (with organ flow controlled by resistance), room temperature (27°C–32°C), open venous drainage, and membrane oxygenation to maintain stable oxygenation and acid-base balance (Figure 1). As a perfusate, we used fresh autologous plasma (hematocrit, 10%), with periodic addition of glucose. The perfusate did not contain white cells or platelets.

Preparation of the perfusate: first, the perfusion system (including the pump and all collateral circuit) was primed
using 200 cc of Dulbecco’s Modified Eagle’s Medium (Lonza, Walkersville, MD). In the meantime, the donor blood obtained after procurement of the limb was centrifuged at 3500 RPM for 15 minutes to remove white blood cell and platelet fractions. Once those fractions were removed, packed red blood cells and plasma were mixed in 1 to 2 ratio, along with dextran to reach the colloid osmotic pressure of 20 to 30 mm Hg. Colloid osmotic pressure was measured using osmometer (Wescor Inc., Logan, UT) before this mixture was added to the pump. Throughout the experiment, we gradually removed 160 mL of perfusate every 2 hours, and substituted with replacement perfusate with 10% hemoglobin with similar parameters of plasma oncotic pressure. This exchange removed toxic metabolites from the system in the absence of liver and kidney. We also measured perfusate glucose levels every 2 hours and substituted the perfusate with either glucose (1 cc D50%) if the level was less than 4.5 mmol/L, or with insulin (2 units) if glucose was above 14 mmol/L.

The perfusion apparatus consisted of an RM3 pulsatile perfusion pump (Waters Medical Systems, Minneapolis, MN) and a commercially available Terumo oxygenator (Baby Capiox RX; Terumo, Tokyo, Japan) 0.5 m² connected to a 70% O₂, 5% CO₂ gas tank. The gas mixture to the membrane lung was 5% CO₂, 95% O₂. The perfusate was fresh pig plasma and red blood cells, supplemented with glucose to maintain perfusate glucose in the range of 100 to 200 mg/dL. Pulsatile perfusion at room temperature (27°C) was maintained with a pressure limitation of 60 to 80 mm Hg. During perfusion, the limb was prepared in sterile fashion and maintained in an antiseptic Dakins solution dressing, and the perfusate was continuously sterilized with ultraviolet light. Flow was measured continuously, and vascular resistance was calculated frequently as an indicator of successful perfusion. The following parameters of perfusion were measured throughout organ perfusion: pressure, flow, resistance, the fiber in a solution containing a high concentration of Ca²⁺ to relax. Fiber bundles of approximately 4 to 6 mm in length and 0.5 to 0.75 mm in diameter were dissected from muscle biopsies. Bundles were placed in a relaxing solution containing Ca²⁺ chelating agents in a pH-buffered solution that causes muscle fibers to relax. Fiber bundles 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.

**Statistical Analysis**

Data are presented as mean ± SEM. Differences between the control and perfusion groups were tested using paired t-test.

**Single-Muscle Contractility Testing**

The contractility of permeabilized muscle fibers was tested as previously described. Muscle biopsies were taken parallel to the longitudinal axis of muscle fibers sized 10 mm in length and 5 mm in width (Figure 2). Biopsies were immediately placed in a relaxing solution containing Ca²⁺ chelating agents in a pH-buffered solution that causes muscle fibers to relax. Fiber bundles of approximately 4 to 6 mm in length and 0.5 to 0.75 mm in diameter were dissected from muscle biopsies. Bundles were placed in stretching solution to further permeabilize the cell membranes for 30 min, and then in storage solution for 16 hours at 4°C, followed by storage at −80°C. On the day of single-fiber testing, bundles were thawed slowly on wet ice, and individual fibers were pulled from bundles using fine forceps. Fibers were then placed in a chamber containing relaxing solution and secured at one end to the servomotor (Aurora Scientific, Aurora, ON) and at the other end to a force transducer (Aurora Scientific) using 2 ties of 10-0 monofilament nylon suture at each end. Using a laser diffraction measurement system, fiber length was adjusted to obtain a sarcomere length of 2.7 μm. The average fiber cross-sectional area was calculated assuming an elliptical cross-section, with diameters obtained at 5 positions along the fiber from high-magnification images at 2 different views (top and side). Maximum fiber isometric force (F₀) was elicited by immersing the fiber in a solution containing a high concentration of Ca²⁺ and adenosine triphosphate (ATP). Specific force of fibers (sF₀) was calculated by dividing F₀ by 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.
RESULTS

All animals in group 1 displayed satisfactory flow to the extremity after transplantation and remained hemodynamically stable. In group 2, after 6 hours of cold storage and transplantation, blood flow was established only in large size vessels (brachial and radial arteries), there was no flow in microcirculation or in muscle planes.

All recipients remained hemodynamically stable. Perfused limbs initially showed a higher resistance within the first 3 hours ($P < 0.05$), which later returned to normal (Figure 2). Overall weight gain at the end of perfusion was statistically significant compared to limb weight before pump perfusion ($P < 0.05$). No significant gain was observed between the beginning and end of reperfusion ($P = 0.05$; Figure 3).

Among all measured parameters in Figure 3, $K^+$, pH, $pCO_2$, and $pO_2$ did not show any statistically significant change before (on pump perfusion) or after transplantation (Figure 4, $P > 0.05$). Lactate levels however showed a gradual increase on pump perfusion ($P < 0.05$) and returned to normal levels after the transplantation (during reperfusion).

Single-muscle fiber contractility testing showed a gradual decline in the cold preservation group, whereas in the pump perfusion group, contractility remained close to normal controls during the perfusion and reperfusion afterward. In the control group (Figure 5, right side), muscle contractility shows a statistically significant decline at 12, 18, and 24 hours after the amputation compared to the contralateral side, as seen on $F_o$ and $sF_o$ values ($P < 0.05$). In the pump perfusion group, muscle contractility returned close to contralateral limb values at 24 hours ($P > 0.05$; Figure 5, left column).

DISCUSSION

In this study, we demonstrated the feasibility of using an ex situ warm perfusion system to keep limb allografts viable up to 12 hours. This is demonstrated by ongoing observable muscle contraction in response to electrical stimulation throughout the reperfusion phase, as well as marked improvements in $sF_o$ values at the end of 12 hours of reperfusion.
FIGURE 4. Data in left column show metabolic parameters of limb perfusion when the limb was attached to ex situ perfusion. Data in right column show metabolic parameters of recipient after transplantation of the limb. Error bars represent standard error of means (SEM).
Using alternating doses of insulin/glucose and changing the perfusate every 2 hours, we were able to maintain potassium, pH, and lactate at constant levels. Oxygen delivery and consumption were also both well maintained under room temperature (27°C–32°C).

Current standard of care for the preservation of vascularized composite tissue allografts is static cold storage at 4°C using UW solution. This method has been widely used in solid organ transplantations since the 1960s. The first static storage solutions were introduced by Collins et al.17 and were subsequently modified throughout the 1980s as EuroCollin’s solution.18 Cooling slows metabolic processes and prolongs the time during which the organ can be deprived of oxygen without loss of viability. However, energy consumption due to metabolic activity is not halted but reduced and, ultimately, the limitation of cold storage is determined by mitochondrial damage and subsequent ATP depletion.19 In cold storage, metabolism is slowed 1.5- to 2-fold for every 10°C drop in temperature,20 and anaerobic metabolism leads to depletion of ATP stores and build up of acidotic environment.21 To prevent and restore energy charges, normothermic preservation methods gained popularity in recent years. Studies have shown maintenance of normal metabolic function as in liver, kidney, and lung transplant models.13,22,23 In this study, we used blood at room temperature (27°C–32°C) and were able to maintain stable oxygen delivery and consumption after transplantation and 12 hours of reperfusion. This is further supported in the current study by measures of muscle force production at the cellular level. Limbs on pump perfusion had smaller decreases in sF₀, and when the limb was reattached, the force values improved markedly. This is in stark contrast to the limbs on cold perfusion, which rapidly lost sF₀ and did not regain force production once the limb was reattached. The recovery of force production is likely due to improved muscle cell survival and a reduction in apoptosis. As muscle tissue death can limit the viability of a limb after reattachment, this suggests that not only does pump perfusion improve muscle force production acutely, it also likely contributes to long-term survival of the transplanted limb.

Initial perfusion experiments using blood were mostly focused on hemodynamic parameters of limb perfusion.24,25 Blood, or hemoglobin as an oxygen carrier, has not been used for decades because of its potential to propagate metabolic overload and stimulate oxidative metabolism. It has been shown on many animal models as well as in the clinical setting that the use of blood as an artificial oxygen carrier is safe and reliable. Few studies however have shown the effective use of normothermic blood on composite tissue allografts. Constantinescu et al.26 showed successful perfusion of amputated swine limbs up to 12 hours. In their feasibility study, they used a modern extracorporeal heart-lung circuit and autologous blood at 32°C for perfusion. Their results indicated effective perfusion of the limbs as evidenced by steady mean arterial perfusion, high pH, and low lactate levels.26 The study however did not include the replantation of the limb to another recipient, and therefore did not account for the ischemia-reperfusion injury. Later, the same group perfused swine forelimbs for 12 hours and replanted to another animal to observe changes associated with ischemia/reperfusion injury.27 Using histopathology and markers of inflammation, they demonstrated that extracorporeal circulation had a negligible effect on reperfusion-induced injuries.

![Graphs showing muscle contractility](image)

**FIGURE 5.** Arrow on x-axis indicates the time of transplantation. After transplantation, biopsies were obtained at first, sixth, and 12th hours. Graphs in the left column show data regarding muscle contractility within the first 12 hours on pump perfusion and 12 hours after the transplantation. Data on the right show muscle contractility of control group, which was subjected to 6 hours of cold preservation and transplantation afterward.
Our findings are similar and further show not only survival but also promising functional recovery as evidenced by single-muscle fiber contractility testing.

Limitations of the current study include limited reperfusion period (12 hours). As a result, we were able to obtain the information regarding the initial phase of the reperfusion injury. To observe its long-term effects on nerve regeneration and limb function, we need to conduct a separate set of experiments to keep recipient animals alive under low dose immunosuppressants until after the nerve regeneration is completed.

The animal model presented in the current study is closer to human upper extremities in terms of its metabolism and circulatory hemodynamics. The method has a potential to application in limb replantation and transplantation.

REFERENCES