Advances in Limb Preservation: From Replantation to Transplantation

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Over the course of the last 60 years, microsurgical techniques, instrumentation, operating microscopes, and suture materials have all been perfected. Microsurgery training became part of the standard curriculum in plastic, orthopedic, and hand surgery programs. Despite those advances, limb replantation and transplantation are still surgical emergencies owing to limits in composite tissue viability under ischemia. Amputated parts, particularly containing large volumes of muscle, have to be revascularized within 4 hours in order to prevent permanent tissue damage. Static cold storage is the current standard to prolong ischemia time with limited success. Our research for the last 7 years has focused on extending ischemia time prior to revascularization. Our long-term goal is to make replantation and transplantation procedures elective. The following essay is the summary of our efforts. *(J Hand Surg Am. 2020;45(7):626–637. Copyright © 2020 by the American Society for Surgery of the Hand. All rights reserved.)*

Key words *Ex situ* perfusion, ischemia-reperfusion injury, replantation, transplantation, vascularized composite tissue allografts.

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THE ORIGIN OF ORGAN AND TISSUE preservation research goes back to the beginning of the 20th century.[1,2] The first experiments were performed on human extremities by Carrell and Lindbergh,[3,4] who observed the potential for cold storage to preserve tissues. They designed a machine that circulated sterile, pulsatile fluid within a leg to remove toxic metabolites.[3,4] Although a composite tissue was used to develop the first perfusion machine, in the clinical setting, machine perfusion was first used to transplant a kidney in 1968, and perfected in solid organ transplantations over the course of the last 5 decades.[5]

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Most of the experimental work on limb preservation was focused on studying the mechanism of reperfusion injury using various preservative solutions, primarily for single-infusion or static preservation. Until recently, removal of the toxic metabolites from the circulation on an ongoing basis or the delivery of oxygen and other nutrients to composite tissues has not been investigated. For the last 7 years, our group has focused on prolonging limb survival using hypothermic and normothermic machine perfusion. Building on the foundations of organ preservation research, our goal is to perform replantation and transplantation procedures in an elective setting. To achieve this goal, we developed 3 experimental models including a large animal model (swine forelimb),[6] a human forearm/hand allograft model,[7] and a small animal model (rat hindlimb).[8] This approach allowed us to study the short- and long-term effects of preservation and perfusion on extremity viability and function.

WHAT ARE THE LIMITATIONS OF THE STATIC COLD STORAGE?

Extremity parts consist of heterogeneous tissues including skin, muscle, tendon, nerve, bone, and vessels with different metabolic thresholds to

ischemia. In particular, muscle and endothelium are highly susceptible to ischemia and cell death.[9] The current standard of care for amputated parts is cold storage.[10] This is a static process and reduces the metabolic rate 1.5- to 2-fold for every 10°C drop in temperature.[11] It is practical, inexpensive, and effective, as long as ischemia time does not exceed a critical point. Prolonged static cold storage (SCS) has detrimental effects on the microcirculation, particularly during the reperfusion phase when blood flow to the limb is restored.[12] At the cellular level, SCS initially reduces metabolism by inhibiting enzymes operational in oxidative phosphorylation.[11,13] When oxidative phosphorylation is slowed, adenosine triphosplate (ATP) production is also reduced, leading to the depletion of energy stores. Depleted energy stores, in the absence of oxygen, result in anaerobic metabolism, which in turn accumulates lactate. Excess lactate and depletion of ATP stores results in deterioration of the Na^+/K^+-ATPase pump, cellular edema, and membrane disruption.[14–16] As leukocytes return to the microcirculation during reperfusion, edematous or disrupted endothelial cells and myocytes are targeted for repair or removal.[17] Leukocyte involvement also leads to the activation of the immune system.[18,19] In the case of transplantation, acute and chronic rejection episodes become more prevalent.[19–21]

How long the tissue survives under cold ischemia depends on its metabolic activity and energy stores. Previous studies have demonstrated that muscle and endothelial cells incur irreversible damage at 4 hours of warm ischemia and 6 hours of SCS.[9] Yet, in hand allografts, cold preservation time was reported to be 5.5 hours on average, extending up to 13 hours in some cases. Particularly, allografts containing large volumes of muscle do not appear to do well after prolonged cold storage.[10] In at least 1 report, severe coagulative myonecrosis, which is the hallmark of cold ischemia injury in the muscle, was reported as early as after 7 hours of SCS of a hand allograft.[22] In that case, despite the maintenance of hand and forearm viability, function was gradually lost in 6 months following transplantation.

Based on this clinical experience and accumulated evidence, it is clear that parts containing large volumes of muscle are more directly affected by prolonged cold preservation than those more distal amputations. The current standard of care (SCS) is effective for a short time but prolonged cold storage has detrimental effects on survival and long-term function of the amputated part. Strategies to sustain and improve the viability and function of composite tissues prior to revascularization have the potential to be the next major leap in extremity reconstruction. To achieve this goal, we used *ex situ* perfusion systems developed on solid organ transplantation models and adapted them to 3 extremity models. The system consists of a roller pump, an oxygenator, a filtration system, a heating/cooling circuit, and tubing. We adjusted and tested each of these components to determine the ideal temperature, perfusate, perfusion pressure, and perfusion mode for the specific model. Throughout these experiments, we tested various combinations of red blood cells, plasma, and synthetic perfusates.

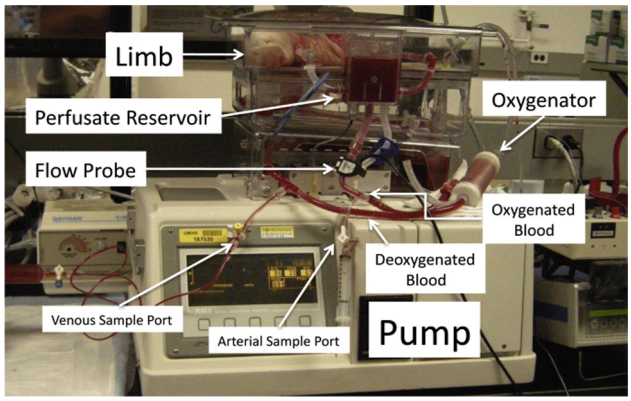

FIGURE 1: Swine forelimb perfusion system consists of a temperature-controlled limb chamber, an oxygenator (Baby Capiox-Rx; Terumo, Tokyo, Japan), a pulsatile perfusion pump (Waters Medical Systems, Minneapolis, MN), a perfusate reservoir for open drainage, and a heat exchanger to maintain temperature at 27°C to 32°C.

SWINE FORELIMB MODEL

Based on hemodynamic similarities to humans, we developed our first model on swine forelimbs.[6] A semi-open system was adapted at near-normothermic (30°C–33°C) temperatures using red blood cells as the perfusate (Fig. 1). Amputated forelimbs were cannulated at the brachial artery and connected to the *ex situ* perfusion system at 30 mL/min flow rate with a mean system pressure maintained around 62 mm Hg. Limbs perfused under these pressures had minimal third space loss and edema, gaining only 1.32% weight after 24 hours of continuous perfusion. These results represented a 10-fold optimization of the reported rates of 20% to 50% weight gain in other extremity perfusion setups.[23,24]

Next, we transplanted the perfused forelimbs to observe the metabolic changes associated with ischemia reperfusion injury. Six perfused limbs in the control group were infused with the University of

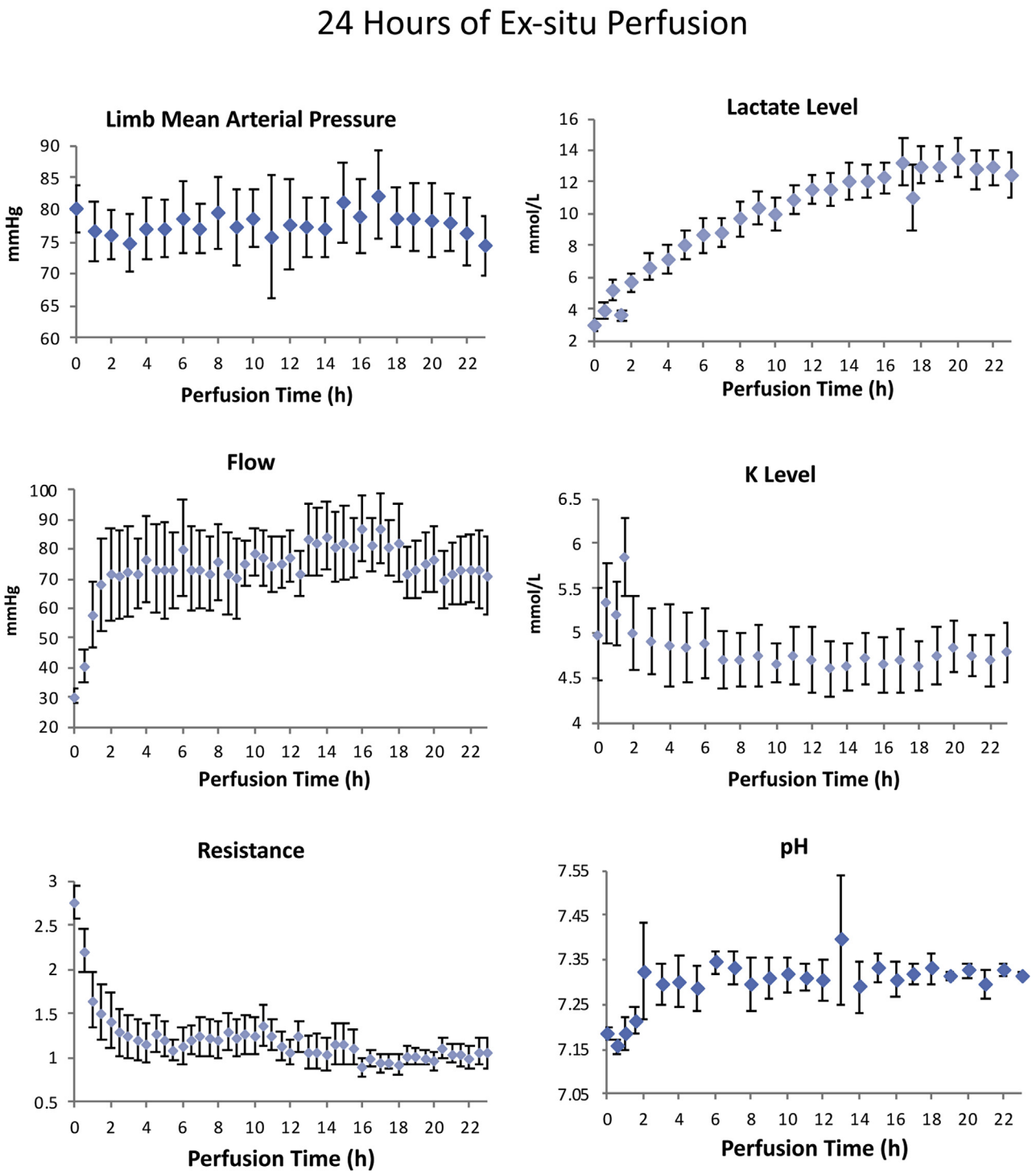

FIGURE 2: Graphs show changes in perfusion parameters over the course of 24 hours of limb perfusion before the limbs were transplanted. Gradual increase in lactate levels is a sign of ongoing metabolic activity switching slowly toward anaerobic respiration.

Wisconsin solution and stored at 4°C for 4 hours prior to transplantation. In the experimental groups, we gradually increased the perfusion time from 6 hours to 24 hours.[6,25]

At 12 hours posttransplantation, all limbs preserved in SCS immediately lost their contractility with significant increases in K^+ and lactate levels, and a decrease in pH. In the experimental group, all hemodynamic parameters and electrolytes remained stable during the *ex situ* perfusion (Fig. 2) and the reperfusion period (Fig. 3). Histology showed minimal signs of muscle damage in the perfused limbs whereas cold-

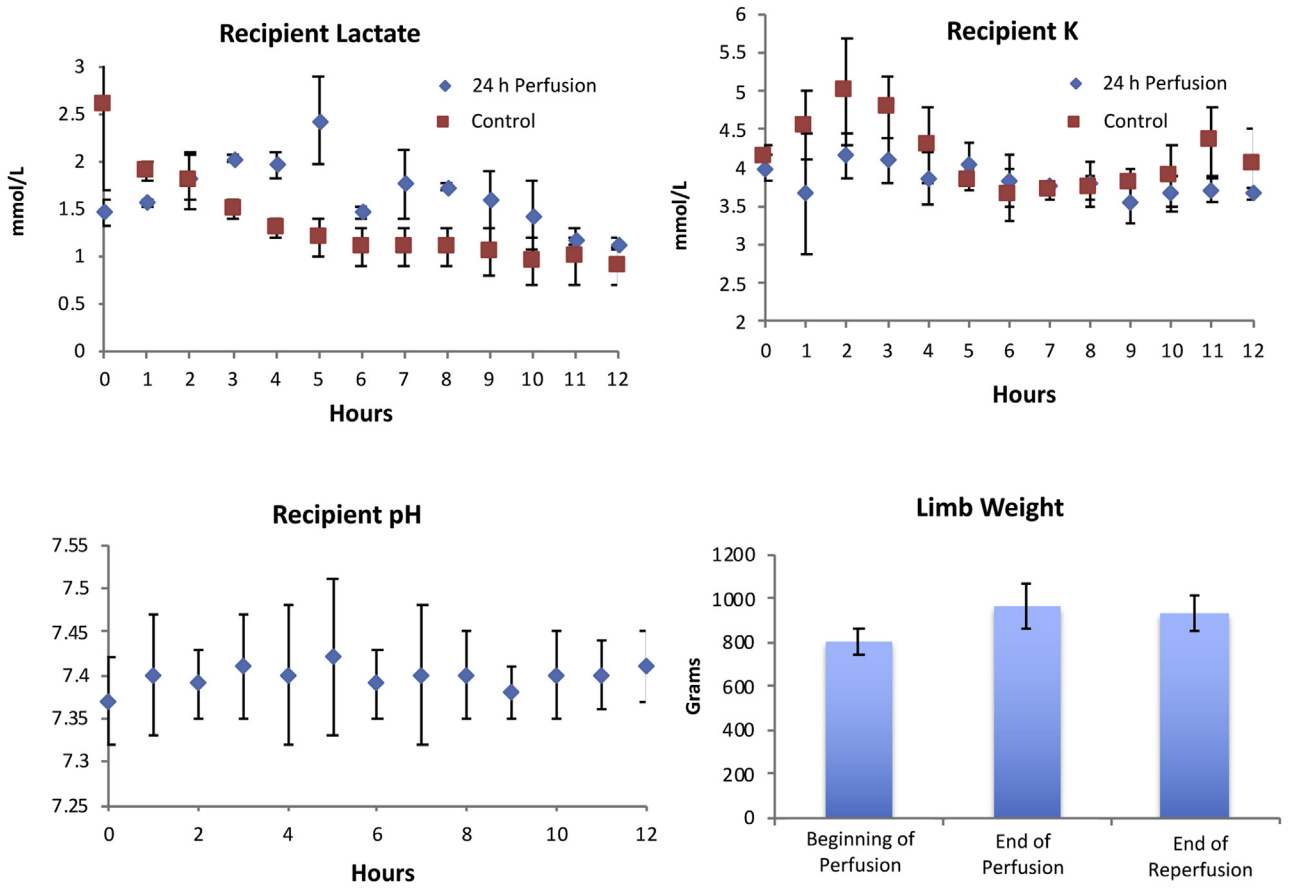

FIGURE 3: Graphs show changes in recipients within the first 12 hours following transplantation. Limbs perfused for 24 hours are in blue, the control group is in red. Initially high lactate level gradually returns to normal. Limb weight gain does not exceed 5% after 24 hours of *ex situ* perfusion and 12 hours of reperfusion.

preserved limbs revealed widespread structural injury. Contractile properties of the smallest functioning muscle unit (single−muscle fiber contractility testing) also showed normal contraction force compared with the contralateral side, whereas limbs preserved in cold storage failed to generate as much force (Fig. 4). Finally, TUNEL (terminal deoxynucleotidyl transferase dUTP [deoxyuridine triphosphate] nick end labeling) staining showed minimal or no apoptotic muscle cells in the *ex situ* perfusion similar to no-injury controls (Fig. E1; available on the *Journal*'s Web site at www.jhandsurg.org).

Altogether these results demonstrated successful extension of limb viability after 24 hours of near-normothermic pulsatile *ex situ* perfusion using diluted red blood cells. This was our first demonstration of an entire forearm (distal to the elbow) maintaining its viability for 24 hours without cardiopulmonary circulation.

HUMAN HAND AND FOREARM MODEL

Next, we developed a portable device to be used in the clinical setting to extend human forearm allograft viability. The *ex situ* perfusion system contained all the elements previously mentioned (Fig. 5 and Video A; available on the *Journal*'s Web site at www.jhandsurg.org).[7] To test the system, 5 human forearm allografts disarticulated at the elbow were procured from donors after brain death and connected to machine perfusion within 75 minutes, on average. We used diluted banked blood under near-normothermic temperatures (30°C−33°C) in a pulsatile fashion. After 24 hours of *ex situ* perfusion, extrinsic flexors (Video B; available on the *Journal*'s Web site at www.jhandsurg.org) as well as intrinsic muscles of the hand (Video C; available on the *Journal*'s Web site at www.jhandsurg.org) continued to have contraction against gravity. All other metabolic parameters remained steady throughout the

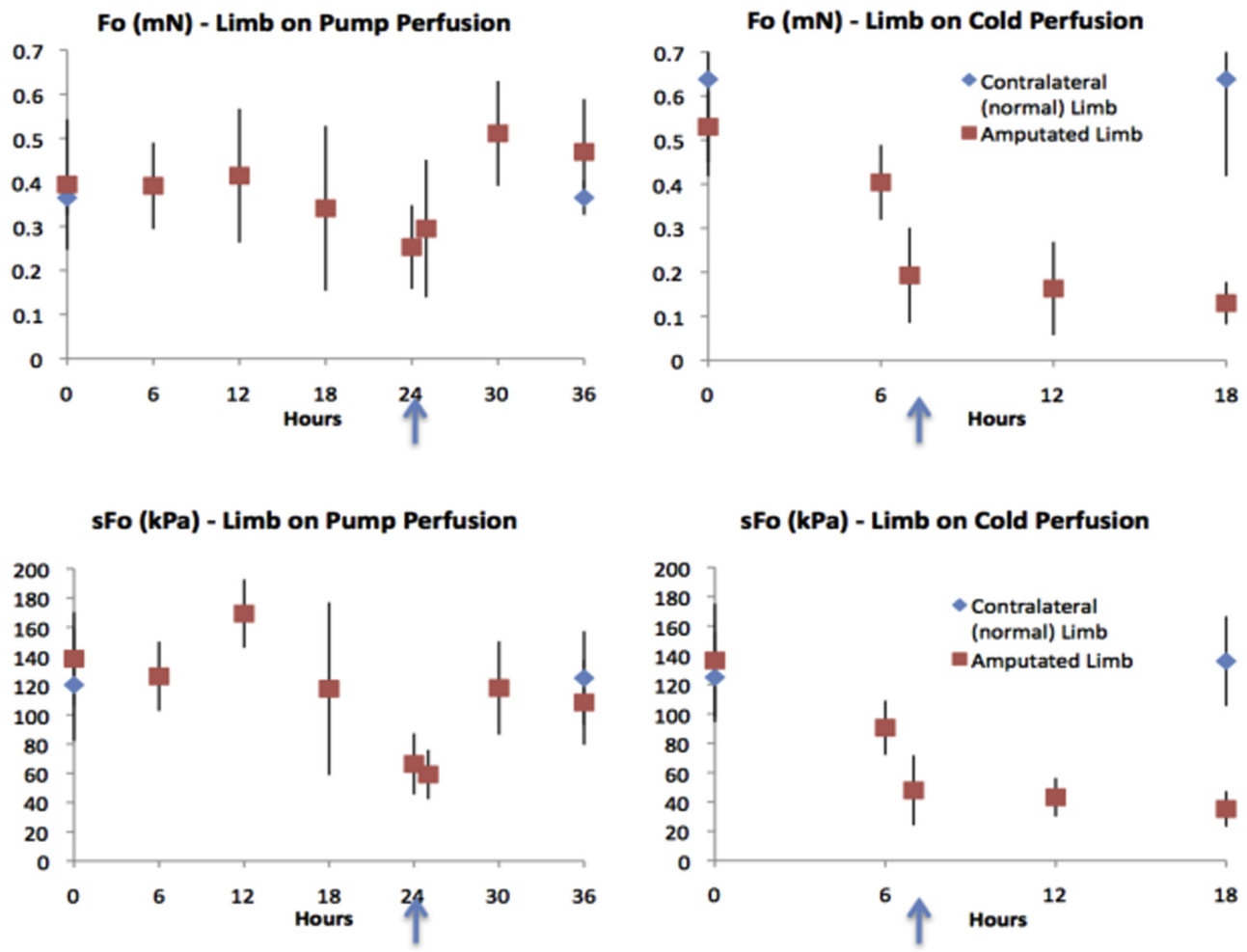

FIGURE 4: For single—muscle fiber contractility testing, 1 end of the skinned flexor carpi ulnaris muscle fiber is secured to a servomotor (Model 322C; Aurora Scientific, Aurora, Ontario, Canada) and the other end to a force transducer (403A; Aurora Scientific). Each fiber length was adjusted to obtain a sarcomere length of 2.5 μm using a laser diffraction measurement system. Maximum fiber isometric force (F_o) was elicited by immersing the fiber in a high concentration Ca^{2+} and ATP solution. Graphs show maximum isometric force (Fo) and specific force (sFo). Muscle samples were obtained every 6 hours until the end of reperfusion at 36 hours (24 hours of *ex situ* perfusion + 12 hours of reperfusion). Arrows indicate the time of transplantation. Graphs on the left side show the *ex situ* perfusion group revealing equal muscle force compared with no-injury controls, whereas samples from cold-preserved limbs (right column) show complete loss of contractile properties.

perfusion for 24 hours (Fig. 6). During this time, peripheral vascular resistance remained low and overall weight gain in the limb was no more than 3% with minimal barotrauma on histology.

This was the first demonstration of viability up to 24 hours on a human forearm allograft without a cardiopulmonary circulation. These results were unprecedented and promising to demonstrate short-term effects of *ex situ* perfusion. In the next step, we decided to study the long-term effects of *ex situ* perfusion, particularly on neuromuscular regeneration in a cost-effective manner, and adapted our system to the rat hindlimb transplantation model.

RAT HINDLIMB MODEL

Rat hindlimb allograft transplantation is an established model to observe signs of reperfusion injury and to measure neuromuscular regeneration.[26–29] Limb transplantation within the same strain (isograft) does not require immunosuppression, which eliminates the effects of acute and chronic rejection on reperfusion injury. Our first question was to test whether the rat hindlimb allograft model was sensitive enough to demonstrate changes associated with SCS after transplantation and replantation procedures. For that, amputated rat hindlimbs were flushed with heparinized saline and stored at 4°C for 6 hours prior to transplantation, similar to the global

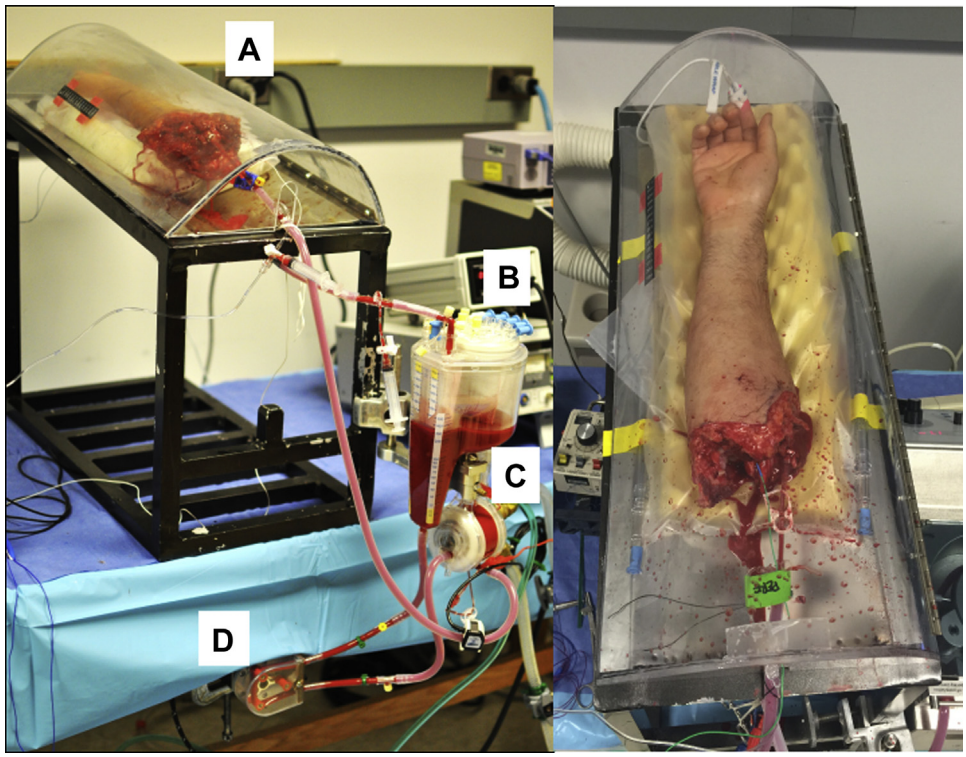

FIGURE 5: **A** Limb chamber consists of a custom-made frame with fiberglass lid with 15° inclination to collect blood draining openly. **B** Reservoir with **C** Oxygenator (Baby Capiox-Rx). **D** Roller Pump (Shiley Roller Pump; Stockert Instruments, Munich, Germany; or M pump; MC3, Ann Arbor, MI). Pulse oximetry was used continuously to monitor oxygen saturation. Circuit flows were controlled through the pump, with target flow set to 6% to 8% of the donor's estimated cardiac output. Systolic pressure was maintained at 110 mm Hg; circuit flow was adjusted, when necessary, to stay below this limit. Temperature within the chamber is maintained at 30°C to33°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. 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, and 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.

average of cold ischemia time for hand transplantation. Control groups (n = 6 in each) included normal limb, sciatic nerve transection/repair (no transplantation), immediate transplantation (no preservation), and static warm storage (at room temperature for 6 hours). At the completion of sciatic nerve regeneration at 12 weeks, all groups underwent needle EMG, extensor digitorum longus (EDL) muscle force measurements, histology (muscle and nerve), and muscle metabolomics analysis.

Results showed that the SCS group had significantly decreased EDL muscle force, and significantly increased muscle injury severity score, compared with immediate transplantation and sciatic nerve repair-only groups (Fig. 7).[30] This demonstrated that SCS failed to protect muscle structure in the long term. The SCS group compared with normal muscle also had reduced EDL force similar to adult human hand transplant patients (Fig. 8). Muscle metabolomics analysis at the end of ischemia showed no change in energy charge status across groups. However, ATP, phosphocreatine, and ATP/ADP (adenosine diphosphate) ratio showed a gradual decrease in all limbs preserved in room temperature (Fig. E2; available on the *Journal*'s Web site at www.jhandsurg.org) [31]. Glycolytic pathway was well preserved in the SCS group, whereas purine degradation products and redox cofactors remained low (Fig. E3; available on the *Journal*'s Web site at www.jhandsurg.org). Interestingly, succinate levels appeared to have correlated best with the muscle injury severity scores and force generation.

These results were parallel to those observed in clinical hand transplantation cases in which almost all hand allografts stored at cold temperatures up to 6 hours survived, but they did not show equal

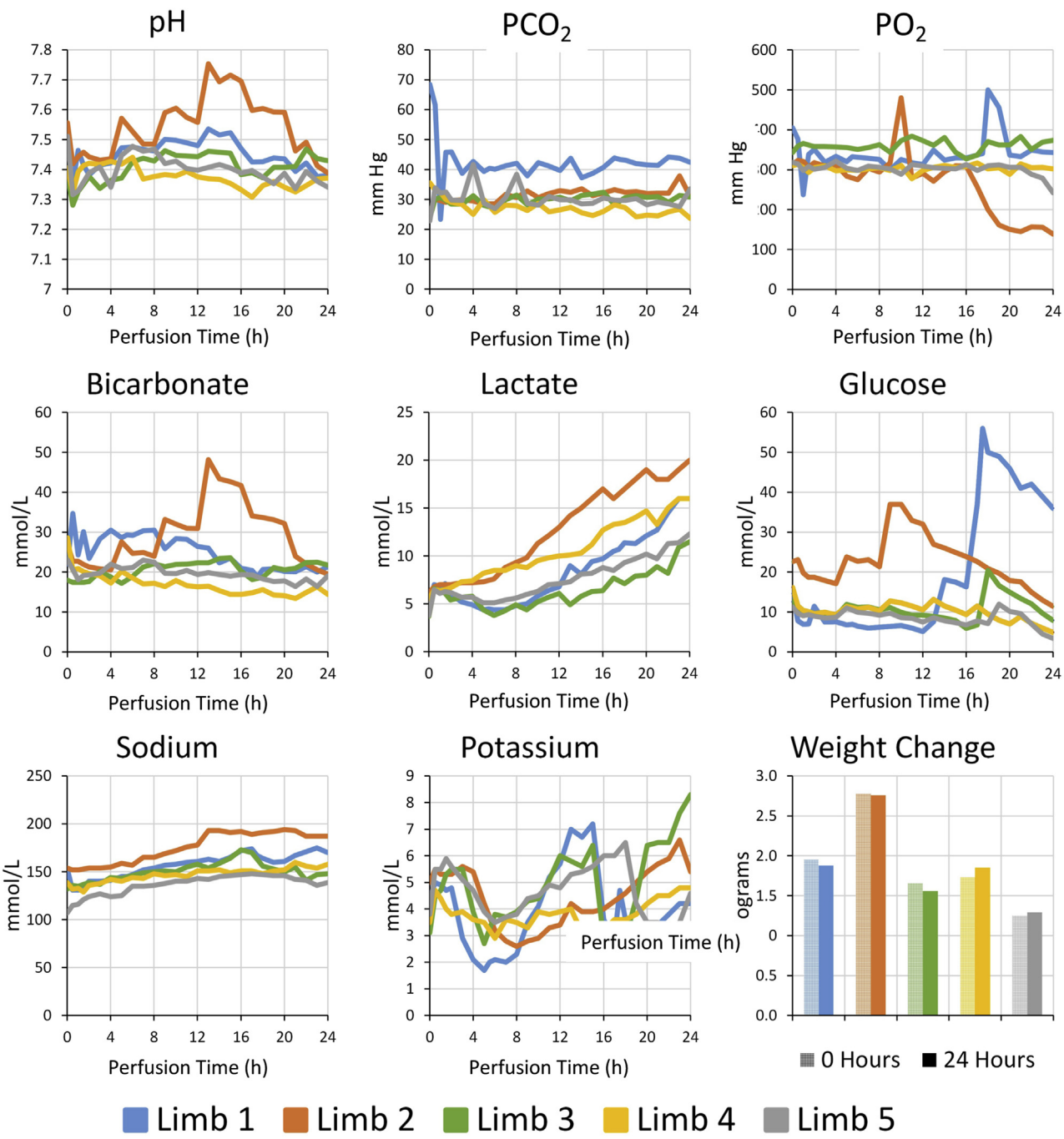

FIGURE 6: All 5 limbs were procured from brain-dead, heart-beating patients. Average cold ischemia time was 75 minutes between procurement and beginning of the perfusion. Donors of limb #1 (blue) and #2 (orange) had diabetes with partial insulin resistance resulting in unexpected increases in blood glucose. Hemodynamic variables remained stable throughout 24 hours of *ex situ* perfusion. Lactate increase over time was controlled with perfusate exchange as there was no filter within the circuit. Despite a gradual increase in lactate, pH remained steady. PCO_2, partial pressure of carbon dioxide; PO_2, partial pressure of oxygen.

function compared with others with shorter ischemic times. Unlike solid organ transplantations, vascularized composite allografts (VCA) appeared to have well-preserved energy stores on metabolomic analysis despite extensive muscle injury and reduction in force. The muscle metabolomic markers used to assess transplant viability in solid organ transplantations were not useful in this model. In summary, the rat hindlimb allograft transplantation model was confirmed to be an appropriate model to study reperfusion injury in VCAs.

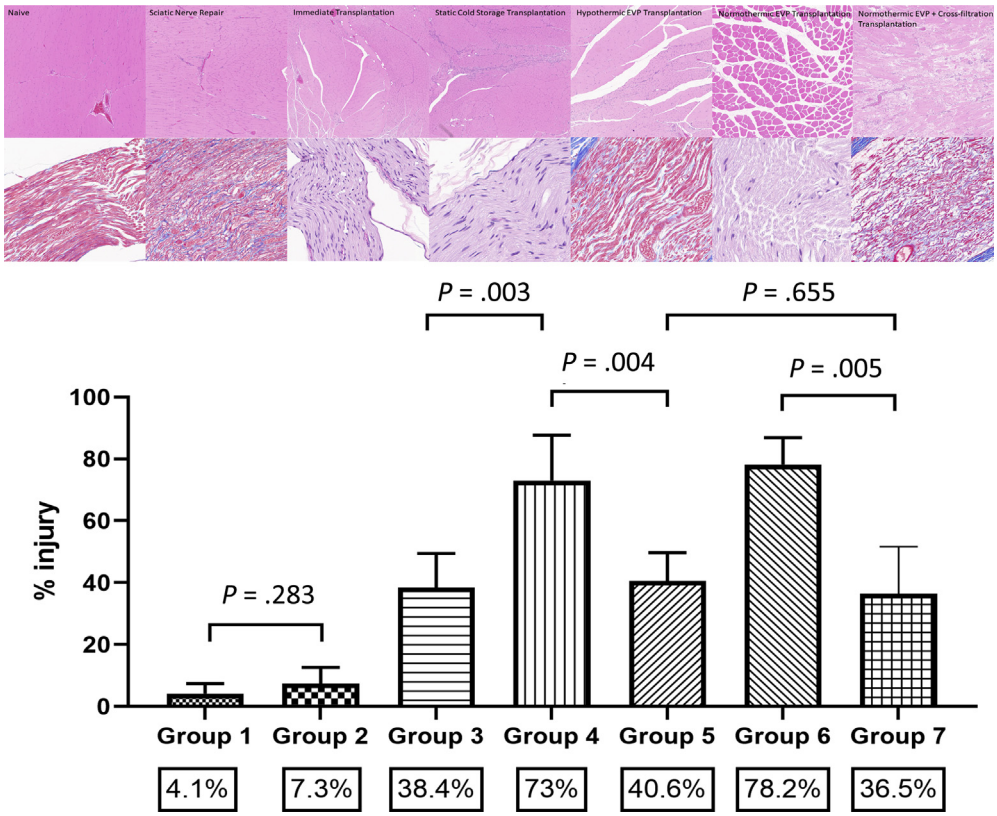

FIGURE 7: Top, Muscle (first row) and nerve (second row) histology. Graphs show the muscle injury severity score. Extensive muscle necrosis and axonal degeneration were observed in groups 4 and 6. Near-normothermic *ex situ* perfusion (group 7) and hypothermic machine perfusion (group 5) groups provided better protection for the muscle. However, hypothemic machine perfusion group (5) had extensive axonal loss, as seen on nerve samples, significantly worse than those seen in group 7. Group 1: naive; group 2: sciatic nerve transection/repair; group 3: immediate transplantation; group 4: SCS transplantation; group 5: hypothermic machine perfusion transplantation; group 6: near-normothermic *ex situ* perfusion transplantation without plasma; group 7: near-normothermic transplantation with fresh plasma.

Next, we developed a miniature *ex situ* perfusion system adapted to the animal model (Fig. 9).[8] During its development phase, we determined the ideal vessel diameter (iliac artery), cannula size (22-gauge), physiological perfusion pressure (50 ± 20 mm Hg), and the ideal mean perfusion flow rates (1 ± 0.3 mL/min). This approach resulted in minimal shear stress with only 3% ± 0.5% weight increase after 6 hours of perfusion. With continuous hemofiltration rate of 6 mL/h; lactate and potassium remained low at 4.3 ± 1.25 mmol/L and 6.3 ± 1.17 mmol/L, respectively. Continuous hemofiltration provided with a more effective method of waste product clearance than frequent plasma exchange.

Using parameters established in our pilot study, we compared multiple *ex situ* perfusion modalities with SCS. These included hypothermic (10°C−15°C) perfusion using histidine-trytophan-ketoglutarate solution, near-normothermic (30°C−33°C), and subnormothermic (20°C−22°C) perfusions using red blood cells and plasma. All amputated limbs were perfused for 6 hours and transplanted. At 12 weeks, muscle and nerve histology, muscle metabolomics, and EMG measures were compared.

In hypothermic machine perfusion at 10°C to 15°C, all limb recipients survived. Muscle histology showed mild to moderate myocyte damage, comparable with those seen in the immediate transplantation group, but significantly better than the SCS group (Fig. 7).[32] Interestingly, findings in EMG were not parallel to those seen in histology and showed reduced amplitude and latency in the hypothermic machine perfusion group, significantly lower than those in the immediate transplantation group (Fig. 8). This group also displayed reduced force generation in maximum tetanic and twitch forces. Muscle metabolomics analysis of amino acid profile in gastrocnemius muscle samples, however, did not show statistically significant differences in terms of muscle composition between groups (Figs. E4, E5; available

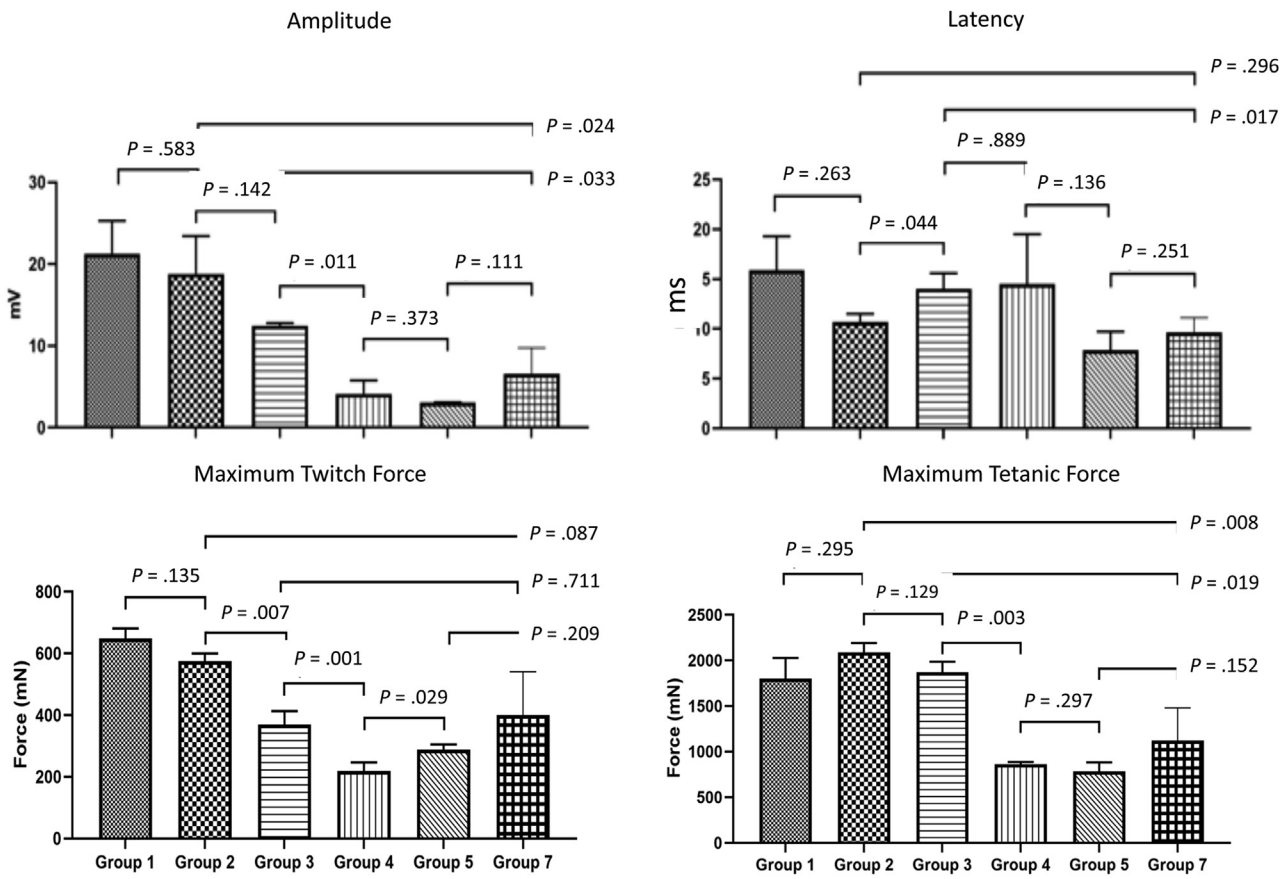

FIGURE 8: Group 1: naive; group 2: sciatic nerve transection/repair; group 3: immediate transplantation; group 4: SCS transplantation; group 5: hypothermic machine perfusion transplantation; group 7: near-normothermic transplantation with fresh plasma exchange. Note that there were no survivors at 12 weeks in group 6.

on the *Journal*'s Web site at www.jhandsurg.org). Altogether these results demonstrated that the hypothermic machine perfusion was better than the current standard of care (SCS), and protected muscle cells from structural injury compared with an immediate transplantation group.[32] But, as shown in EMG and histology, it adversely affected the myelin sheath thickness and its regeneration potential at hypothermic temperatures. Prolonged hypothermic machine perfusion appeared to affect the nerve and the muscle tissue, a finding that may have important implications in hand transplantation and replantation.

In near-normothermic (30°C−33°C) machine perfusion using red blood cells, there were no survivors beyond postoperative day 2 owing to severe reperfusion injury and multiple organ failure. Unlike swine forelimb muscles, rat hindlimbs suffered from extensive structural damage. This was likely due to rodents' limited ability to clear waste products and a higher metabolic rate per muscle cell. In order to reduce the metabolic rate and lessen the reperfusion injury, we brought down the perfusate temperature to subnormothermic level at 20°C to 22°C and added freshly filtered plasma to red blood cells using a live animal (Fig. 10). This intervention resulted in 100% survival. At 12 weeks, subnormothermic *ex situ* perfusion with red blood cells and plasma revealed only 36% muscle myocyte injury, a value less than those immediately transplanted limbs (38%) with 1 hour of ischemia. Furthermore, sciatic nerve amplitude, latency, maximum twitch, and tetanic force measurements were uniformly better that those hypothermic, near-normothermic, and SCS groups (Figs. 7, 8). Overall, the long-term effects of reperfusion injury at 12 weeks were comparable with those limbs transplanted immediately.

In conclusion, the protective effects of subnormothermic *ex situ* perfusion is only realized when oxygen delivery through red blood cells was combined with fresh plasma. This ideal combination provided the best potential for muscle and nerve regeneration in the long term.

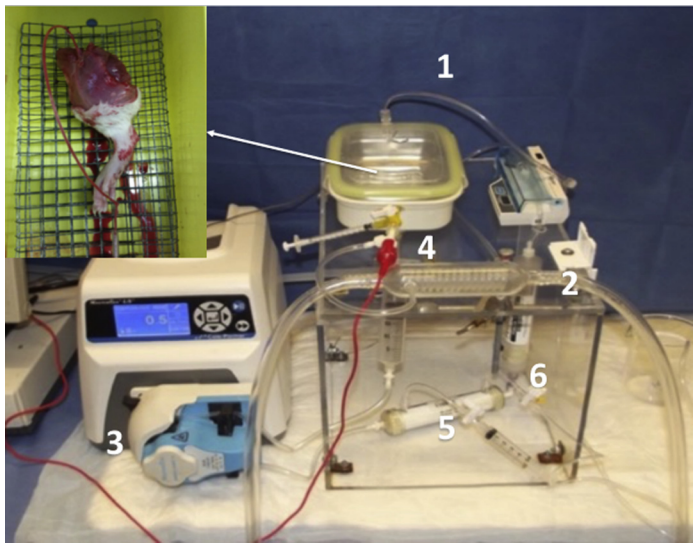

FIGURE 9: Mini *ex situ* perfusion system including (1) VCA chamber (Leak-proof Container, SE-560; Steeltainer, Quebec, CA), (2) a coiled glass heat exchanger connected to a cardiopulmonary bypass heat regulator (TCM 400 MR, Terumo Sarns TCM II Heater Cooler; Sarns, Tokyo, Japan), (3) peristaltic roller pump (Masterflex L/S peristaltic pump w/ easy-load 3 pump head, Pump: HV-07528, Pump head: HV-77800; Cole-Parmer, IL), (4) venous reservoir (Luer-Lok 30-mL syringe, 302832; Becton, Dickinson, and Company, Franklin Lakes, NJ), (5) silicone membrane oxygenator (PDMSXA-1000; PermSelect, Ann Arbor, MI), (6) hemofilter (Minntech Hemocor, HPH-Junior; Medtronic, Wynnewood, PA) with plasma outflow and inflow pumps (Alaris Infusion Pump, 7230, version 4.54; Alaris/Carefusion, San Diego, CA), and 22-gauge intravenous cannula for arterial cannulation (inset). All components were connected using a 1/8-inch internal diameter polyvinyl chloride tubing (Nalgene 180 Clear Plastic PVC Tubing; ThermoFisher, 8000-9020, Waltham, MA) and barbed tubing connectors (Harvard Apparatus, 72-1406, Holliston, MA). All other parts are commercially available and can be sterilized using ethylene oxide.

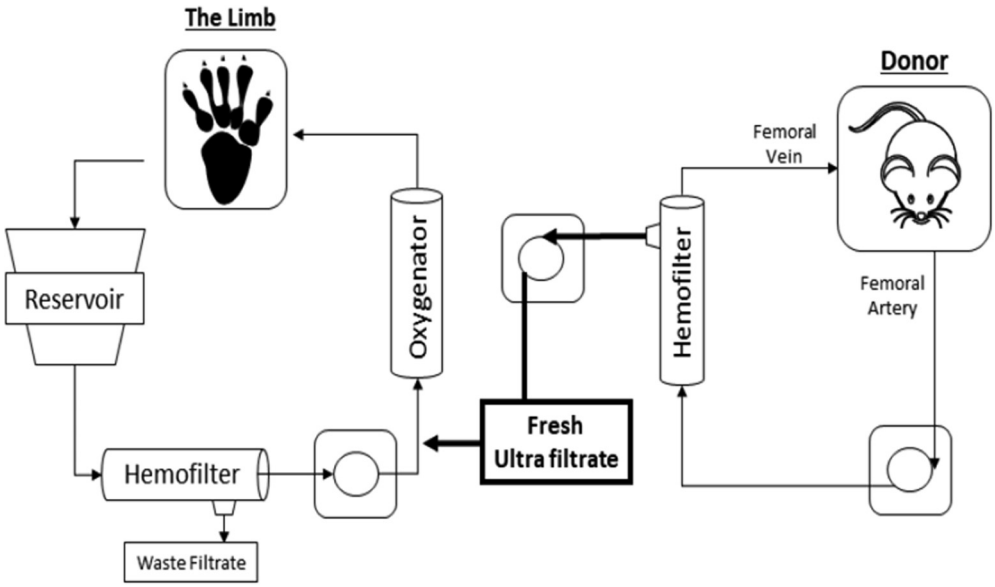

FIGURE 10: While the limb is being perfused (on the left), a second circuit is connected to the *ex situ* perfusion system to enrich the perfusate by adding freshly filtered plasma from a live animal.

SUMMARY OF FINDINGS

Our research over the course of the last 7 years yielded many findings on extremity preservation. For the first time, we have been able to demonstrate the successful extension of VCA survival up to 24 hours without cardiopulmonary circulation. A summary of these findings includes

- In VCA replantation and transplantation, survival does not equate to function. Although the difference between the 2 may not be as pronounced in distal level amputations, more proximal amputations containing muscle and long segments of nerves heavily rely on the regenerative capacity of ischemic tissues.
- The current standard of care, SCS, has detrimental effects on muscle and nerve regeneration in as early as 6 hours. Our research highlights long-term changes associated with prolonged cold storage on muscle and nerve tissue.
- The hypothermic (10°C−15°C) machine perfusion with a synthetic perfusate histidine-tryptophan-ketoglutarate solution successfully removes toxic waste products and prevents muscle injury better than the SCS in the rat hindlimb model. Hypothermia, however, adversely affects the nerve and the muscle tissue. During this process, muscle succinate levels appear to correlate better with muscle injury than other energy markers.
- Near-normothermic (30°C−33°C) *ex situ* perfusion using diluted red blood cells alone fails to prevent reperfusion injury in the rat hindlimb transplantation model. This finding suggests that mere delivery of oxygen is not enough to support limb viability.
- Subnormothermic perfusate temperatures (20°C−22°C) and the addition of fresh plasma exchange to red blood cells provide excellent protection for both nerve and muscle tissue, better than those observed at hypothermic perfusion and SCS.
- It is feasible to maintain forearm and hand allograft viability up to 24 hours using a portable *ex situ* perfusion system developed in our laboratory.
- The swine forelimb allograft model appears to represent all hemodynamic characteristics of human forearms and can be used in future testing.

FUTURE DIRECTIONS

The perfusion technology developed in our laboratory allows us to perfuse extremity parts for up to 24 hours with excellent oxygen exchange at the microcirculatory level. In testing the upper limits of this viability, we perfused limbs up to 50 hours and failed to prevent significant weight gains beyond 36 hours of perfusion due to loss of vasomotor tone. Despite the maintenance in neuromuscular contractility, limbs gained over 50% of their weight after 48 hours on machine perfusion. Interestingly, this phenomenon is also observed in brain-dead donors connected to life-support machines. In isolated brain death, if apnea is prevented by mechanical ventilation, vital organ function gradually progresses from normal to failure in a reproducible fashion.[33] The window of organ procurement is usually 48 hours between brain death and organ failure.[34] Also, an isolated organ perfused with blood at 37°C follows an accelerated course of the same physiological sequence: normal function, loss of vasomotor tone, edema, and organ failure. Although the cause of organ failure in the absence of brain function is not exactly known, it has been proposed that it is likely due to lack of a hormone or a protein normally released from the midbrain that functionally stabilizes endothelial permeability for 48 hours preventing capillary leakage, edema, and organ failure.[35] Unfortunately, the replacement of (known) hormones released from the mid-brain, such as antidiuretic hormone and others, does not prevent capillary leakage, suggesting the presence of an unknown molecule playing an important role. Although we do not know the size and the shape of this hypothetical molecule, we demonstrated that the addition of plasma containing small molecular weight proteins to red blood cells made significant improvements of the overall viability and neuromuscular regeneration as good as those seen on immediately transplanted limbs. Identification of such a molecule will not only have the potential to make replantation and VCA transplantation elective but will be ground-breaking in solid organ transplantation as well.

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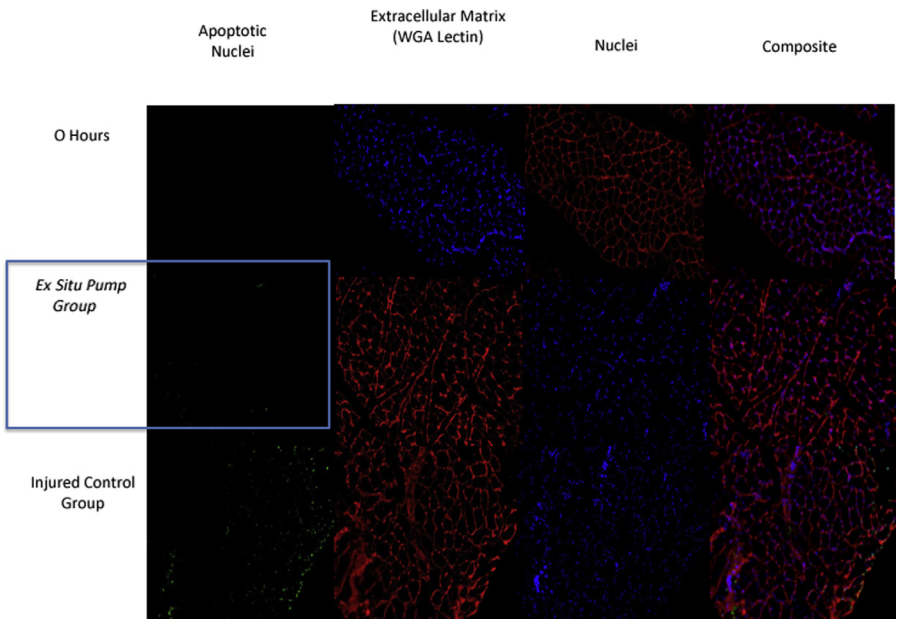

FIGURE E1: TUNEL staining shows apoptotic nuclei in green fluorescent clusters. After 24 hours on pump perfusion and 12 hours of reperfusion, no green cell clusters are seen (blue rectangle), similar to 0 hours sample. Method: Muscle biopsies are placed in TissueTek optimum cutting temperature (OCT) and snap frozen in liquid nitrogen; 10-μm sections on a cryostat are labeled with a Click-It TUNEL assay (Invitrogen, Waltham, MA), which marked apoptotic nuclei with the green fluorophore AF488. Sections were also be treated with the blue fluorescent nuclear dye Hoechst 33342 to identify total nuclei, and the red fluorescent extracellular matrix (ECM) marker wheat germ agglutinin (WGA)-lectin-AF555 to identify intramyocellular versus extramyocellular nuclei.

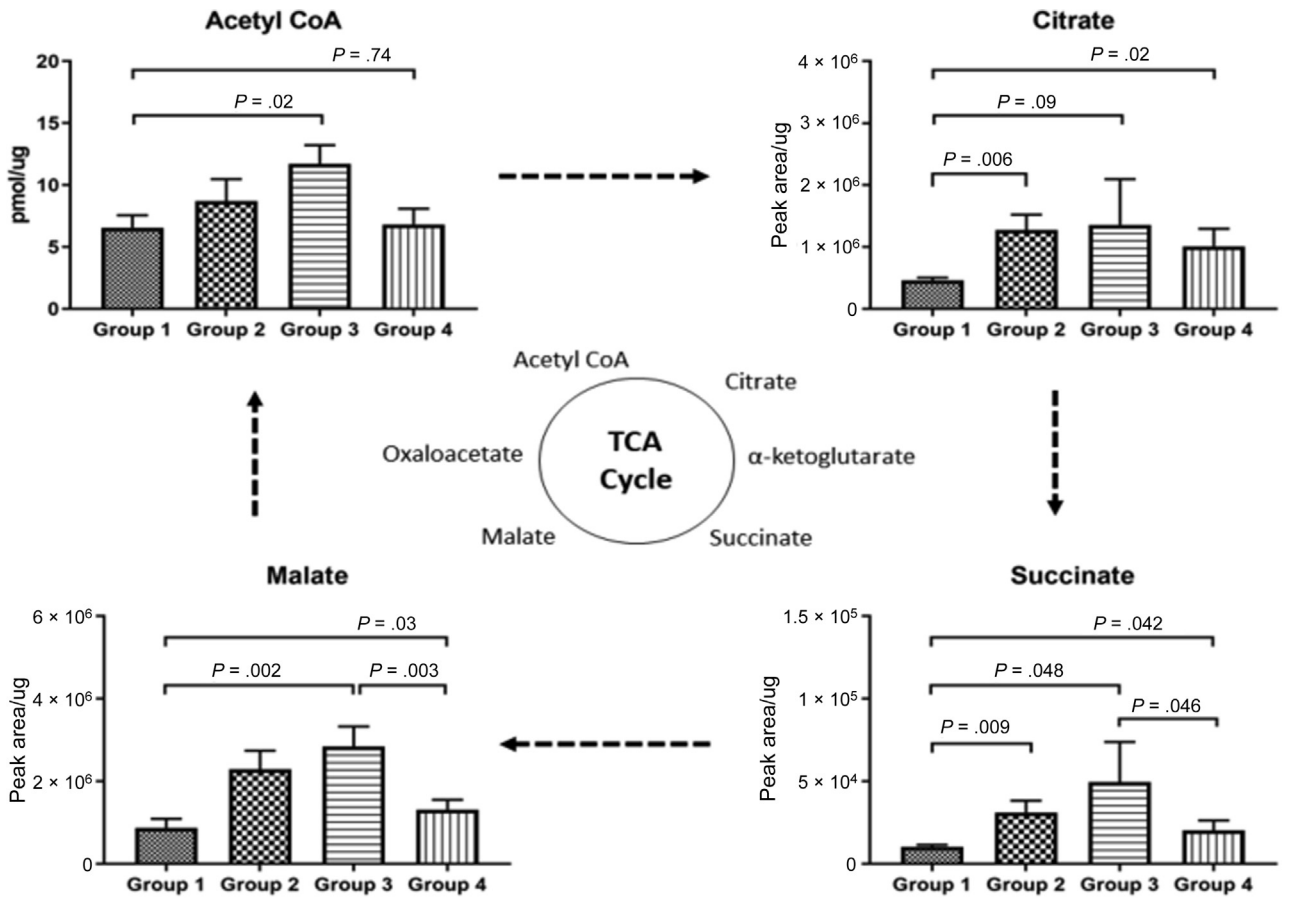

FIGURE E2: Tricarboxylic acid (TCA) metabolome profiling of VCA preservation. Note that static cold preservation appears to keep succinate levels lower than other experimental groups. Group 1: naive; group 2: warm ischemia at room temperature for 2 hours; group 3; static warm storage at room temperature for 6 hours; group 4: SCS at 4°C for 6 hours. Acetyl CoA, acetyl coenzyme A.

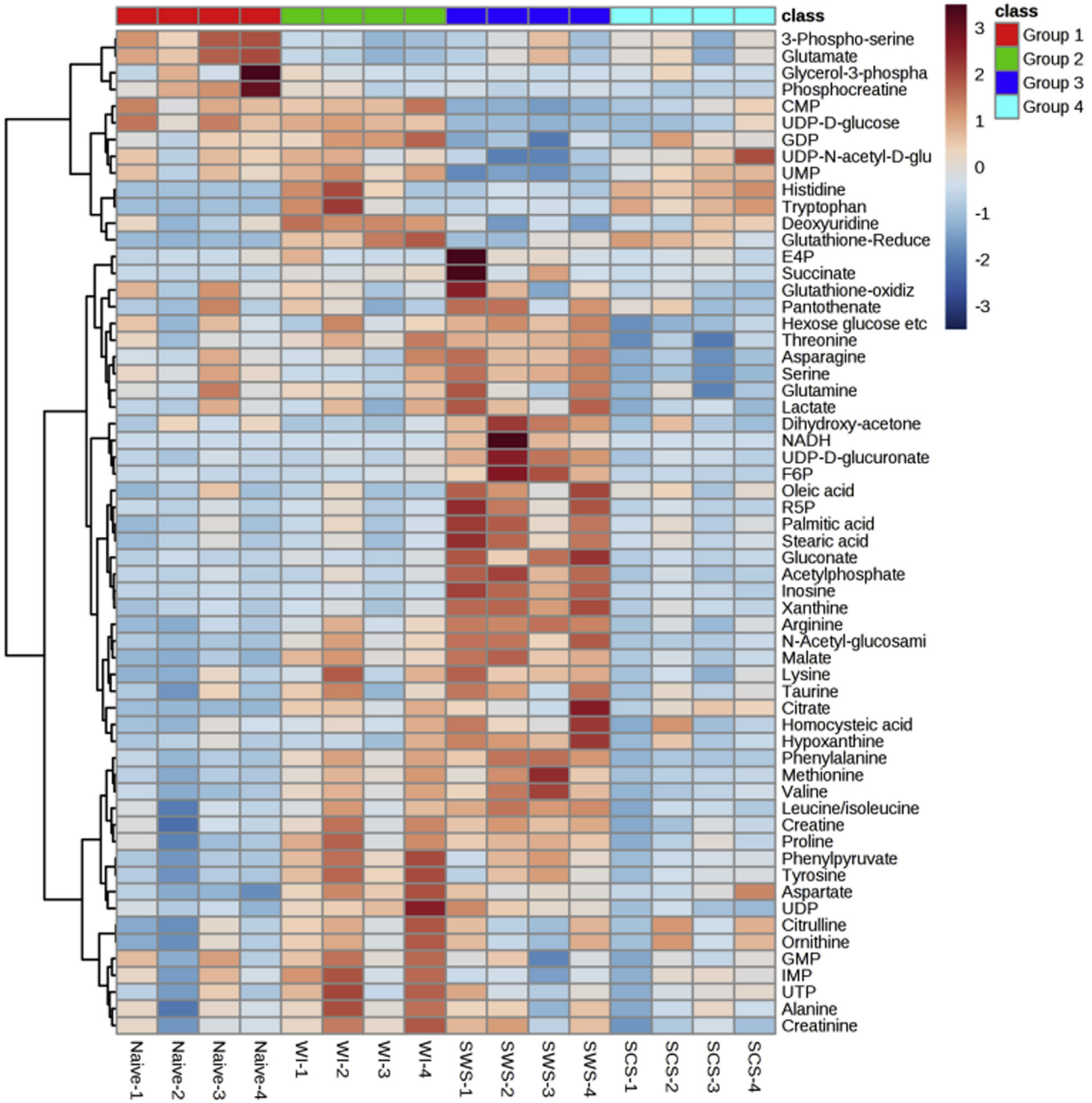

FIGURE E3: Heat map analysis shows mean metabolite concentrations of gastrocnemius muscle samples obtained from the naive control (group 1), static warm storage (SWS) for 2 hours (group 2), SWS for 6 hours (group 3), and SCS for 6 hours (group 4). Means were scaled to facilitate comparison. Each row represents the scaled average concentration of the metabolite that is indicated on the right of the heat map. Each column represents a muscle sample from any of the given groups. Blue heat map coloring indicates a lower metabolite concentration and red indicates a higher metabolite concentration. Cluster analysis was performed on the scaled metabolite concentrations, and the resulting dendrogram is shown at the left of the heat map. CMP, cytidine monophosphate; E4P, erythrose 4-phosphate ; GDP, guanosine diphosphate; IMP, inosine 5'-monophosphate; NADH, nicotinamide adenine dinucleotide, reduced form; R5P, ribose 5-phosphate; UDP, uridine diphosphate; UMP, uridine monophosphate; UTP, uridine triphosphate; WI, warm ischemia.

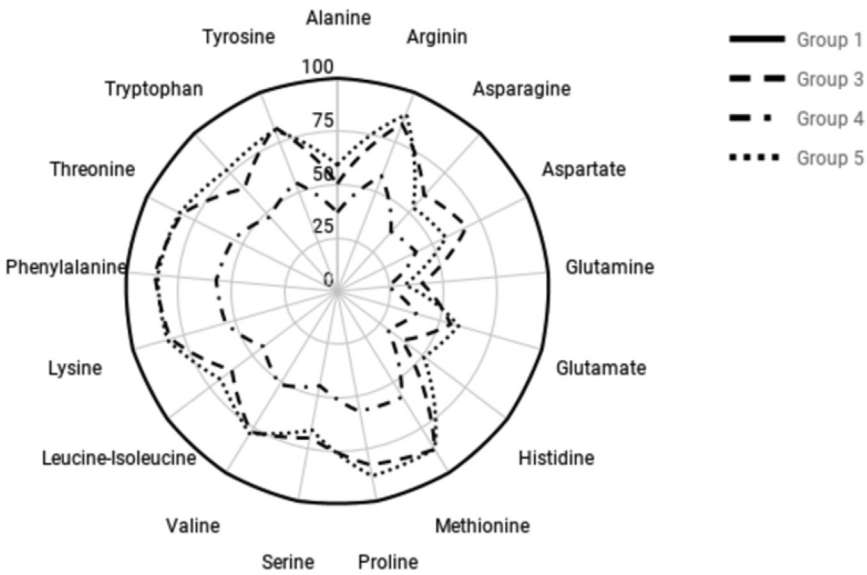

FIGURE E4: Metabolomic analysis of essential amino acids is shown in 4 groups. Group 1: normal (naive) muscle, control; group 3: immediate transplantation; group 4: SCS transplantation; group 5: hypothermic *ex situ* limb perfusion and transplantation. Metabolomic analysis of all amino acids contained in muscle samples obtained 12 weeks following transplantation is shown. ANOVA, analysis of variance.

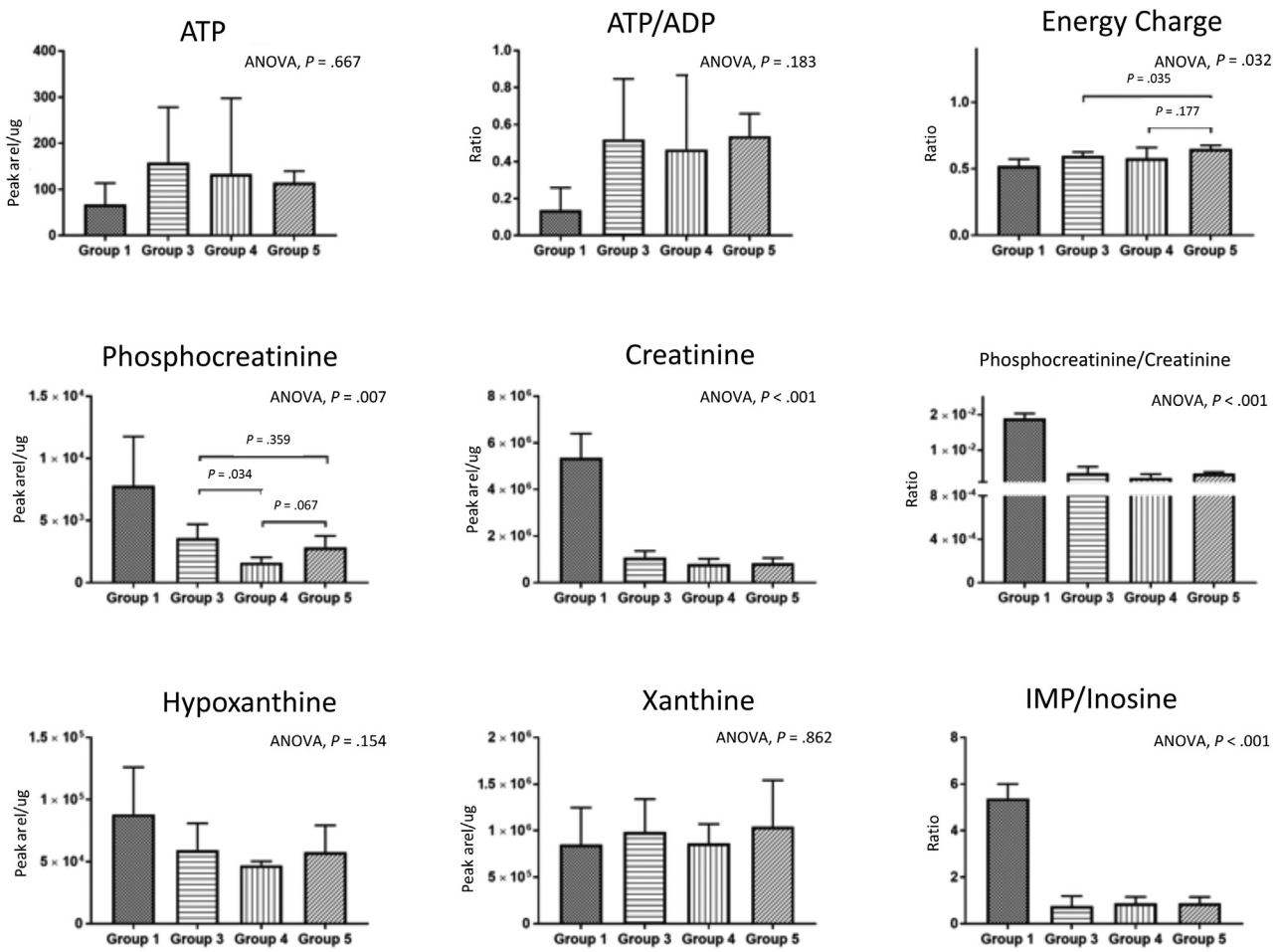

FIGURE E5: Metabolomic analysis of key elements in energy production and storage are shown in 4 groups. Group 1: normal (naive) muscle, control; group 3: immediate transplantation; group 4: SCS transplantation; group 5: hypothermic *ex situ* limb perfusion and transplantation. Metabolomic analysis of all amino acids contained in muscle samples obtained 12 weeks following transplantation is shown.