NAVAL MEDICAL RESEARCH UNIT SAN ANTONIO

# Development of a Non-Human Primate (Rhesus Macaque) Model of Upper Limb Vascularized Composite Allotransplants

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**ABBREVIATIONS**

| | |
|---|---|
| SOT | Solid Organ Transplantation |
| IRI | Ischemia Reperfusion Injury |
| UW | University of Wisconsin Solution |
| NHP | Non-Human Primate |
| ECMO | Extracorporeal Membrane Oxygenation |
| QNO | Quadrox-I Neonatal Oxygenator |
| SOC | Standard of Care |
| VCHR | Venous Cardiotomy Hardshell Reservoir |
| CSP | Cold Static Preservation |
| CVCR | Custom Venous Collection Reservoir |

## EXECUTIVE SUMMARY

**Background:** Traumatic limb amputations represent a substantial challenge facing many civilian and combat wounded. The goal of vascularized composite allotransplantation (VCA) research is to successfully reconstruct complex donor tissues providing long-term functionality and improved quality of life. Despite advancements in VCA research to date, a tissue preservation strategy that successfully mitigates ischemia reperfusion injury, preserves graft functionality, and provides long-term graft survival remains imperfect.

**Objective:** To develop a non-human primate (NHP) model of upper limb vascularized composite allotransplant, that allow for evaluation of tissue preservation and reperfusion strategies.

**Materials and methods:** Upper extremities were procured from male rhesus macaques for technical refinement (n=12) and model (n=5). Cold storage preservation with University of Wisconsin solution for 12 hours at 4°C was performed, followed by reperfusion of the limbs using extracorporeal membrane oxygenation (ECMO, Maquet, Rastatt, Germany) and a pulsatile perfusion pump with warm (37°C at 45±10 mmHg) autologous whole blood. Tissue biopsies and blood were evaluated at baseline (BSLN), pre-reperfusion (R0), and post-reperfusion (R60).

**Results:** Despite encountered complexities, the results shown here are gathered from n=5 model extremities. Significant changes: (1) compared to BSLN lactate and potassium were significantly increased at R60, (2) Base deficit significantly decreased at R0 and R60, and (3) Cytokines IL-8 and sCD40-L were significantly increased at R60. Histopathological analysis improved after reperfusion.

**Conclusions:** This unique model of preservation using non-human primate (NHP) extremities represents a first of its kind in transplantation literature. This protocol establishes a baseline for a standardized NHP limb preservation and is the first step in developing an ex vivo pre-transplantation assessment tool providing statistical analysis of projected graft function and survival.

## INTRODUCTION

Traumatic limb amputations represent a substantial challenge facing many patients in both military and civilian populations.[1] Within the realm of reconstructive transplantation, the specialized area of vascularized composite allotransplant (VCA) research is pursuing the goal of successful transplant of complex, biomechanically functional donor tissues in order to improve patients' long-term function, wellness, and quality of life.[2-4] There is often a prolonged period of time (4-13 hours) from tissue procurement to transplant during which special care must be taken to protect the donor tissue from ischemic injury and subsequent reperfusion injury. This barrier has also been experienced in solid organ transplantation (SOT) (e.g. kidney and liver) with increased success over the past decades due, in large part, to improvements in ex vivo tissue preservation strategies.[5]

Unlike solid organs, VCAs, including extremities and facial tissue, represent complex composite tissues largely composed of bone, muscle, nerves and epithelium which are extremely sensitive to prolonged ischemia times.[6-8] Despite advancements in tissue preservation in the field of SOT, a preservation strategy that successfully mitigates ischemia reperfusion injury (IRI), maintains graft functionality, and prevents acute and chronic graft failure has yet to be created for VCA.[7, 9] This clinical gap significantly constrains the available VCA donor pool, and hence the number of patients that would benefit from VCA reconstruction. In order to increase our success with VCA, improvements in *ex vivo* tissue preservation strategies are required.

Tissue preservation strategies for solid organs have been extensively investigated and date to the early 20th century.[10, 11] Conversely, VCA preservation strategies are yet to be extensively studied and hence remain unrefined.[6, 12] Current efforts in VCA preservation rely on the extrapolation of solid organ preservation experience and techniques. Solid organ research has demonstrated that optimal preservation heavily depends on the type of solution utilized and the temperature at which the tissue is kept during the ex vivo preservation phase.[5, 8, 13]

Efforts to develop preservation strategies for VCA tissues from full limbs have been initiated.[14-16] However, standardization of a proper preservation and reperfusion technique that can maintain tissue integrity, reduce ischemic damage for prolonged periods and extend the potential use for transplantation have yet to be developed.

In the current study, we developed a protocol using standard-of-care (SOC) cold static preservation (CSP) at 4 °C with Belzers' University of Wisconsin solution (UW) combined with

an autologous blood based recovery and reperfusion strategy utilizing a recirculating pump. We based our study on the hypothesis that *ex-vivo* VCA preservation and reperfusion strategies would minimize ischemic damage and therefore extend the time of VCA viability for potential replantation. This model was developed in order to assess the effects of SOC tissue preservation on non-human primate (NHP) upper extremities prior to transplantation. This NHP model was used due to its anatomical, physiological and molecular similarities with human upper extremities, which would allow comparison and translation of outcomes to clinical SOC. Male NHP were utilized in this protocol for two reasons: (1) males comprise the majority of battlefield injuries, and (2) males, regardless of species, exhibit more deleterious complications and higher mortality trauma and hemorrhage than females.[17] The overall goal of this work was to develop a model that sets the standard for VCA preservation strategies that allow for prolonged preservation of upper extremities, mitigate cold ischemia and reperfusion injury to optimize graft survival.

## MATERIALS AND METHODS

### *Subjects*

Male, non-human primates (NHPs, *Macaca mulatta*) weighing 8-12 kilograms and 5-12 years old were used in accordance with the protocol approved by the Institutional Animal Care and Use Committee at the 711$^{th}$ Human Performance Wing, Joint Base San Antonio-Fort Sam Houston, as well as ACURO approval and conducted in accordance with the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animals Resources, National Research Council, National Academy Press, 2011. All procedures were performed in facilities accredited by the AAALAC, i.

All upper limbs used in this study were obtained at the completion of a separate, approved research protocol relating to poly-traumatic hemorrhagic shock, via tissue sharing. Upper limbs were surgically procured following completion of the hemorrhagic shock protocol by trained surgeons while the NHPs remained under deep inhalational anesthesia with isoflurane (1.0% - 5.0%). All animals were subsequently euthanized. Twelve limbs were used during technical refinement of different aspects of the protocol and five limbs limbs were used in studies to assess the effects of the preservation and reperfusion technique.

***Procedures***

*Surgical Procedure for Limb Procurement and Preservation*

A diagrammatic summary of the experimental protocol for limb procurement, storage, and reperfusion is provided (Fig. 1). Briefly, under sterile conditions the deltoid muscle's humoral attachment was released with the bulk of the muscle being reflected cranially, followed by the release of the tendons attaching the bicep brachii, coracobrachialis, and triceps brachii muscles to the scapula. The glenohumeral and coracohumeral ligaments were sharply released and the humeral head was disarticulated from the glenoid cavity. The brachial artery and vein were both ligated and transected; the artery was then cannulated with an angiocatheter immediately prior to ischemia. Lastly, while still under anesthesia, the animal was exsanguinated in accordance with the American Veterinary Medical Associations' Guidelines on Euthanasia. Whole blood was collected in a blood donor bag containing anticoagulant citrate phosphate dextrose adenine solution at a 1:10 ratio and stored at 4°C. The volume of blood collected from each animal was recorded for subsequent use in the reperfusion protocol.

The brachial artery of the amputated limb was flushed intravascularly with 100 mL of University of Wisconsin (UW) solution (Waters Medical System, Rochester, MN, USA), approximately twice the intravascular volume of the NHP limb. The tissue was then wrapped in UW soaked surgical sponges to reduce desiccation, placed in a sterile bag, and stored at 4°C for 12 hours. After 6 hours the upper limb was removed from cold storage, flushed with 100 mL of fresh UW preservation solution, and returned to cold storage for the remaining 6 hours of preservation.

As alternative solutions for limb preservation, the suspended animation agents hydrogen sulfide ($H_2S$, Faraday Pharmaceuticals, INC) and hydrogen selenide ($H_2Se$, Fred Hutchinson Cancer Research Center, FHCRC), were initially suggested to be used as preservation agents. For procurement of both of these agents ($H_2S$, $H_2Se$), LP-CRADAs were prepared and executed between NAMRU-SA and Faraday Pharmaceuticals, INC ($H_2S$) and between NAMRU-SA with FHCRC ($H_2Se$). However, procurement of $H_2Se$ was not possible since FHCRC communicated to NAMRU-SA that there were complications related to the safety of handling of $H_2Se$ by research technicians. Therefore, $H_2Se$ was not used as a preservation agent the proposed studies.

On the other hand, in spite of multiple attempts by members of NAMRU-SA to communicate with Faraday Pharmaceuticals, INC, regarding guidelines on the appropriate

dose/concentration handling of $H_2S$, no response was ever received. As a result, initial experiments utilizing $H_2S$ were based on historical concentrations matching published data from studies conducted by the Co-PI. These studies used approximately 4.08 mM for preservation of skin flaps in a porcine model. However, these procedures were performed under a chemical fume hood, which was not feasible for an entire upper extremity. An isolated attempt was conducted using this concentration for a 12 hour preservation period in an NHP upper extremity. It was determined that this level exceeded safety levels of the agent when used outside of a chemical fume hood. Therefore further investigation for safe and effective concentrations was performed in the literature. According to previous published reports for preservations of solid organs, lower concentrations of $H_2S$ with an average of 150 µM $H_2S$ were effective. One NHP extremity was evaluated for 24 hours preservation utilizing the150 µM $H_2S$ preservation, although technical issues related to the procedure confounded the data and is not reported.

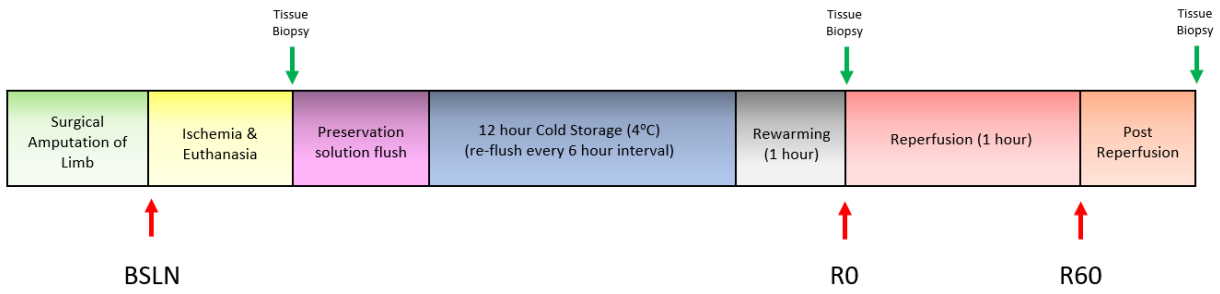



**Fig. 1. Diagrammatic representation of the timeline for the storage and reperfusion of the amputated limb.** Experimental design is graphically depicted including time points of amputation, preservation solution flush, cold storage, and reperfusion. Whole blood was drawn for labs (blood chemistry (CHEM), arterial blood gas (ABG), and cytokine analysis at baseline (BSLN), pre-perfusion (R0), and post reperfusion (R60).

*Reperfusion*

Upon completion of the cold storage period (12 hours), the upper limb was gradually warmed over 1 hour by a combination of thermal lamps and a heat pad. An extracorporeal membrane oxygenation (ECMO, Maquet, Rastatt, Germany) circuit was established (Fig. 2). Briefly, the ECMO circuit was equipped with a water cycle system with multiple, non-permeable tubes attached to an external heat source (water bath). Within the oxygenator, cross linked tubing

allowed heat exchange between the water and blood. In order to account for heat loss from the bath to the blood while preventing thermal shock hemolysis, the water bath was set at a cautionary 39°C. Autologous whole blood was connected to the ECMO reservoir which was cycled for a period of 10 minutes ± 5 to allow for equalization of temperature and oxygenation. A standard of 1.5 mL of calcium gluconate was added to the whole blood to counteract any residual effects due to the use of anticoagulant during blood collection. An implantable thermocouple probe (Physitemp, Clifton, NJ) was placed into the biceps brachii muscle for continuous monitoring of internal limb temperature. Vascular resistance was negated by the development of a customized venous collection reservoir (CVCR) by our group. Upon attachment of the brachial artery to the ECMO, the limb was positioned at an incline over the CVCR. Blood collected at the CVCR was then recirculated through the ECMO and used for reperfusion. Reperfusion was performed for one hour with a maintained perfusion pressure below physiological pressures (mean arterial pressures of 45 mmHg ± 10) to minimize blood cell lysis from the ECMO circuit and monitored by in-line pressure transducer in the brachial artery.

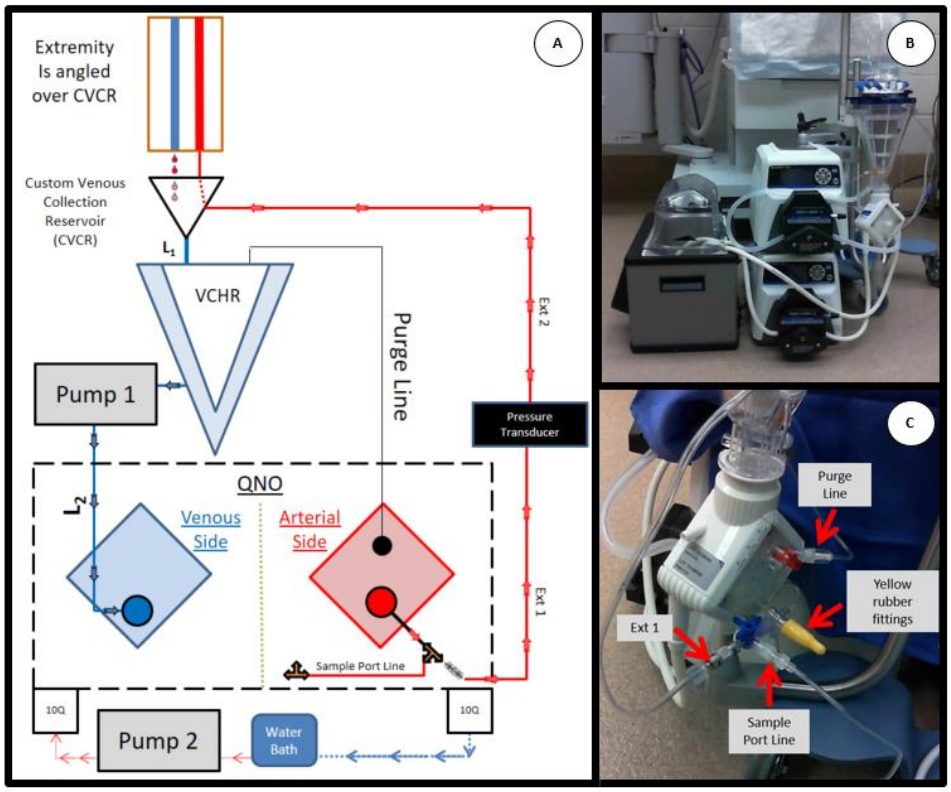



**Fig. 2. Schematic depiction of the ECMO circuit**. **A.** The diagram includes the venous cardiotomy hardshell reservoir (VCHR), fluid pumps, custom venous collection reservoir (CVCR), water bath, oxygenator (QNO). **B.** Standardization of ECMO setup. Pump 1 (Top)

responsible for the artificial peristalsis for the circuit system while Pump 2 (bottom) circulates warm water through the oxygenator. **C.** Arterial side of oxygenator.

*Blood and Tissue Samples*

Whole blood samples were collected from each animal at the time of exsanguination (baseline, BSLN), at the end of cold storage (R0), and one hour following the completion of reperfusion (R60). Whole blood and tissue samples were stored at 4°C and -80°C until analysis.

*Analysis of Blood Chemistry, Cytokines and Chemokines*

Analysis of blood chemistry and arterial blood gas were undertaken using a Catalyst Dx Chemistry Analyzer (IDEXX Laboratories, Inc., Westbrook, MN, USA) and a GEM Premier 4000 (Instrumentation Laboratory, Bedford, MA) according to the manufacturer's instructions. Systemic inflammatory cytokines and chemokines in serum were quantified using a Milliplex non-human primate cytokine magnetic bead panel (Premixed 23 Plex - Immunology Multiplex Assay, EMD Millipore, Billerica, MA, USA). Bead fluorescence was measured using the Bio-plex 200 system plate reader and results were analyzed using Bio-Plex Manager Software Version 6.1 (Bio-Rad Laboratories, Hercules, CA, USA).

*Histopathological Analysis*

Tissues biopsied at BSLN were biceps brachii, brachial plexus, and axillary vessels. Biopsies collected at R0 were brachioradialis, radial nerve, and radial vessels. At R60, the brachioradialis, ulnar nerve, and brachial vessels were acquired. Tissue samples were formalin fixed, sectioned, and stained using hematoxylin-eosin (H&E). Tissue sections were analyzed by a certified veterinary pathologist for evidence of alterations in gross morphology, degeneration (pale, swollen, and vacuolated myofibers), and necrosis in muscular, neural, and vascular tissues.

*RNA Expression Analysis*

Muscle, nerve, and vascular tissues were biopsied at BSLN, R0, and R60. Samples were then stored at -80°C for RNA extraction and subsequent RNA expression analysis. RNA was isolated from tissue biopsies using RNAzol® RT (Sigma-Aldrich, St. Louis MO) according to manufacturer instructions. Tissues biopsied at BSLN were biceps brachii, brachial plexus, and axillary vessels. Biopsies collected at R0 were brachioradialis, radial nerve, and radial vessels. At

R60, the brachioradialis, ulnar nerve, and brachial vessels were procured. Isolated RNA was used for first strand cDNA synthesis followed by BioMark Fluidigm® gene expression analysis, performed by the Bioanalytics and Single-Cell (BASiC) Core at the University of Texas Health Science Center San Antonio using the Biomark™ HD system (Fluidigm, South San Francisco, CA, USA). Gene expression of 94 genes was evaluated using TaqMan® probes **(Supplementary Table 1)**. The data were analyzed using the Singular™ Analysis Toolset (Fluidigm) at the BASiC core. Data obtained as CT values was normalized to the mean of the housekeeping gene: glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The delta-delta (ΔΔ) CT method was used to calculate the fold change expression of each gene compared to BSLN levels. This normalization to BSLN was performed as tissue specific for nerve, muscle, and vasculature.

*Nerve conduction studies*

Nerve conduction studies were performed in NHP limbs. Even though an electrical stimulus was applied, no electrical response was obtained by either visual inspection or by analysis of the nerve conduction velocity (NCV) data. In fact, when analysis was performed, no clear electrical signal was able to be distinguished from background noise. However, a possible explanation for the lack of observed electrical activity was due to either tissue ischemia or the nerve was severed during limb procurement. Additionally, metabolic changes and/or electrolyte depletion as well as changes in temperature could have affected the potential for electrical conduction in the extremity.

*Statistical Analysis*

Data are shown as mean ± the standard error of the mean (SEM). Statistical comparisons were performed using two-way ANOVA followed by the Bonferroni's multiple comparisons test with $p<0.05$ considered significant (Prism 7.0, GraphPad Software, Inc., La Jolla, CA, USA).

## RESULTS

*Physiologic Monitoring*

Following removal from CSP, the upper extremities were gradually warmed using heating lamps and pads for one hour reaching a mean temperature of 20.1°C ± 6.4. Following reperfusion for 60 minutes, limb temperature increased to an average of 27.8°C ± 6.3, but did not reach the

normal physiological temperature for NHPs (38°C). Blood was immediately rocked in 4°C after collection to cease degradation and metabolic activity. Despite 1 hour of rewarming in the 37°C incubator, the blood temperature at R0 averaged 24.2°C ± 1.7.

Analysis of the autologous whole blood immediately prior to its use in reperfusion and post-reperfusion indicated a significant increase in lactate (3.38 mmol/L ± 0.27 *versus* 9.53 mmol/L ± 0.55, $p<0.0001$), as well as from BSLN (2.46 mmol/L ± 0.42). A significant decrease in base deficit from R0 compared to BSLN (-11.96 mmol/L ± 0.77 *versus* -0.60 mmol/L ± 0.95 *v*), R60 (-20.68 mmol/L ± 1.58) from R0, and R60 from BSLN was also appreciated. A significant increase in potassium at R60 (12.70 mmol/L ± 1.53) when compared to time points BSLN and R0 (4.88 mmol/L ± 0.59 and 3.84 mmol/L ± 0.21, respectively) was analyzed (Fig. 3).

The donor blood used in the ECMO circuit displayed evidence of acute anemia, with a mean hematocrit of 17.02% ± 2.88 prior to collection, 17.84% ± 3.86 pre-reperfusion (stored on a rocker platform for 12 hours), and 17.44% ± 4.13 post-reperfusion. Mean total hemoglobin was 7.18 g/dL ± 0.71 prior to blood collection, 7.7 g/dL ± 0.82 pre-reperfusion, and 7.98 g/dL ± 0.88 post-reperfusion. Despite the anemia, the blood used in circuit was able to be assayed consistently for physiologic and immunologic markers using standard methods. We did not observe any significant change in hematocrit between pre- and post-reperfusion. Blood oxygen saturation was monitored during reperfusion, but the results showed no significant change.

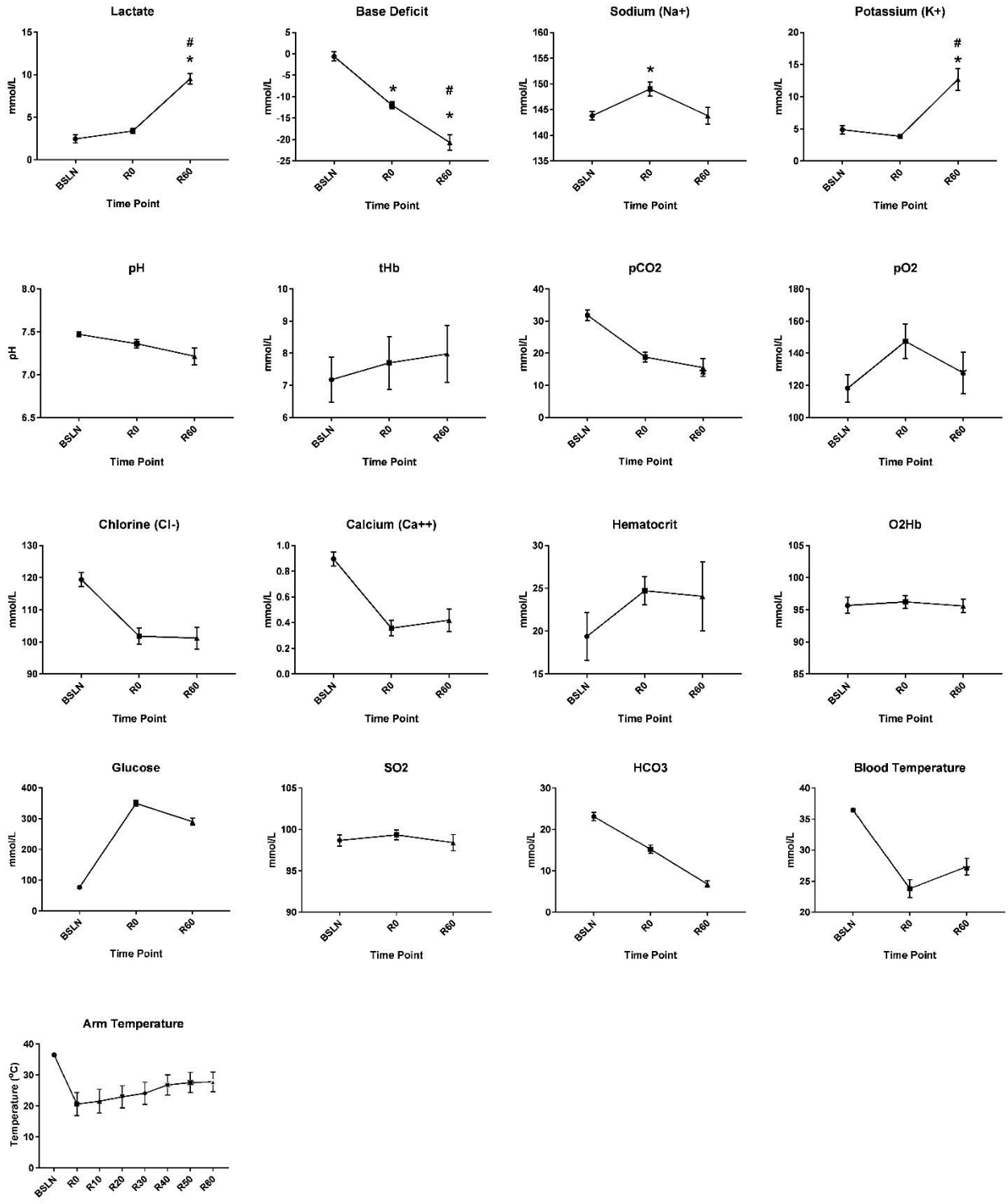



**Fig. 3. Blood gas measurements shows redistribution of metabolic by products.** Data show the mean ± SEM for blood lactate, base deficit and pH for the blood in the ECMO circuit at

baseline, pre-reperfusion (R0), and post-reperfusion (R60). Statistical analysis was performed by two-way ANOVA and $p \leq 0.05$ was considered to be a statistically significant different when compared to BSLN (*) and compared to R0 (#).

*Cytokine Analysis*

We next examined the inflammatory response to the CSP on the limb. We queried the donor blood in the ECMO circuit pre-reperfusion and compared it to the blood post-reperfusion. The examined cytokine analysis successfully detected the presence of ten cytokines and chemokines (out of 23 assayed). The majority of the detectable cytokines remained quantifiably constant pre- and post-reperfusion without statistical significance. However, the analysis did show a significant increase in Interleukin 8 (IL-8) and soluble CD40 ligand (sCD40L) at R0 (409.96 ± 37.82 pg/mL and 924.01 ± 96.83 pg/mL, respectively) and R60 (546.5 ± 40.13 pg/mL and 1535.41 ± 163.93 pg/mL, respectively) compared to BSLN (187.22 ± 49.37 pg/mL and 52.35 ± 14.24 pg/mL, respectively). The cytokine sCD40L also showed a significant increase at (R60) compared to (R0). Increased TGF-α levels were observed at R0 (9.87 pg/mL ± 5.03) and R60 (17.31 pg/mL ± 6.43) compared to BSLN (2.08 pg/mL ± 0.48) (Fig. 4).

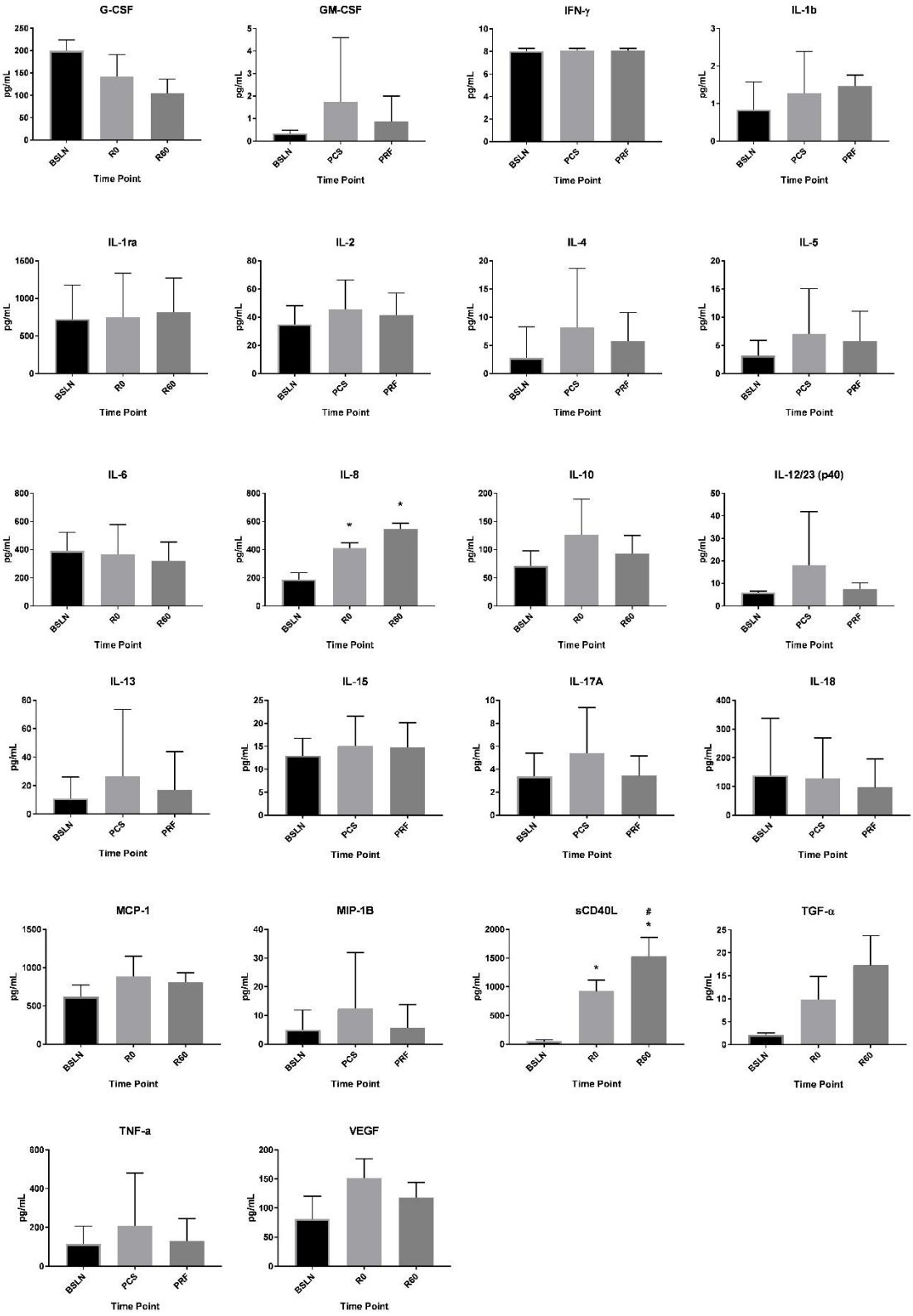
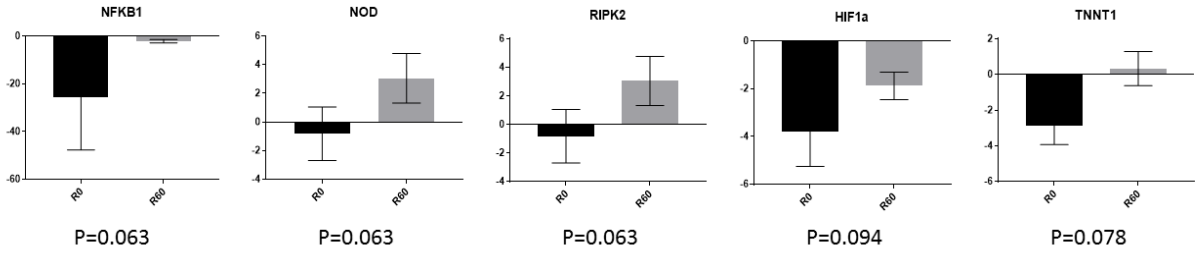

G-CSF
GM-CSF
IFN-γ
IL-1b
IL-1ra
IL-2
IL-4
IL-5
IL-6
IL-8
IL-10
IL-12/23 (p40)
IL-13
IL-15
IL-17A
IL-18
MCP-1
MIP-1B
sCD40L
TGF-α
TNF-a
VEGF
pg/mL
Time Point
BSLN
R0
R60
PCS
PRF

**Fig. 4. Effect of cold storage and reperfusion on cytokines and chemokines production.** Cytokine and chemokines were measured by multiplex immunoassay using serum samples isolated from donor whole blood. Data are the mean concentration (pg/mL) ± SEM. Statistical analysis was performed by two-way ANOVA and $p \leq 0.05$ was considered to be a statistically significant different when compared to BSLN (*) and compared to R0 (#).

*Gene Expression*

In the genetic expression analysis we examined the differential expression of 94 different genes (Supplementary Table 1) in tissues obtained from the limbs at BSLN, R0, and R60. Fold change analysis following ΔΔCT calculations revealed no significant differences in the expression of genes following preservation and reperfusion with autologous whole blood. However, a trend for an increase in fold changes was observed in in five (5) genes at R60 compared to R0. This included genes involved in an inflammatory response such as NFKB1 (-2.18 ± 0.69 *versus* --25.54 ± 22.18), NOD1 (3.05 ± 1.73 *versus* -0.83 ± 1.87), and RIPK2 (-3.05 ± 1.73 *versus* -0.83 ± 1.87); the apoptosis/hypoxia associated gene HIF-1a (-1.88 ± 0.58 *versus* -3.79 ± 1.46) and the muscle specific genes TNNT1 (-0.33 ± 0.95 *versus* -2.89 ± 1.04) (Supplementary Figure 1).




**Supplementary Figure 1: Effect of cold storage and reperfusion in muscle gene expression:** Levels of gene expression are reported as mean fold changes ± SEM for vasculature for muscle tissue. Statistical analysis was performed by the Wilcoxon test (non-parametric T-test) with $p \leq 0.05$ considered significant between R0 *versus* R60.

*Histology*

Histopathological assessment allowed evaluation of the effects of cold storage on the limb with pre-procurement results compared to the effect whole blood reperfusion had on the tissue at time point R60. We observed no significant differences in the histologic score pre- and post-

reperfusion in any of the nerve or vasculature. In skeletal muscle, significant damage was observed at R0 (1.67 ± 0.28) compared to BSLN (0.36 ± 0.2). However, significant improvement in the histological score was appreciated when comparing R60 (0.75 ± 0.30) compared to R0; suggesting that the reperfusion and rewarming with blood restores the recovery of the ischemic limb (Fig.6).

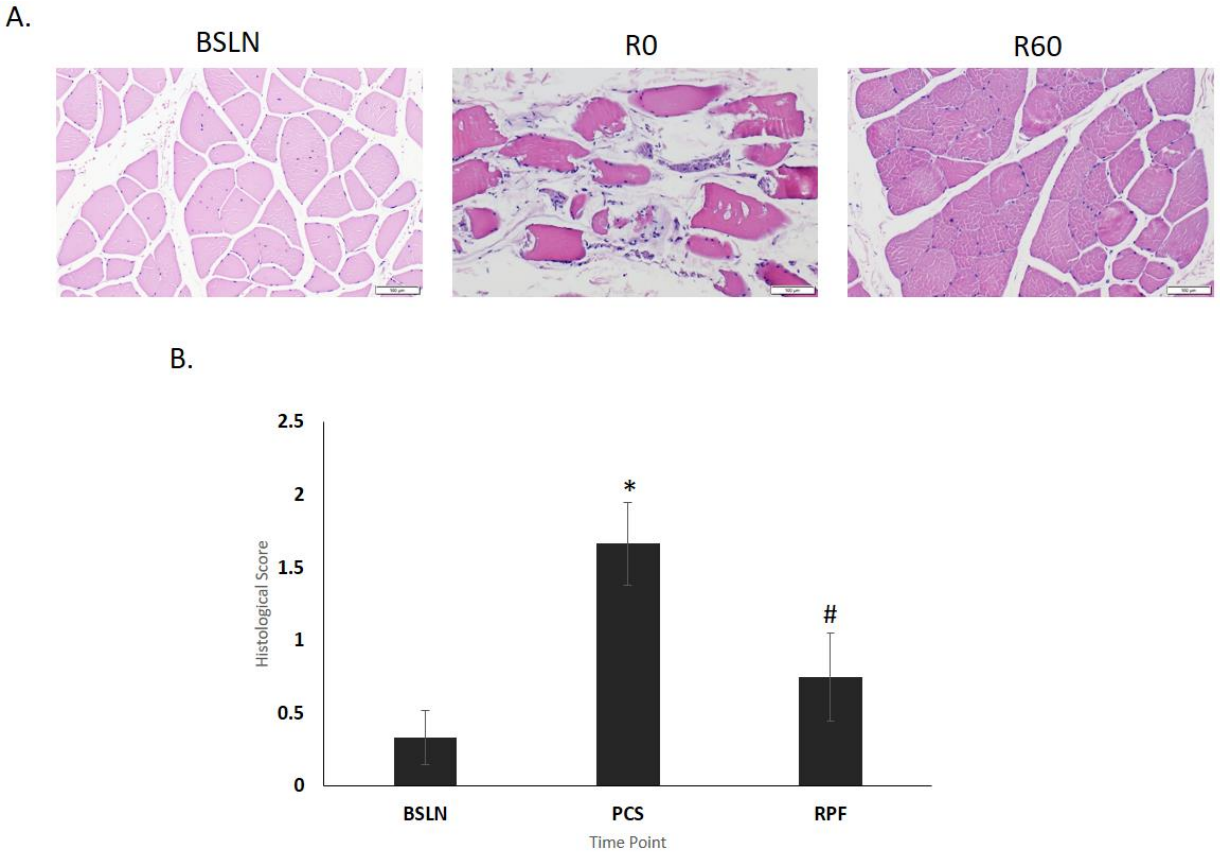



**Fig. 6. Histopathological analysis of muscle tissue.** The tissue slides were randomized and read blinded by a veterinary pathologist. **A**. In representative histology images, please note mild multifocal necrosis at R0 compared to BSLN and R60. Muscle images collected at 20X. Necrosis is characterized by hypereosinophilia, shrunken (hypercontracted/fragment myofibers with loss of cross striations and pyknotic nuclei. **B.** Histologic scores were 0 = Normal (0% necrosis), 1 = minimal changes (1 – 5% necrosis), 2 = mild (5 - 25% necrosis), 3 = moderate (involvement of 25 -50%), 4 = Marked (50 – 75% necrosis), 5 = Severe (75 - 100% necrosis). (*) denotes $p \leq 0.001$ compared to BSLN; (#) denotes $p<0.05$ compared to PCS.

## DISCUSSION

As the first study design of its kind in the transplantation field, one can appreciate the complexities and outcomes of NHP VCA research. The uniqueness of this model can primarily be seen in the use of autologous whole blood as a reperfusate compared to other successful preservation studies in which allogeneic blood or blood products were used for reperfusion assessment.[12, 16, 18] Autologous whole blood was incorporated as the reperfusion fluid to restore oxygenation of the amputated limb, rather than type-matched allogeneic whole blood. This design was set into place to avoid initiation of potential allogeneic immune responses. The significant changes seen in serum parameters during the study were a decrease in base deficit following reperfusion and a significant increase in serum potassium. With this unique study design these findings appear to be consistent with the mobilization of metabolic byproducts from the ischemic isolated limb during cold storage rather than the additional compounding factor of an allogeneic response. We did not observe any significant change in hematocrit between pre- and post-reperfusion suggesting that the increased potassium was not due to hemolysis but to extracellular and intracellular environmental changes in the VCA due to the specific ex vivo preservation technique.

These findings were further supported by the examination of the inflammatory response to the CSP on the limb through examining an exhaustive list of cytokines present after preservation (R0) and post-reperfusion (R60). By evaluating the donor blood in the ECMO circuit pre-reperfusion (R0) compared to the blood post-reperfusion (R60) the statistically elevated levels of IL-8 and sCD40Lwere observed (Fig.4). IL-8 is a cytokine found pre-formed in the alpha granules of endothelial cells and is released upon endothelial damage.[19] The increase in IL-8 levels would suggest the release of the preformed cytokine since the cold storage of the limb would decrease transcription and translation of the IL-8 mRNA. sCD40L is shed by platelets following activation[20] and the increase seen in the current study may be due to platelet activation in response to the endothelial injury in the post-reperfusion (R60) blood sample. An increased trend was observed in TGF-$\alpha$, an inflammatory cytokine expressed in a variety of cells including macrophages and granulocytes. Granulocytes are increased in the donor blood in response to the injury/hemorrhage and resuscitation protocol used in the donor prior to limb procurement and blood collection. These results show that while there was release of preformed inflammatory mediators, there was no significant increase in the majority of other stereotypic inflammatory

markers such as IL-6, IL-1β, and TNF-α in response to the cold storage after the 1 hour of autologous whole blood reperfusion. The lack of expression of inflammatory cytokines may be due to a timing issue as the increase in the inflammatory responses following an initiating event such as injury occurs within hours to days with cytokines at first being locally expressed,[21] and thus the clinical applicability of these results may be limited to VCA. For example, in hemorrhage one of the first cytokines to be measurably upregulated is IL-1α at 2 hours post-hemorrhage in the lungs.[22] In this model we were assaying the perfused blood at 1 hour since the intent of the current study was to use the blood based reperfusion as a method to "recover" the limb from the cold storage. In addition to tissue revitalization, it may be possible to extend the recovery/reperfusion phase beyond 1 hour with additional assay time points as a potential method to evaluate the limb for functional and immunologic status prior to transplant. Despite the described shortcomings, the changes in inflammatory markers appear to be caused by the changes in the limb from the cold storage technique.

Even though no significance was observed from the gene expression analysis, a trend for upregulation of some genes involved in the inflammatory response (NFKB, RIPK2 and NOD1) and hypoxia (HIF-1α) was observed following reperfusion. NFKB encodes the NF-Kβ transcription factor, which upon diverse stimulus initiates an inflammatory cascade including transcription of cytokines and genes involved in an immune response and apoptosis. It has been shown that both RIPK2 and NOD1 are strong activators of NF-Kβ. HIF-1α encodes the prototypic hypoxia inducible factor, which is a master regulator of gene transcription in response to hypoxia. Interestingly, HIF-1α also modulates the expression of other genes involved in energy metabolism, cellular proliferation, apoptosis, and inflammation under oxygen deprived conditions. Future studies will look into preservation strategies longer than 12 hours as well as preservation agents that can enhance the protection of VCA tissues for transplantation allowing research to pinpoint the desirable specific genes expressed maximizing cell proliferation and recovery from ischemia while minimizing extensive cellular apoptosis. This will be further supported by the use of TUNEL staining to correlate genetic expression and the extent of cellular apoptosis the VCA is undergoing.

Lastly, improvement in the pathological score of muscle from R0 to R60 suggests that the reperfusion and rewarming with autologous whole blood improved the recovery from CSP for 12 hours and likely reduced IRI at the muscular level. These data suggest that while the rewarming and reperfusion of the limb with donor whole blood was not ideal, there were positive improvements in the limb following 12 hours of cold storage. Furthermore, the lack of any significant upregulation of inflammatory cytokines would suggest that the use of autologous blood as a reperfusion fluid may improve the physical, physiological, and immunological status of the limb potentially impacting graft function after transplantation.

As indicated above this newly developed NHP VCA model took extensive iterations for refinement. During the study multiple factors limited our capabilities to equate physiological temperature in the blood and limb. Structural regulation maintained the reperfusion room at an approximate ambient temperature of 19°C which severely hindered the rewarming of the limb to the desired 38°C. We speculate that reduced blood oxygen saturation was possibility due to limb hypothermia, as sub-physiological temperatures are known to reduce the dissociation of oxygen from hemoglobin.[23] In addition, the pre-procurement hemorrhagic shock model likely had an effect on our cytokine and genetic expression graft profile. This shortcoming could be seen as a negative in the civilian VCA donation environment, but from a combat casualty setting this model is the first assessment of a deceased soldier in the field acting as a donor for other wounded warriors.

## CONCLUSION

The study outlined above demonstrates a method to recover an amputated NHP forelimb preserved using the clinical standard of care CSP/UW protocol. This work may serve as a baseline to compare other ischemia mitigation strategies, as no model of its kind exists to date. If serving as a baseline for the current clinical 'standard of care' protocol- based on lessons learned from solid organ transplantation, future work may be able to expand the transplant pool. Future work may include alternates to UW solution, specifically tissue preservation agents such as hydrogen iodide, hydrogen sulfate and hydrogen selenide.[24-26] Additionally, the use of oxygen carriers and/or blood substitutes may improve tissue preservation times and outcomes. Each of the above are ongoing areas of interest and research in our lab. This data will serve as a critical starting point and baseline for this future work.

The major difference in this study to clinical standard of care organ preservation techniques is the addition of an autologous blood-based reperfusion component rather than allogeneic blood. This NHP model allows one to evaluate the limb's physiologic and immunologic status without the compounding effect of the antigenic mismatch seen in allotransplantation. Therefore, the preservation technique alone can be singularly evaluated without being diluted by the immunological response to allogenicity. We plan on extending the scope of future studies to include different preservation solutions (oxygen and non-oxygen carrying), varying temperatures, and longer reperfusion phases to enable a more detailed physiologic and immunologic examination of the VCA.

## MILITARY SIGNIFICANCE

Major limb amputation is a common devastating traumatic injury seen in combat casualties. Statistics reveal that as of June 2015, there have been 1,645 cases of major limb amputations from Operation Iraqi Freedom (OIF), Operation New Dawn (OND) and Operation Enduring Freedom (OEF).[27] As a consequence of injuries to these vascularized composite tissues, a tremendous physical, psychological and economic burden is placed not only on the combatant, but their families and the operational readiness of the DoD. Currently, for limb loss, the most utilized restorative option is the use of prosthetics, which only partially restore mobility and functionality. The specialized field of vascularized composite allotransplantation (VCA) has the goal of transplanting donor limbs onto wounded warriors with extremity amputations to return full function and potential for return to duty. However, in spite of scientific and technological advancements in the field of VCA, the field of VCA has still encountered difficulties in standardization of prolonged tissue preservation procedures (beyond 6 hours). This limited preservation time reduces the time to transport and transplant the donated limb and effectively reduces the number of available donor limbs that could potentially be beneficial for a combat casualty. In the work presented here, we developed and standardized an NHP model of upper limb Vascularized composite allotransplant (VCA). In this model, we evaluated and standardize a technique for cold storage preservation NHP VCA tissues, followed by reperfusion with autologous whole blood. The parameters measured here can be considered foundational in terms of physiological, immunological and histological outcomes, which serve as baseline for following

studies evaluating different preservation/reperfusion agents. This study provides sufficient pre-clinical data that can easily be translated to human clinical applications, including the evaluation of ischemia/reperfusion injury in amputated extremities and preparation of donor VCA tissues prior to transplantation.

| Gene | Assay ID | | Gene | Assay ID | | Gene | Assay ID | | Gene | Assay ID |
|---|---|---|---|---|---|---|---|---|---|---|
| ACTA1 (HK) | Hs00559403_m1 | | CD70 | Rh02838000_m1 | | TXNRD1 | Rh04343960_m1 | | PTN | Rh02802821_m1 |
| ACTB (HK) | Rh03043379_gH | | CFLAR | Rh02846875_m1 | | MAPK8 | Rh01548508_m1 | | PTPRC (CD45) | Hs04189704_m1 |
| ADIPOQ | Rh02788052_m1 | | CHRM2 | Hs00265208_s1 | | MCL1 | Rh02879360_m1 | | RAGE | Rh02865606_m1 |
| ADORA2A | Rh02902837_m1 | | GPX1 | Rh01028922_g1 | | MDK | Hs00171064_m1 | | RIPK2 | Rh02846684_m1 |
| AKT1 | Rh02834327_m1 | | CRADD | Rh02914729_m1 | | mTOR | Hs00234508_m1 | | RPLP0 (HK) | Hs02992885_s1 |
| ATG8 (GABARAP) | Hs00925899_g1 | | CX3CR1 | Hs01922583_s1 | | MYD88 | Rh02842312_m1 | | RPS6KB1 | Rh00923436_m1 |
| BAD | Rh02848299_m1 | | CXCL1 | Rh03456656_mH | | SEPP1 | Rh03811846_m1 | | RTN4 | Rh02802344_m1 |
| BAX | Rh01016551_g1 | | DAPK1 | Rh02856080_m1 | | MYH1 | Rh04255418_m1 | | S100A6 | Rh02801773_m1 |
| BBC3 (PUMA) | Hs00248075_m1 | | DIABLO | Rh02853950_m1 | | MYH2 | Rh02929567_m1 | | S100B | Rh02799138_m1 |
| BCL2 | Hs00608023_m1 | | EP300 | Rh02838656_m1 | | MYOD1 | Rh02913647_m1 | | SGCA | Rh02826245_m1 |
| BDNF | Rh02788328_s1 | | FAS | Rh02787979_m1 | | MYOT | Rh02861830_m1 | | SLC2A4 | Rh02837153_m1 |
| BFAR | Rh02801062_m1 | | FASLG | Rh02787582_m1 | | NEB | Rh02854810_m1 | | SOD1 | Rh02787593_g1 |
| BID | Hs00609632_m1 | | FBXO32 | Rh02852339_m1 | | NFKB2 | Rh02621752_m1 | | SOD2 | Rh03063062_s1 |
| CAPN2 | Rh00965092_m1 | | FGF2 | Rh02787883_m1 | | NFKB1 | Rh02847287_m1 | | TLR4 | Rh01060206_m1 |
| CASP1 | Rh02992859_gH | | GAPDH (HK) | Rh02621745_g1 | | NOD1 | Rh02859608_m1 | | TNNI1 | Rh02845037_m1 |
| CASP3 | Hs00234387_m1 | | GDNF | Rh01047953_m1 | | NOL3 | Hs00358724_g1 | | TNNT1 | Rh01088194_m1 |
| CASP7 | Rh01029848_m1 | | HIF1a | Rh00936379_m1 | | NOS1 | Rh02829260_m1 | | SEPHS2 | Rh03811831_s1 |
| CASP8 | Rh02851256_m1 | | HMGB1 | Hs01590761_g1 | | NOS2 | Rh02789787_m1 | | TP53 | Rh02789310_m1 |
| CASP9 | Rh02837912_m1 | | BAG1 | Hs00185390_m1 | | NOS3 | Hs01574659_m1 | | TRAF3 | Rh02845108_m1 |
| CCL13 | Rh02857529_m1 | | HSPA1A | Hs00359163_s1 | | NRCAM | Rh02836109_m1 | | TRIM63 | Rh00822395_m1 |
| CCL2 | Rh02621753_m1 | | KMT2A | Hs00610538_m1 | | OSM | Rh02877363_m1 | | UTRN | Hs01126016_m1 |
| CCL22 | Hs01574247_m1 | | LEP | Rh02788316_m1 | | PPARGC1A | Rh02864710_m1 | | VCAM1 | Rh02837371_m1 |
| CD27 | Rh02800118_m1 | | LTA | Rh02829188_g1 | | PPARGC1B | Hs00991677_m1 | | | |
| CD40 | Rh02621776_m1 | | MAPK1 | Rh01046827_m1 | | PPP3CA (Calcineurin) | Rh00949962_mH | | | |

**Supplementary Table 1. RNA probes (94 genes) used to evaluate gene expression.**

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**14. ABSTRACT**

**Background**: Traumatic limb amputations represent a substantial challenge facing many civilian and combat wounded. Despite advancements in VCA research to date, a tissue preservation strategy that successfully mitigates ischemia reperfusion injury, preserves graft functionality, and provides long-term graft survival remains imperfect. **Objective**: To develop a non-human primate (NHP) model of upper limb vascularized composite allotransplant, that allow for evaluation of tissue preservation and reperfusion strategies. **Materials and methods:** Upper extremities were procured from male rhesus macaques (n=17). Cold storage preservation with University of Wisconsin solution for 12 hours at 4°C was performed, followed by reperfusion of the limbs using extracorporeal membrane oxygenation and a pulsatile perfusion pump with warm autologous whole blood. **Results:** The results shown here are gathered from n=5 extremities. Significant changes: (1) compared to BSLN lactate and potassium were significantly increased at R60, (2) Base deficit significantly decreased at R0 and R60, (3) Cytokines IL-8 and sCD40-L were significantly increased at R60. Histopathological analysis improved after reperfusion. **Conclusions:** This protocol establishes a baseline for a standardized NHP limb preservation and is the first step in developing an ex vivo pre-transplantation assessment tool providing statistical analysis of projected graft function and survival.

**15. SUBJECT TERMS**
Extremity, vascularized composite allotransplantation, non-human primate, preservation, reperfusion.

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