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TITLE: A Novel Application of Normothermic Machine Perfusion for Face Recovery to Reduce Intragraft Inflammation and Optimize Organ Viability

PRINCIPAL INVESTIGATORS: John Brassil, Gerald Brandacher, M.D.

CONTRACTING ORGANIZATION: Functional Circulation LLC
Johns Hopkins University School of Medicine

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John Brassil E-mail: jbrassil@functionalcirculation.com
Gerald Brandacher M.D. E-Mail: gbranda2@jhmi.edu

E-Mail:

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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Functional Circulation LLC
1810 Maple Avenue
Northbrook, IL 60062

Johns Hopkins University
733 N. Broadway, Ste. 117
Baltimore, MD 21205-1832

8. PERFORMING ORGANIZATION REPORT NUMBER

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14. ABSTRACT

Warriors who suffer from combat-related injuries. Face transplantation is currently the only treatment option to fully restore devastating craniofacial injuries with functional and anatomical equivalents by replacing "like-with-like" tissue. Recent advances in microsurgical techniques and immunosuppressive protocols have enabled wider application of face transplantation with highly encouraging results. However, the current gold standard in tissue preservation – static cold storage on ice – is insufficient to preserve facial allografts for more than a few hours. Advancements in the field of VCA regarding matching and allocation, desensitization, and potential tolerance induction are all within reasonable reach to achieve; these are, however, constrained by limited preservation time. Thus, this project applies normothermic machine perfusion (NMP), as practiced clinically in vital organ transplant, to the specific and unique requirements of the face to increase tissue viability and to reduce both ischemia reperfusion injury and inflammatory potential. Initial trials showed excellent feasibility of surgical flap harvest as well as vascular cannulation. Grafts tolerated 24 hours of perfusion without any gross signs of tissue ischemia or necrosis. Weight change from experimental start to the end of perfusion ranged from 6% to 16% in our trial flaps with minimal visible edematous changes. H&E analysis showed normal appearing skin in both the flaps after 24 hours of perfusion. Biochemical analysis showed little chemical change in the monitored analytes to suggest tissue ischemia or cell death.

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1. Introduction

Vascularized composite allotransplantation (VCA) holds much promise to improve the quality of life for our Wounded Warriors who suffer from combat-related injuries. Face transplantation is currently the only treatment option to fully restore devastating craniofacial injuries with functional and anatomical equivalents by replacing *"like-with-like"* tissue. Recent advances in microsurgical techniques and immunosuppressive protocols have enabled wider application of face transplantation with highly encouraging results. However, the current gold standard in tissue preservation – static cold storage on ice – is insufficient to preserve facial allografts for more than a few hours. Advancements in the field of VCA regarding matching and allocation, desensitization, and potential tolerance induction are all within reasonable reach to achieve; these are, however, constrained by limited preservation time. Thus, this project applies normothermic machine perfusion (NMP), as practiced clinically in vital organ transplant, to the specific and unique requirements of the face to increase tissue viability and to reduce both ischemia reperfusion injury and inflammatory potential. Initial trials showed excellent feasibility of surgical flap harvest as well as vascular cannulation. Grafts tolerated 24 hours of perfusion without any gross signs of tissue ischemia or necrosis. Weight change from experimental start to the end of perfusion ranged from 6% to 16% in our trial flaps with minimal visible edematous changes. H&E analysis showed normal appearing skin in both the flaps after 24 hours of perfusion. Biochemical analysis showed little chemical change in the monitored analytes to suggest tissue ischemia or cell death.

2. Keywords

VCA, normothermic machine perfusion, sub-normothermic machine perfusion, face transplantation, preservation

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3. Accomplishments

- **What were the major goals of the project?**

Site 1:

The Site 1 goals for this Year 1 reporting period were the implementation of Specific Aim 1, to design and produce perfusion systems that apply sustained normothermic, pulsatile perfusion, active disinfection, and ergonomic support to enable 24-hour, prolonged preservation of faces. This implementation consisted of 1) providing two normothermic perfusion systems adapted to experimental requirements, 2) performing verification tests and installing those systems, 3) supporting their operation (on site and virtually) during the experiments, and 4) providing revisions to those systems in response issues identified in their use (Subtask 6).

Site 2

The major goals for Site 2 during the Year 1 reporting period were focused on collaboration with Site 1 to work towards completion of SA 1 (to design and produce a perfusion system that applies sustained normothermic, pulsatile perfusion, active disinfection, and ergonomic support to enable 24-hour, prolonged preservation of faces.) Site 2 aimed to obtain Institutional Animal Care and Use Committee (IACUC) and DoD Animal Care and Use Review Office (ACURO) approvals in order to initiate animal experimental work for this project (SA 1, Subtask 2), and to perform swine hemifacial graft explants for testing on the new perfusion device (SA1, Subtask 3/4). Site 2's work provided critical information for the revision/ optimization of the perfusion machines under SA1, Subtask 6.

Specific Aim 1: Design and produce a perfusion system that applies sustained normothermic, pulsatile perfusion, active disinfection, and ergonomic support to enable 24-hour, prolonged preservation of faces.	**Timeline**	**Site 1** (Initiating PI)	**Site 2** (Partnering PI)	**Progress**
Major Task 1: Development of benchtop normothermic perfusion systems adapted for 24-hour perfusion of the face.	Months	Functional Circulation (Brassil)	Johns Hopkins (Dr. Brandacher)	%
Subtask 1: Design and develop two normothermic perfusion systems for concurrent preservation of porcine hemifaces including component design for temperature modulation, pressure control, oxygenation, automation, and combination disposable/reusable perfusion circuit with passage of Assembly and Factory Acceptance Test	1-3	X		100
Milestone #1: Perfusion systems ready for Major Task 1, Subtask 3				

Subtask 2: Submit documents for Institutional Animal Care and Use Committee (IACUC) and DoD Animal Care and Use Review Office (ACURO) approvals	1-3		X	100
Milestone #2: IACUC and ACURO approval obtained				
Subtask 3: Provide and support the perfusion systems for initial testing of the perfusion system on porcine hemifaces	4-6	X		100
Subtask 4: Perform hemiface graft explants for testing on new perfusion device [4-6 hemifacial grafts]	4-6		X	80
Subtask 5: Perform biochemical and histological testing on grafts and control tissues at specified timepoints (hours 1, 2, 6, 8, 20, 24) during perfusion and at the experimental end [H&E, IHC, glucose, lactate, pH]	4-6		X	90
Subtask 6: Review results from Subtask 3-5 and revise perfusion system as necessary to optimize tissue preservation	7-8	X	X	90
Milestone #3: Perfusion systems ready for Major Task 2				
Specific Aim 2: Perform orthotopic transplantation of SLA-matched and mismatched porcine hemifaces.				
Major Task 2: Explant, preserve, transplant, and manage and observe recovery of swine hemiface recipients (n = 25 recipient swine, n = 13 donor swine)				
Subtask 1: Provide and support two perfusion systems to perform hemiface preservation	8-24	X		
Subtask 2: Perform orthotopic facial transplantation using a swine leukocyte antigen (SLA) defined minipig model with major histocompatibility match with tacrolimus maintenance using grafts stored for 12 hours in static cold storage (Group I; control group) [5 recipient pigs total]	8-11	X	X	
Subtask 3: Perform orthotopic facial transplantation using a swine leukocyte antigen (SLA) defined minipig model with major histocompatibility match with tacrolimus maintenance using grafts perfused for 12 hours of NMP (Group II; control group) [5 recipient pigs total]	11-14	X	X	
Subtask 4: Perform orthotopic facial transplantation using a swine leukocyte antigen (SLA) defined minipig model with major histocompatibility match with tacrolimus maintenance using grafts perfused for 24 hours of NMP (Group III; experimental group) [5 recipient pigs total]	15-18	X	X	
Subtask 5: Perform orthotopic facial transplantation using a swine leukocyte antigen (SLA) defined minipig model with major histocompatibility match with tacrolimus maintenance using grafts perfused for 24 hours sequentially comprising of NMP, hypothermic machine perfusion, and NMP (Group IV; experimental group) [5 recipient pigs total]	19-22	X	X	
Subtask 6: Perform orthotopic facial transplantation using a swine leukocyte antigen (SLA) defined minipig model across a full major histocompatibility mismatch with induction therapy and tacrolimus maintenance using grafts perfused for 24 hours sequentially comprising of NMP, hypothermic machine perfusion, and NMP (Group V; experimental group) [5 recipient pigs total]	22-24	X	X	
Subtask 7: Perform protocol skin biopsies from healthy pig skin, experimental, and control groups and evaluate histopathological changes (H&E, IHC).	8-24		X	
Subtask 8: Perform biochemical monitoring during graft perfusion (Glucose, lactate, pH)	8-24		X	
Subtask 9: Perform immune cell phenotyping, metabolic monitoring (CBC, LFT, Cr, BUN, glucose), and immune monitoring assays (Luminex, CFSE MLR) on Group V animals	8-24		X	
Subtask 10: Analyze and compare the data from Groups I-V to assess for optimal perfusion system and settings	24	X	X	
Milestone #3: Experimental data collected and analyzed				
Specific Aim 3: Apply the developed approaches of 24-hours normothermic preservation with viability assessment to human faces donated for research.				
Major Task 3: Explant, preserve, and analyze human faces donated for research (n = 2-3)				
Subtask 1: Apply for HRPO and IRB approval	15-24		X	
Milestone #4: HRPO approval received				
Subtask 2: Contract with local OPO to obtain faces donated for research	15-24		X	

Subtask 3: Design, assemble, and qualify portable perfusion system for faces	19-24	X		
Milestone #5: Approvals, agreements, and systems in place for feasibility study of portable perfusion preservation of human faces				
Subtask 4: Recovery, preservation, and analysis of human faces normothermically preserved on the portable system	25-33	X	X	
Subtask 5: Compile Design Outputs for FDA Design History File	33-36	X		
Milestone #6: Feasibility study with human faces complete				
Subtask 6: Write and submit results for publication	33-36	X	X	

- **What was accomplished under these goals?**

Site 1:

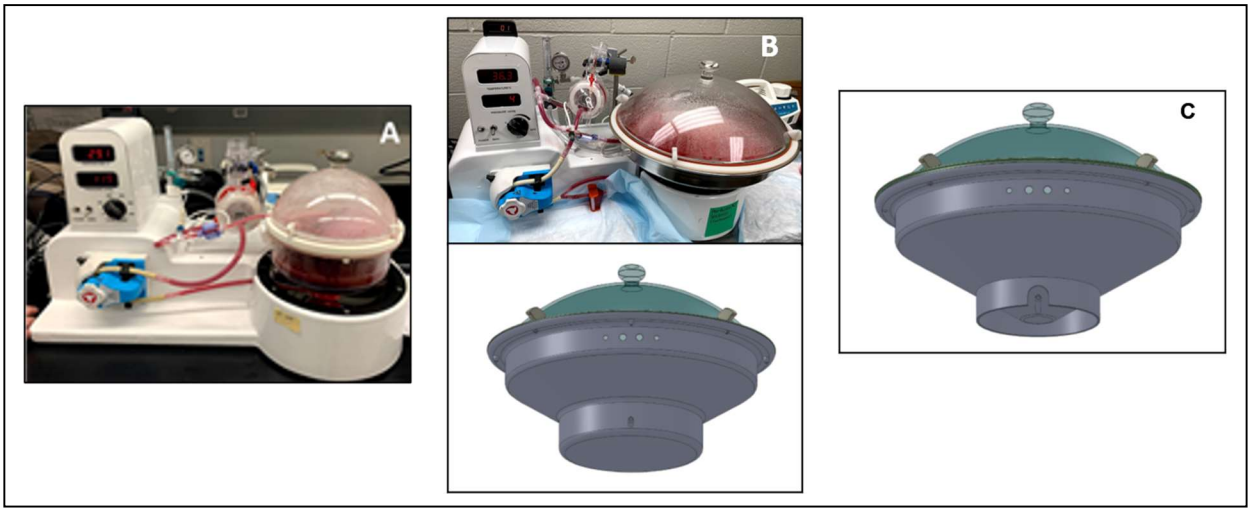
Major Activities

At Site 1, the major activities focused on providing, maintaining, and improving two of our Model 30 perfusion systems to meet the initial and evolving needs of the project. Initially these activities involved designing, procuring, testing, and integrating the elements of the systems required by the SOW. These initial elements included cannulas, pulsation software, revised sealing and sterilization features, normothermic perfusate, temperature-controlled heaters, microprocessor controller, and calibration and verification protocols and tests. Once the systems were verified, Site 1 participated in their installation at Site 2 and in their initial and subsequent use in the SA1 feasibility groups (Subtask 4).

According to Subtask 6, additional design and implementation activities were performed responsive to either identified problems or new requirements uncovered in the feasibility perfusion studies. Additional capabilities were developed and integrated into the perfusion systems including the large 360 mm perfusion bioreactor, 40 micron leukocyte filter, digital filtering of mechanical signals to stabilize the outputs, datalogging with Wi-Fi and USB interfaces, switchable operating mode between continuous and pulsatile perfusion, improved aseptic sealing, and calibrated temperature testing.

Further activity within this year included two dry run studies using the perfusion systems with perfusate but without hemiface flaps. These studies provided the opportunity to observe more carefully the systems mechanically and from a fluidic standpoint. They suggested further modifications to the new larger-sized perfusate reservoir to prevent red blood cell settling at its base (**Figure 1).** These new reservoirs are now on order. The investigators also learned in these dry run studies that the systems' sterilization and sealing prevented gross microbial growth in now multiple 24-hour studies.

Figure 1: Evolution of Reservoir. A) Smaller reservoir on explant day 1. B) Revised larger reservoir with flat bottom. C) Second revision of reservoir with conical base which is currently in production.



Specific Objectives

The **Site 1** specific objectives for this Year 1 were to provide two systems capable of 24-hour normothermic perfusion of porcine hemifaces. The purpose of these systems was to evaluate the perfusion process feasibility in preparation of porcine hemiface transplant using the same process in SA2. Success in the feasibility phase in SA1 depended on adequate maintenance of the perfusion environment such that the hemiface flaps would be substantially viable, practically uninfected by pathogens, and with minimal inflammatory potential. In this bench feasibility phase, histological, biochemical, and blood culture testing measured the status of the preserved hemiface flap toward suitability for proceeding to the transplant phase. Specifically, for Site 1, the characteristics of controlled pressure, flow, temperature, pulsatility, and aseptic environment in a context of overall reliability encompassed the original system requirements. Further objectives were identified indicating a larger-sized bioreactor and datalogging capability, which were also met.

Significant Results or Key Outcomes

The perfusion systems with required modifications have been built and provided to support the initial project requirements and newly agreed requirements around bioreactor size and datalogging.

Other Accomplishments

In addition to providing and supporting two perfusion systems for the identified Aims, Site 1 has accomplished the following additional objectives:

- We performed a bench feasibility test of a dialysis circuit integrated with the Model 30 perfusion system (Appendix 1), responsive to the SA2 anticipated problems and pitfalls in the Project Narrative.
- We designed a revised 360 mm bioreactor to prevent cell aggregation at its base (Figure 1B)
- We accepted responsibility for providing a biochemistry analyzer instrument per SA 1 Subtask 5. We have applied to the GO to transfer that responsibility from Site 2 to Site 1, and meanwhile we have purchased the instrument expecting reimbursement by the grant only if the SOW revision is approved.
- We have received the Design History File Table of Contents from our regulatory consultant, which is the first step towards the ISO 14971 risk analysis indicated in SA3.

Site 2

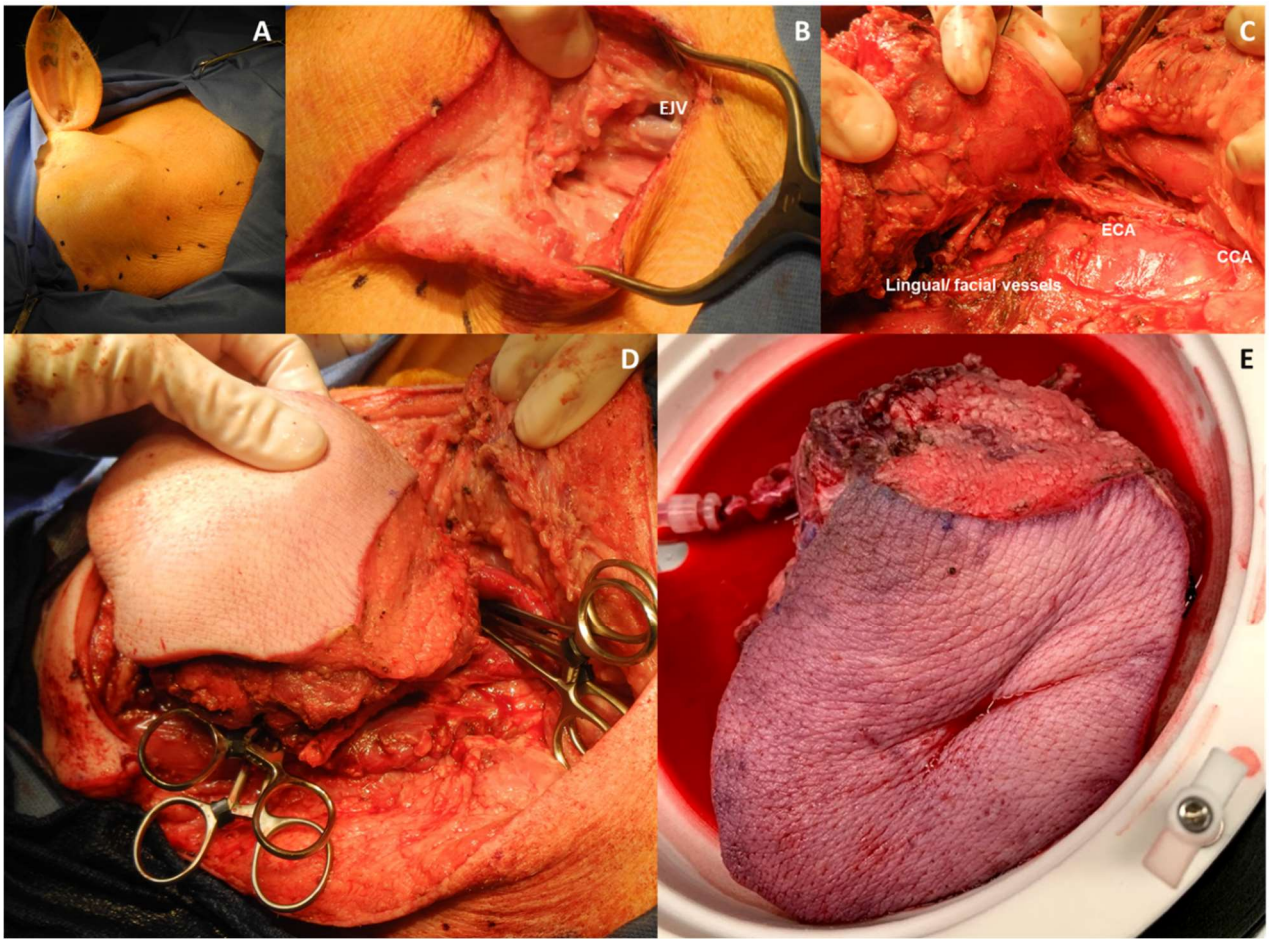
At the start of this reporting period, Institutional Animal Care and Use Committee (IACUC) and DoD Animal Care and Use Review Office (ACURO) approvals were obtained (Milestone #2 under SA1, Subtask 2). This allowed for experimental work on swine hemifaces under SA1, subtask 3 and 4 to be initiated. On February 12, 2020, two hemifacial grafts were explanted from a single pig 25232 as a terminal procedure for a 24 hours perfusion on the first iteration of bioreactors provided by Site 1. This first day provided early feedback on the functions of the bioreactors as well as key surgical insights into the optimal approach for flap harvest in Major Task 2.

Surgically, the focus of this first experiment was exploration of swine hemifacial vasculature with the creation of a more limited flap. With the first hemiface explant (**Figure 2**), the dissection began in the neck, through the sternocleidomastoid and strap muscles with the goal of identifying the external jugular vein (EJV) and common carotid artery (CCA) (**Figure 2A-C**), which together serve as the pedicle for the hemifacial grafts.

Dissection was then carried cranially and anteriorly until the linguofacial vessels could be identified (**Figure 2C**). The flap was then elevated off the underlying bone and soft tissue, and included skin, soft tissue, muscle, and glandular components of the pig lateral face (**Figure 2D**).

Flap was left in place perfused while the dissection of the second hemiface was started so that both flaps could be removed at the same time upon sacrifice of the animal and ischemic time could be minimized. However due to ligation of a maxillary vein during the dissection of this first hemiface graft, the graft appeared to have some element of thrombosis when it was ultimately harvested and placed on the perfusion machine (**Figure 2E**).

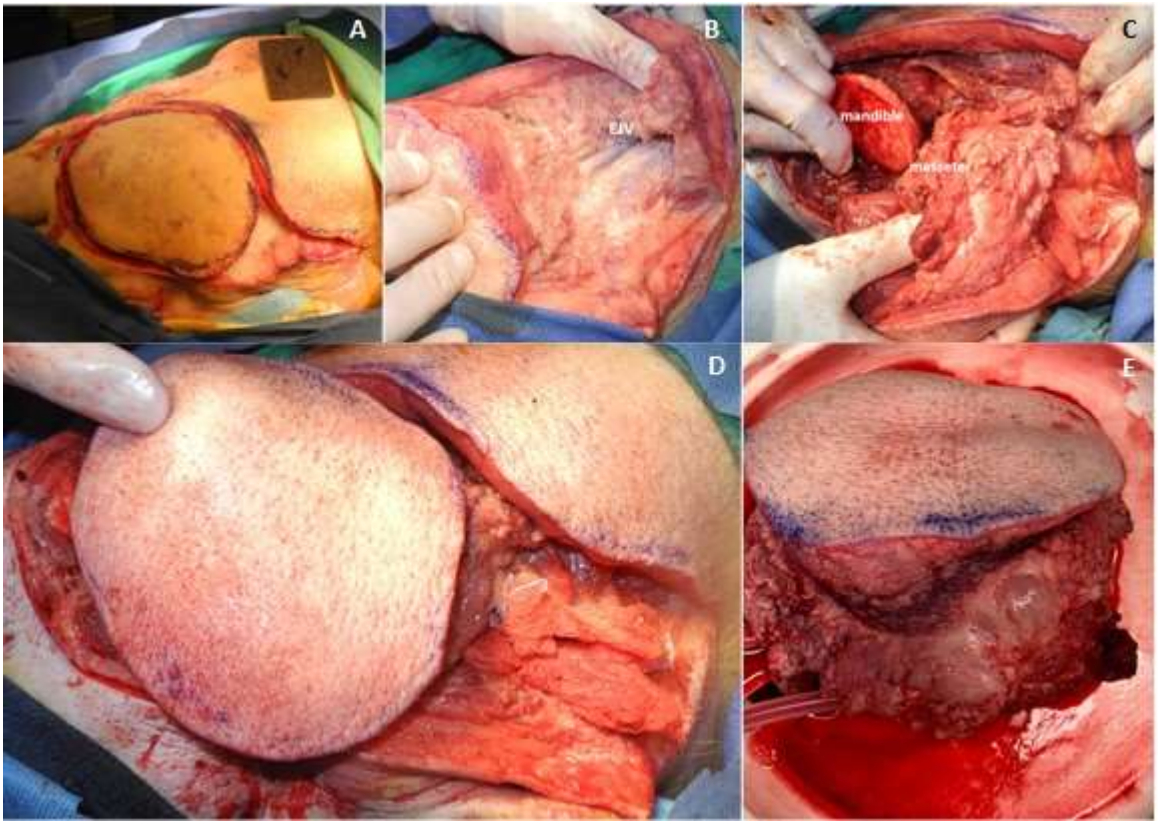
Figure 2: Dissection of Hemi-facial Graft #1 Explant Day 1. A) Skin marking for flap dissection. B) Initial approach from anterior neck to identify EJV. C) Dissection carried forward to isolate common and external carotid arteries as well as lingual and facial arteries and veins. D) Flap immediately after elevation while still being perfused. E) Flap upon connection to bioreactor.



With the lessons learned from the first hemifacial dissection in mind, contralateral dissection was initiated (**Figure 3**). A more anterior approach was leveraged to allow for earlier identification of the EJV and CCA while avoiding a lengthy dissection into the strap muscles and near the airway, which occurred with the first flap dissection (**Figure 3B**). Care was also taken not to ligate the maxillary vein or any other large vein to avoid the thrombosis issues noted with the hemi-facial graft.

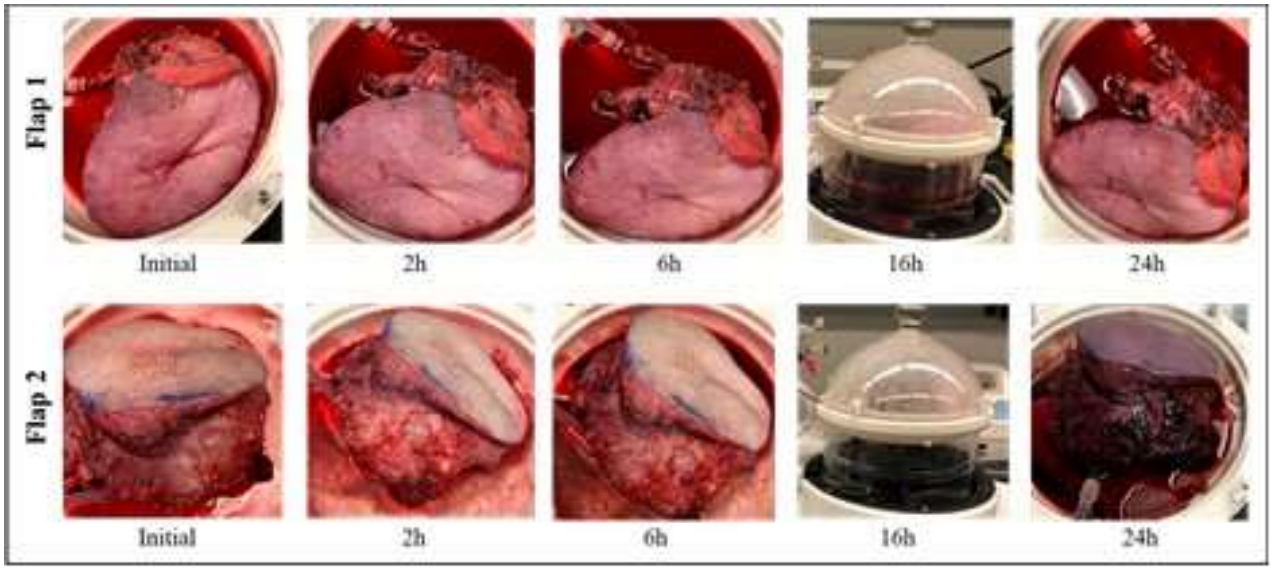
From this point, a similar dissection was pursued to dissect out the course of the linguofacial vessels. However, on this side of the face, the course of the linguofacial vessels was noted to be markedly different from the contralateral side as the vessels coursed behind the mandible. This required a deeper dissection of the tissue until the soft tissue could be elevated off the external aspect of the mandible as well as deep to it (**Figure 3C** and **3D**). The graft upon initiation of perfusion appeared healthy and non-thrombosed (**Figure 3E**).

Figure 3: Dissection of Hemi-facial Graft #2 Explant Day 1. A) Skin marking for flap dissection along with initial incisions along those lines. B) Initial approach more anterior and medial to more quickly identify EJV. C) Dissection carried forward to locate linguofacial vessels however, on this side tissue had to be mobilized deep to the mandible. D) Flap immediately after elevation. E) Flap upon connection to bioreactor.



Both flaps were removed from the animals and blood was collected from the terminal donor animal for perfusate preparation. Flap 1 was then placed on a pulsatile perfusion circuit and perfused for 24 hours. Flap 2 was placed on a continuous perfusion circuit and perfused for 24 hours. Appearance of the grafts over the perfusion period are demonstrated in **Figure 4**.

Figure 4: External Appearance of Hemifacial Flaps over Perfusion Period, Explant Day 1



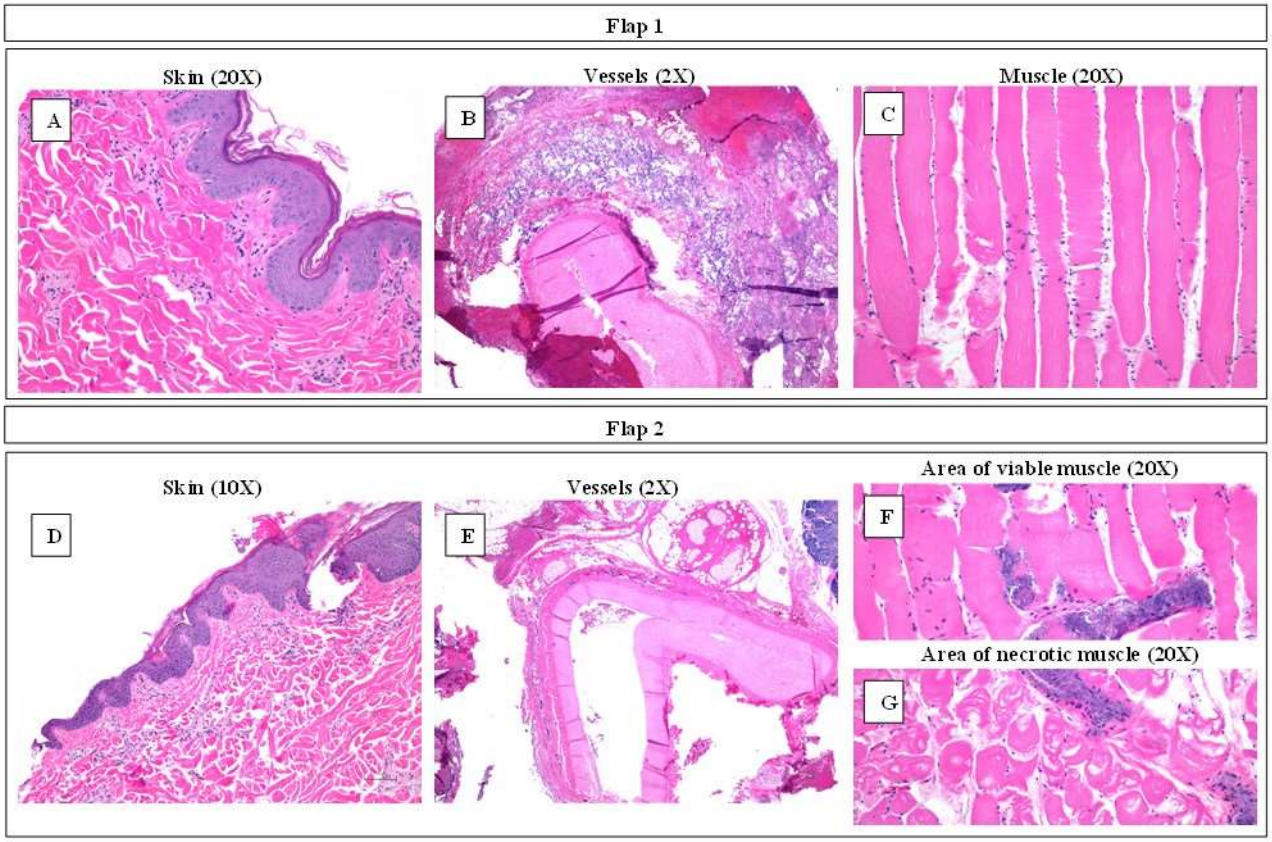
Flap 1, while initially exhibiting skin changes concerning for thrombotic injury, remained stable on the perfusion circuit after 24 hours. Pre- and post- perfusion weights are noted in Table 1, showing that the weight of Flap 1 decreased over the perfusion period. Flap 2 developed a thrombus in the venous outflow tract overnight (**Figure 3**), and weight increased over the perfusion period (**Table 1**). From an etiologic standpoint, it is suspected that a lack of heparin precipitated this issue.

Table 1: Flap Weight Change over Perfusion Period, Explant Day 1

	Flap 1	Flap 2
Initial Weight (g)	344	282
Post Perfusion Weight (g)	254	482
% Change	26% Decrease	82% Increase

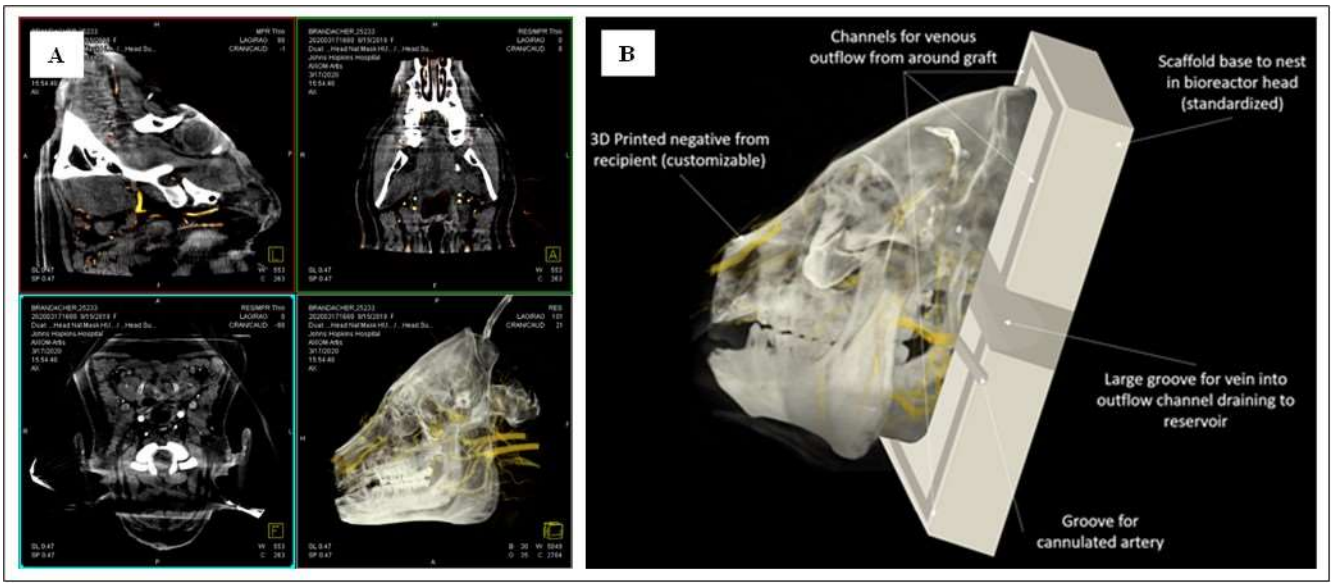
Under Subtask 4, skin, muscle, and vessel samples were taken from the perfused flaps and preserved in formalin. Histological evaluation revealed 100% viable skin and artery as well as 80-90% viable muscle in flap 1 after normothermic machine perfusion for 24 hours with an oxygenated autologous blood-based colloid solution. Flap 2 thrombosed, and unsurprisingly had inferior tissue viability. Flap 2 skin and muscle were 70% and >50% viable respectively. Flap 2 artery, nerve, and lymphatics were 100% viable. Representative tissues from both flaps are shown in **Figure 5**.

Figure 5: Histological Appearance of Flaps After 24 Hours Perfusion, Explant Day 1. A) Flap 1 skin with 100% viable epidermis, dermis, adnexa, and subcutis. B) Flap 1 artery is 100% viable with some surrounding necrosis/ inflammation. C) Flap 1 muscle 80-90% viable. D) Flap 2 skin 70% viable. E) 100% viable Flap 2 artery surrounded by necrosis/ inflammation. Viable nerve and lymphatic tissue. F) & G) Flap 2 with less than 50% viable muscle.



Importantly, this first surgical experience with hemifacial flap explantation revealed significant linguofacial vascular variation between either side of the swine face. A comprehensive literature review was undertaken to better understand swine facial vascular anatomy, but this revealed a lack of published data on this subject. To obtain a better pre-operative view of this critical anatomy, an expert veterinary radiologist was consulted to select an optimal pre-operative imaging strategy. The led to a first trial of cone beam computed tomography angiography (CTA) of a terminal swine with the veterinary radiologist (**Figure 6A**) which demonstrated good vascular granularity as feasibility of future CTAs for pre-op swine donors. As such, a procedure amendment was written and submitted to the Johns Hopkins IACUC for routine preoperative cone beam CTA for donor and recipient swine in this protocol.

Figure 6: Swine Cone Beam CTA. A) Representative images. B) Rendering of example scaffold.



Further this imaging also serves as a basis for creating recipient specific scaffolds that can be attached to the perfusion machine in a modular fashion (**Figure 6B**). To this end we have reached out to experts in 3D printing within Johns Hopkins regarding a collaboration to produce these scaffolds for future experiments.

Unfortunately, at this point in our work, the COVD-19 pandemic led to a shutdown of our research operations, which prevented major animal surgeries from mid-March 2020 to July 6, 2020. This created a 4.5-month delay of study progress on the surgical end. As such, Site 2 focused on revising the perfusion system as necessary to optimize tissue preservation (SA1, Subtask 6).

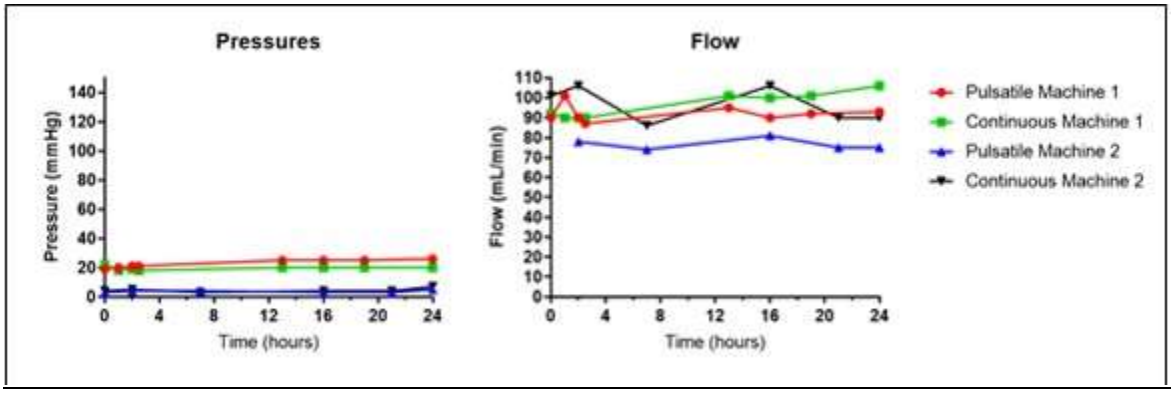
Our first surgical experience also revealed that the initial size of the reservoir for the hemifacial flap and perfusate was too small and would need to be enlarged. Our Site 2 collaborator worked to enlarge the entire reservoir to accommodate a large flap size (**Figure 1A** to **1B**). Further, the shutdown time was used to review literature on optimal perfusates for normothermic perfusion of VCA, and two 24 hour perfusate-only runs of the bioreactors with were performed with the goal of better characterizing the behavior of the perfusate over time.

Both dry runs were conducted with one continuous and one pulsatile circuit. Given concerns for thrombosis and bacterial contamination in the first surgical experience, heparin and broad-spectrum antibiotics were added to the perfusate. We did see a thrombus in the circuit f of the continuous machines running blood from an unusually thrombogenic swine.

Final cultures of perfusates from both dry runs were negative for bacterial growth—an improvement from explant day one, where the perfusate and graft did have bacterial growth visible on histology.

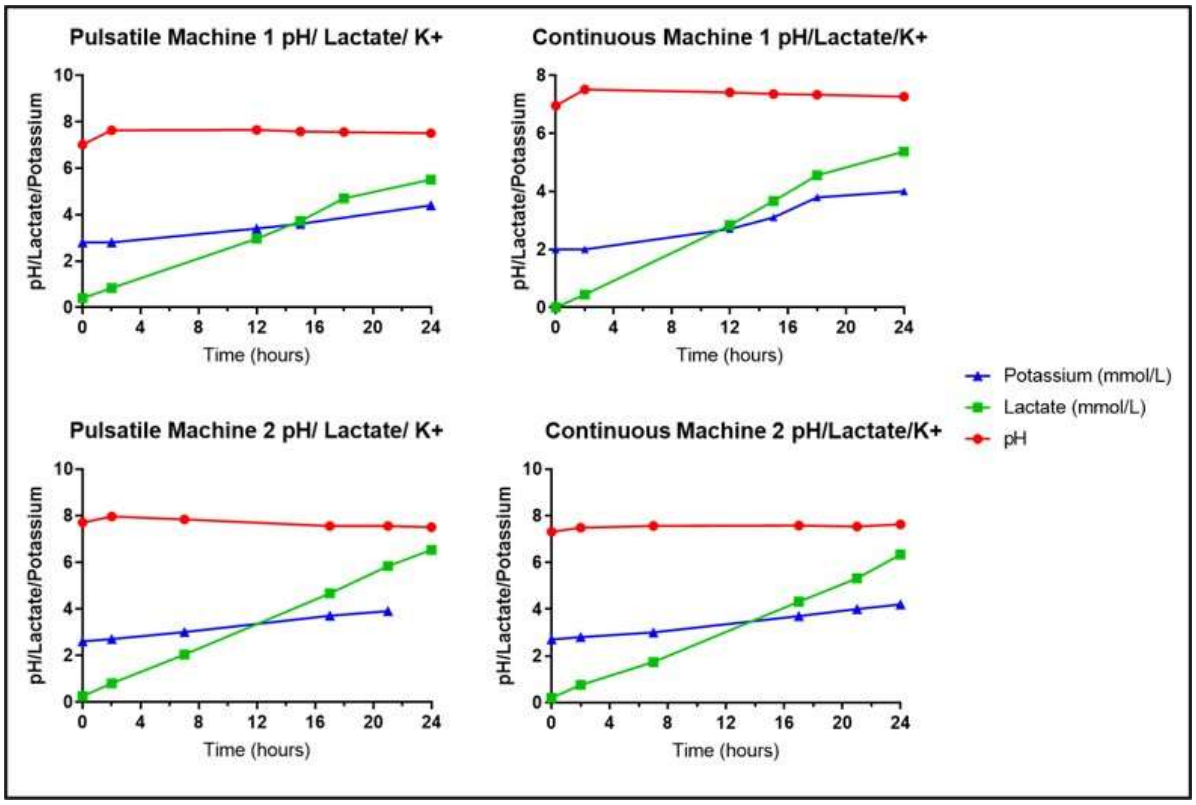
Perfusion hemodynamics and chemistries were monitored for both machines on both dry-run days and are shown in Figures 7 and 8 below.

Figure 7: Pressure and Flow in 24-hr Perfusate Only Runs on Continuous and Pulsatile Pumps



Flow and pressures as demonstrated in **Figure 7** are lower than the typically expected parameters as there is no actual graft in the circuit increasing the resistance.

Figure 8: Key Biochemical Parameters in 24-hr Perfusate Only Runs on Continuous and Pulsatile Pumps



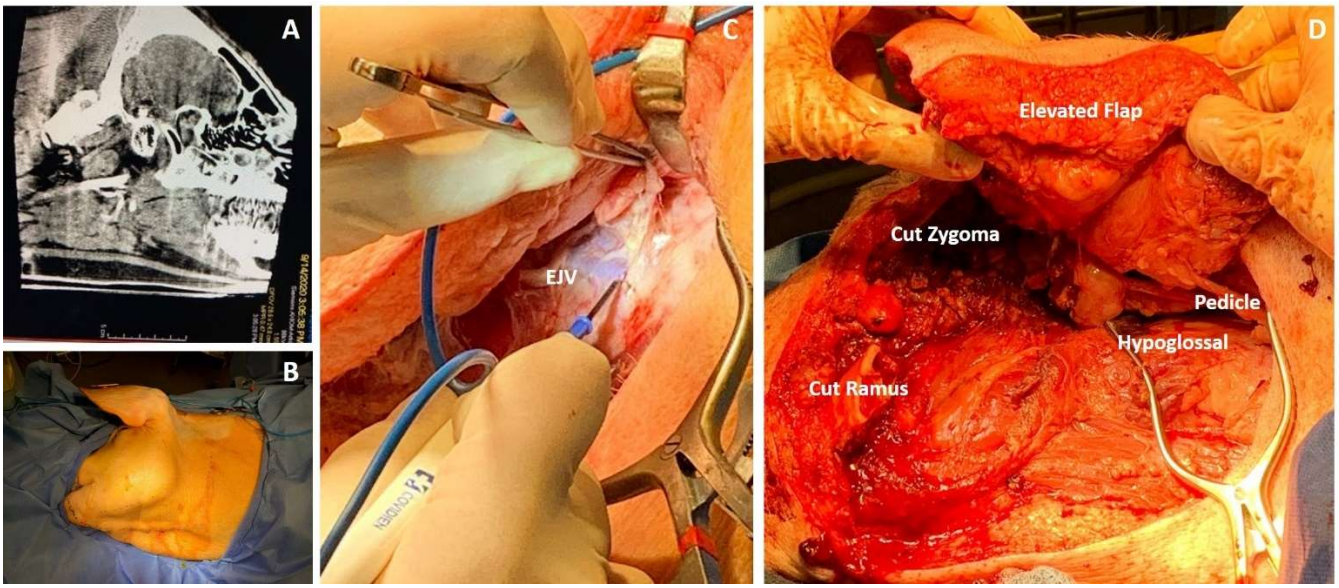
Across both pulsatile and continuous runs, key biochemical parameters were well replicated providing an understanding of the background metabolism of red blood cells alone perfusing through the circuit. One perfusate issue associated with the large reservoir base (**Figure 1B**) was noted in the process of these perfusion runs: erythrocytes were tending to settle in the flat wide reservoir base. As such adjustments are being made with Site 2 to make the base more conical (**Figure 1C**) to allow for better drainage of erythrocytes out of the reservoir.

Upon the reopening of the animal surgical facilities at Johns Hopkins in June, we were able to order swine and prepare for a second set of hemifacial explants. Swine 25300 (**Figure 9B**) was taken to the operating on September 25, 2020 for this purpose. Cone beam CT was performed prior to the explantation (representative image located in **Figure 9A**), which allowed for more extensive planning around vascular anatomy. Flap size was also enlarged to include the ear.

This dissection experience allowed for closer identification of useful landmarks (representative images shown in **Figure 9**). The initial approach was largely similar to the first explant surgery, with dissection to the strap muscles of neck to allow rapid identification of EJV/ carotid artery pedicle (**Figure 9A**). However, the styloid process, medial pterygoid, and zygomatic arch provide useful borders that protect the flap vessels from disruption during dissection (**Figure 9D**).

A more detailed discussion of the surgical procedure will be further provided in the next quarterly report once a full surgical team debrief is completed. This discussion and one more set of hemifacial explants will be completed prior to finalization of the hemifacial explant procedure for SA2 transplants.

Figure 9: Representative Images from Hemifacial Explant Day 2. A) Representative cross section from cone beam CTA. B) Positioning of Swine 25300 for explantation of Flap 1. C) Strap muscles dissected and retracted providing access to the external jugular vein (EJV). D) Elevated hemifacial flap with pedicle still attached.

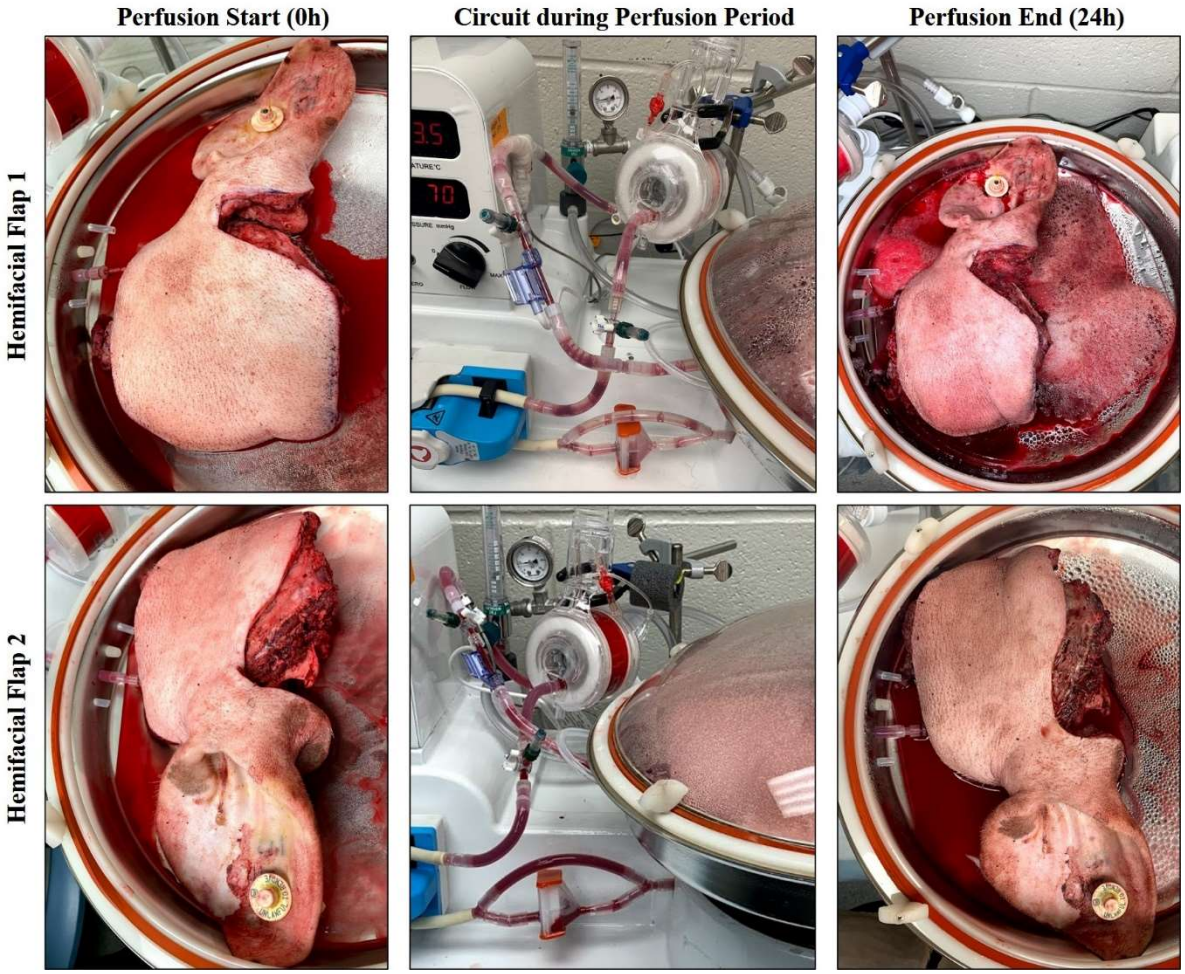


Subsequently, two hemifacial flaps containing the same tissue components as in experiment 1, but including the ear were explanted, flushed, and weighed before being placed in the enlarged reservoir for 24 hour normothermic perfusion with the modified autologous whole blood based perfusate. Flap 1 was placed on a continuous perfusion circuit and Flap 2 was placed on a pulsatile perfusion circuit.

Both machines were set to maintain a target arterial pressure of 50mmHg +/- 10mmHg. Perfusate was modified from the first experiment to contain heparin, antibiotics, and methylprednisolone. In addition, the perfusate was adjusted during the perfusion period to maintain a glucose level >50mg/dL.

Both Flaps upon initiation and termination of perfusion are pictured below in **Figure 10**. Flap 1 on the continuous perfusion pump unfortunately had a technical failure at hour 4 of perfusion which caused a loss of perfusate volume as well as air entry into the circuit (**Figure 10**). Although evidence of frank air in the perfusion circuit was not immediately notable, by perfusion hour 17, the presence of air in the circuit was very pronounced.

Figure 10: Hemifacial grafts pre- and post- perfusion. Top Row (left to right): Hemifacial flap 1 at beginning of perfusion, air entry into circuit, graft with evidence of air embolism and thrombosis. Bottom row (left to right): Hemifacial flap 2 at beginning of perfusion, appropriate appearance of circuit during perfusion, and 24 hour normothermically perfused graft.



An additional 250cc of perfusate was added to the Flap 1 perfusion system at ~hour 20 given concern that the relative lack of fluid in the circuitry could be due to loss of volume; however, at this point significant air had embolized into the graft and resulted in prominent thrombosis at the Flap 1 venous outflow. Weight change and relative size of both grafts is demonstrated in **Table 2** and **Figure 11** below.

Table 2: Flap Weight Change over Perfusion Period, Explant Day 2

	Flap 1	Flap 2
Initial Weight (g)	924	1965
Post Perfusion Weight (g)	1950	1492
% Change	**111% increase**	**24% decrease**

Interestingly, in both experiments, the graft placed on the pulsatile pump lost weight upon completion of perfusion.

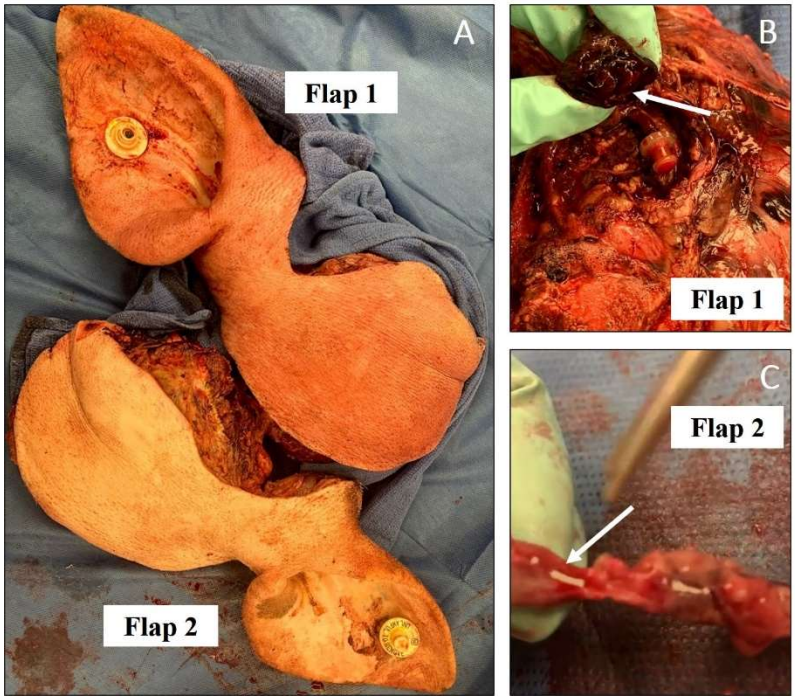
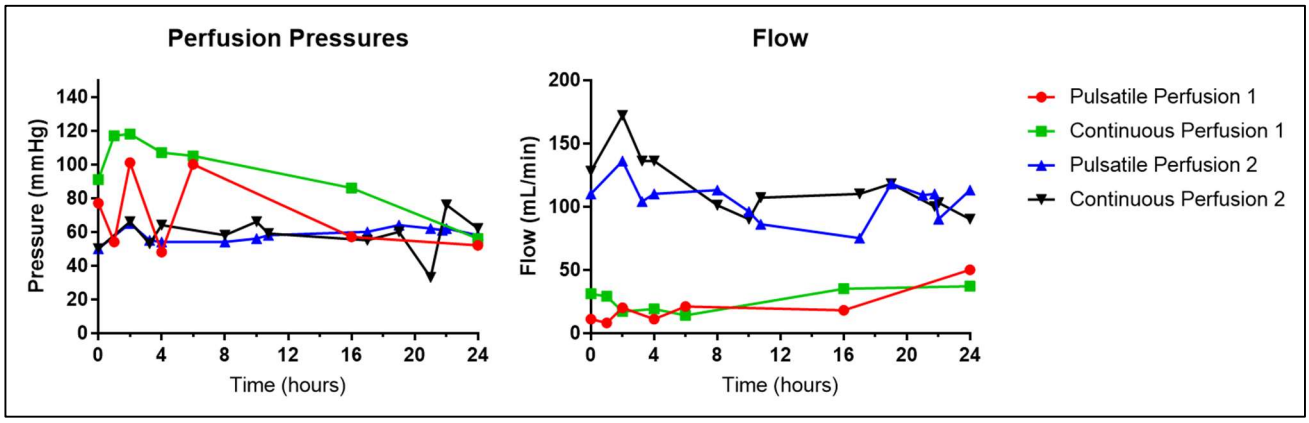


Figure 11: Relative Sizes of Flaps at end of Perfusion Period and Venous Outflow. A) Flap 1 was clearly congested and thrombosed at 24 hours perfusion compared to Flap 2. B) Flap 1 thrombosed venous outflow. C) Flap 2 cross section of venous outflow with no evidence of thrombosis.

Perfusion hemodynamics and chemistries were monitored for both flaps and data from both explant days is shown below in **Figures 12** and **Figure 13**.

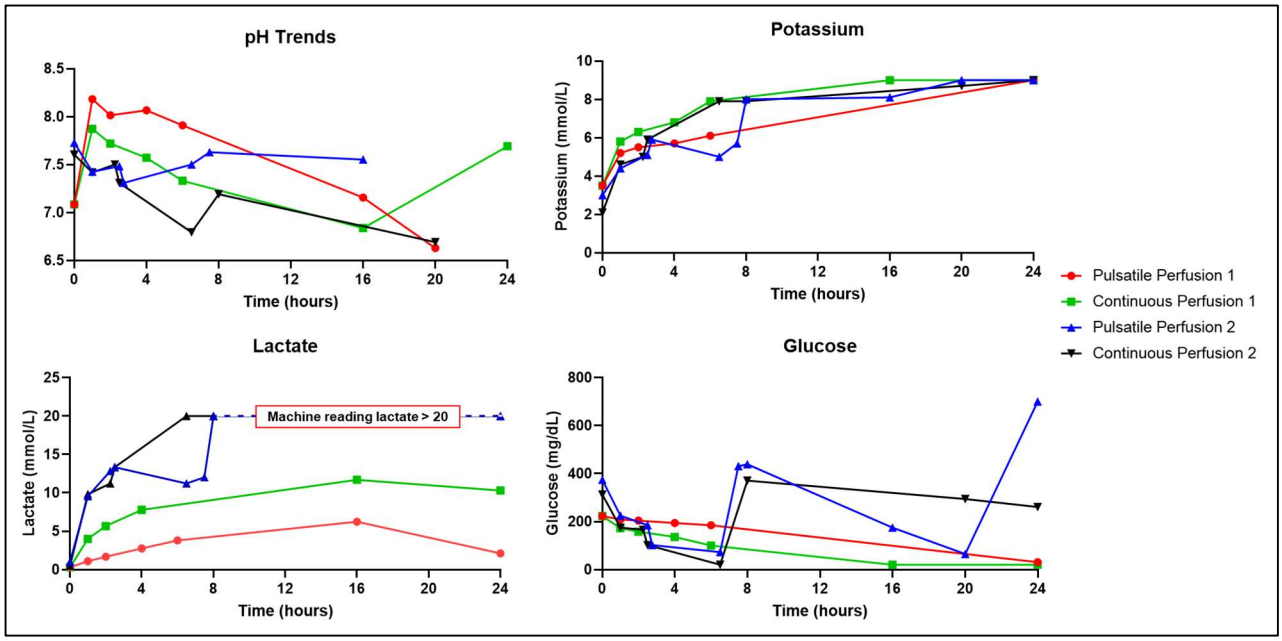
Figure 12: Pressure and Flow in 24-hr Normothermic Perfusion of Hemiface Explants on Continuous and Pulsatile Pumps



Data demonstrates more careful titration of pressures at target of 50mmHg +/- 10mmHg in experiment #2. Flow rates were distinctly higher during experiment #2 compared to experiment #1, which was consistent with the larger reservoir and graft sizes in experiment #2 though perfusate volume was the same.

This same increase in graft size relative to perfusate volume is suspected to be the cause of biochemical differences between the two experiments as well. Compared to experiment #1, the values from experiment #2 are consistent with a faster accumulation of lactate and consumption of glucose (**Figure 13** below).

Figure 13: Key Biological Parameters in 24-hr Normothermic Perfusion of Hemiface Explants on Continuous and Pulsatile Pumps



At this time, histopathological analysis of samples from the second experiment are pending analysis and will be included in the next quarterly report.

- **What opportunities for training and professional development has the project provided?**
  Nothing to report

- **How were the results disseminated to communities of interest?**
  Nothing to report

- **What do you plan to do during the next reporting period to accomplish the goals?**

Site 1:

In the next reporting period Site 1 will perform additional development activities to support the completion of the SA1 feasibility phase and to prepare for immediate transition to the SA2 transplantation phase. This will involve the revised 360 mm bioreactor better suited to prevent cell settling (already on order), plus evaluating and developing where possible additional capabilities for perfusate swap-out, increased perfusate volume, and venous sampling for incorporation in the next feasibility experiment.

In anticipation of SA2 we will design the disposable perfusion circuit as indicated in the project budget and as discussed in the 4.17.19 submission follow-up document

Beyond those specified activities, we will proceed with another dialysis bench study incorporating higher flow to further reduce recirculating lactate while also focusing on hematocrit maintenance to enabled reduced arterial pO_2. Also, we will build an energy delivery device to reduce or eliminate microbial bioburden in recirculating perfusate as indicated in the infection prevention section of the project pitfalls in the Project Narrative.

Site 2:

In the coming reporting period, we will complete the histopathological analysis of experiment #2 samples as well as an in-depth debrief on surgical and machine components of the project. Preparations will be made to complete one more set of explants in order to the complete SA1 feasibility work.

4. Impact

- **What was the impact on the development of the principal discipline(s) of the project?**
  Nothing to report

- **What was the impact on other disciplines?**
  Nothing to report

- **What was the impact on technology transfer?**
  Nothing to report

- **What was the impact on society beyond science and technology?**
  Nothing to report

5. Changes/Problems

- **Changes in approach and reasons for change**
  Nothing to report

- **Actual or anticipated problems or delays and actions or plans to resolve them**

  Site 1:

  At this 1-year anniversary of project RT180059, Specific Aim 1 (SA1), originally planned for 8-months' duration, has met its sample size objectives per the SOW. However, the team wants to add a third experiment to the plan, thereby lengthening SA1 to now end October or November of this year. The main reason is that the increased capacity of the perfusion bioreactor has increased the hemiface flap size accommodated by the system, which in turn necessitates increased perfusate volume. Since perfusate is obtained from the donor pig at the time of explant, new methods must be developed to assure donor viability should more blood volume be needed, while considering the possibility of incorporating transfused blood in the perfusate. These possibilities will be evaluated by the team and evaluated at one more planned feasibility test. The current IACUC allows for such a study and the work to support it are anticipated in Subtask 6 of SA1.

  Meanwhile, preparations are under way to move directly thereafter to SA2. According to the Statement of Work (SOW), SA1 is focused on the design and production of perfusion systems to apply sustained normothermic, pulsatile perfusion, active disinfection, and ergonomic support to enable 24-hour, prolonged preservation of faces. This aim is becoming accomplished using side-by-side perfusion systems, each performing two x 24-hour perfusion studies of explanted porcine hemifaces including in-process and post-perfusion bench evaluation of the process conditions and graft viability. These tests are nearing completion, and the systems will remain ready for SA2 in step with the experimental program.

  The underlying reason preventing SA1's earlier completion has been the curtailment of animal surgery between March and July 2020 at Site 2 (matching the norm throughout US academic laboratories) due to the global pandemic. Although institutional capacity for studies remains reduced due to both procurement and staffing limitations, our team has now re-established its experimental schedule and intends to stay on track for the project scope elements we control.

  Although the experimental surgeries in this Year 1 faced institutional delays, our teams took advantage of the additional opportunity to perform two additional blood-only perfusion studies to evaluate the systems' abilities to generate consistent perfusate flow and maintain temperature, aseptic conditions, and blood cell viability throughout the 24-hour time frame. As a result of these studies and through review sessions by the teams, additional improvements were made to the systems including enlarging the perfusate chamber from 205 mm to 360 mm to accept additional anatomy (e.g., hemiface plus ear), provision of datalogging capability to the software, and

implementation of a tachometer flow meter. As a result, we have a better system and higher overall confidence in its capability.

Site 2:

The primary cause for delays in the previous reporting period was the university wide COVID-19 related shutdown leading to a 4.5-month halt in animal surgeries. We were able to find ways to use this time productively and improve our system. With this experience under our wing and further moving forward we are working to complete final tasks on the SA1 feasibility experiments such that we can continue to progress to SA2 on solid footing.

- **Changes that had a significant impact on expenditures**

  Site 1 has applied to the Grant Officer to move budget responsibility for the biochemistry analyzer from Site 2 to Site 1. This change was detailed in our written request and can be accomplished without change to the original budget taking advantage of below-budget costs for Fringes. The formal request for change in this case was consequent to the change in the SOW and cost for equipment in excess of $5000 ($7350).

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

  The Site 1 anticipated collaboration with Dr. Monbaliu at KU Leuven will be cancelled, primarily due to travel restrictions resulting from the global pandemic. The uncompleted ACURO process, which we initiated will be halted as a result. The purpose of that collaboration, indicated in the problems and pitfalls section of the Project Narrative and supported in the project budget was initially intended to provide the project access to another clinical user of existing normothermic liver preservation systems, whose knowhow might prove helpful should we face problems. Since the writing of the proposal, our access to Dr. Monbaliu is curtailed, while the preliminary technical performance of our system is promising, and better than originally anticipated. Investigators of both Sites 1 and 2 agree that the budget and work originally for Leuven might be better consolidated into the work and team at Site 2. This provides the additional possibility of augmented samples or groups within the existing experimental plan at Site 2, should the scientific opportunities turn out to exceed the technical problems.

- **Significant changes in use or care of human subjects**
  Nothing to report

- **Significant changes in use or care of vertebrate animals.**
  Nothing to report

- **Significant changes in use of biohazards and/or select agents**
  Nothing to report

6. Products

Site 1:

Two existing benchtop perfusion machines have been revised to enable the normothermic perfusion of porcine hemifaces for the aims of this project.

7. Participants & Other Collaborating Organizations

- **What individuals have worked on the project?**

Name:	John Brassil
Project Role:	Principal Investigator
Nearest person month worked:	7
Contribution:	Mr. Brassil manages the Site 1 project including the scientific, business and engineering activities.
Name:	Gerald Brandacher, M.D.
Project Role:	Principal Investigator
Nearest person month worked:	1

Contribution:	Dr. Brandacher has supervised all animal surgeries and discussions to move this project forward.
Name: Project Role: Nearest person month worked: Contribution:	Byoung Chol Oh, D.V.M., Ph.D. Co-Investigator 1 Dr. Oh has been involved in all animal surgeries, post-surgical data analysis and processes, as well as discussions to move this project forward.
Name: Project Role: Nearest person month worked: Contribution:	Damon Cooney M.D., Ph.D. Co-Investigator 1 Dr. Cooney has worked to establish and complete the IACUC protocol. He has made important contributions to surgical planning for the proposed model.
Name: Project Role: Nearest person month worked: Contribution:	Giorgio Raimondi Ph.D. Co-Investigator 1 Dr. Raimondi has made contributions to data analysis for the proposed model.
Name: Project Role: Nearest person month worked: Contribution:	Richa Kalsi, M.D. Research Fellow 1 Dr. Kalsi is involved in all animal surgeries, post-surgical data analysis and processes, as well as discussions to further project refinement and optimization.

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**
-

Gerald Brandacher	Change: Ended – W81XWH-16-C-0212 - Phase II: Novel Super-cooling of Genitourinary Cells and Tissues for Transplant Role: Site PI Effort: 1.08 CM Date: 10/01/2018 – 05/24/2020
Gerald Brandacher	Change: Ended – W81XWH-16-1-0708 - Engineering a Hybrid Thymus to Unravel the Tolerogenic Properties of Vascularize Role: Co-I Effort: .12 CM Date: 09/30/2016 – 06/29/2020

- **What other organizations were involved as partners?**
  Nothing to report

8. Special Reporting Requirements

QUAD CHART: Submitted with attachments.

9. Appendices

Appendix 1: Perfusion Bench Test with Dialysis - Site 1

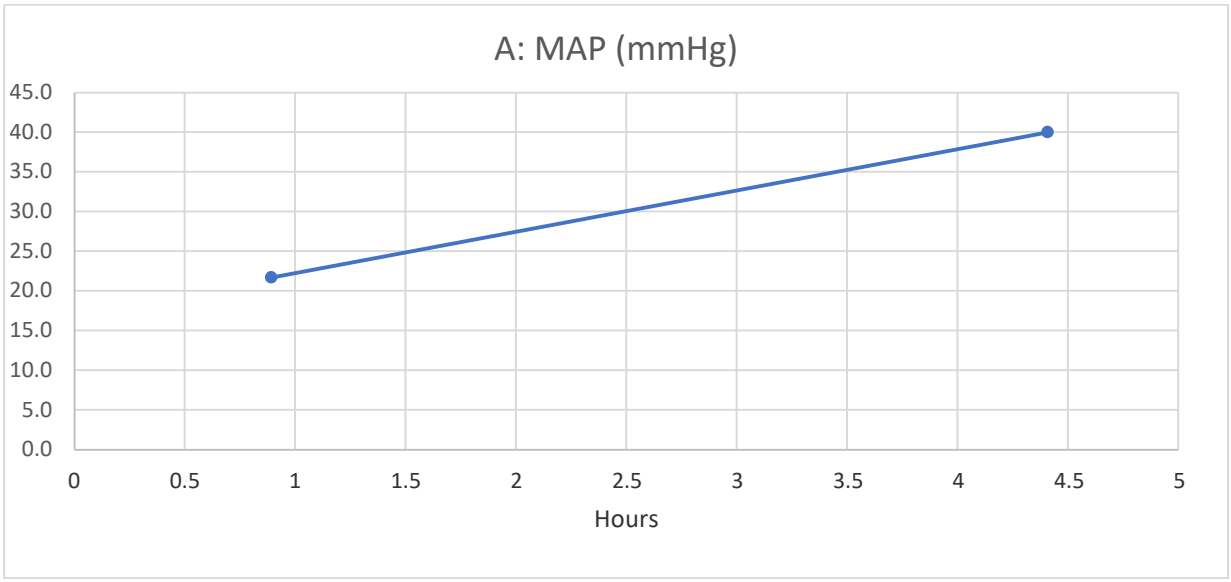
A dialysis circuit was incorporated into the perfusion system and tested on the bench at Site 1 for its ability to remove lactate from the perfusate. Perfusate was prepared by mixing 500 mL abattoir-procured porcine blood with 500 mL RS-I perfusate and circulating it through the dialyzer-augmented circuit at a flow rates of 30 to 75 mL/minute producing MAPs of 21 to 36 mmHg. The RS-I dialysate was chosen to provide comparable electrolyte composition as the perfusate, except for lactate which was added to the perfusate at the start of the experiment. Dialysate was circulated through the dialyzer at flow rate of 64 mL/minute. Transmembrane pressure was adjusted between the dialysate and perfusate circuits to maintain a constant level of recirculating perfusate, which was manually maintained for 4.4 hours. A Fresenius F160NR high flux dialyzer was used in a parallel flow arrangement to simplify priming. Perfusate was oxygenated via a silicone tube helical oxygenator and maintained at 33 to 36 ^{0}C throughout. Samples of perfusate were collected during perfusion, centrifuged, and the supernatant frozen and analyzed for biochemistry at the Iowa State University Veterinary Clinical Pathology Lab. An overview of the setup is in Figure A1-1.

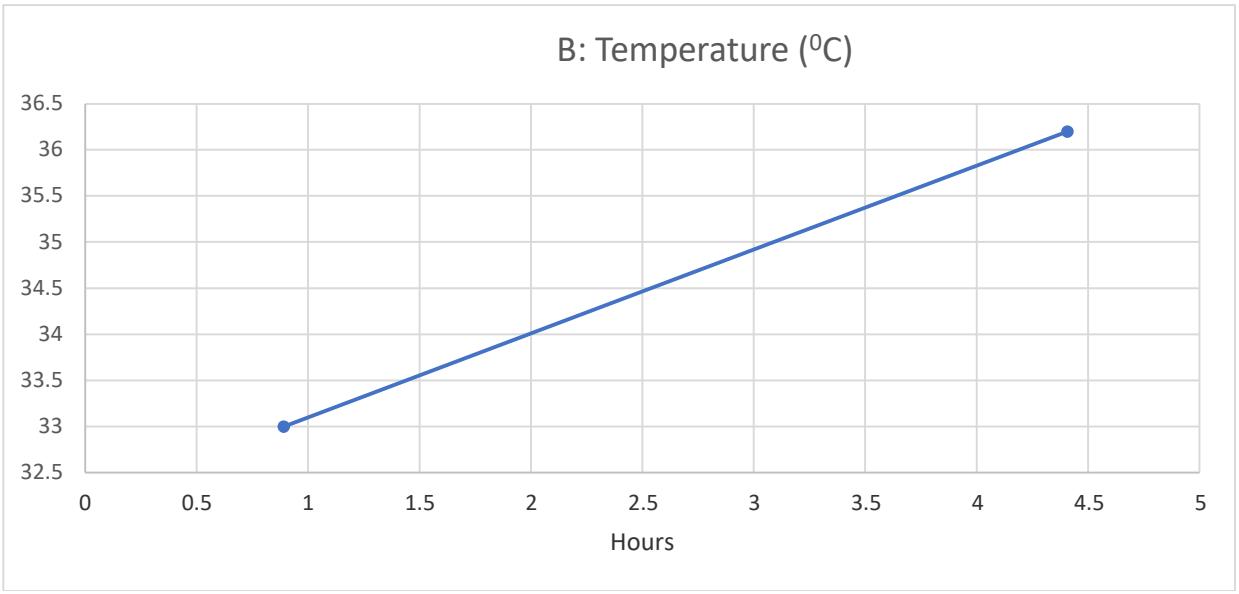
Figure A1-1: Perfusion Machine with Dialysis

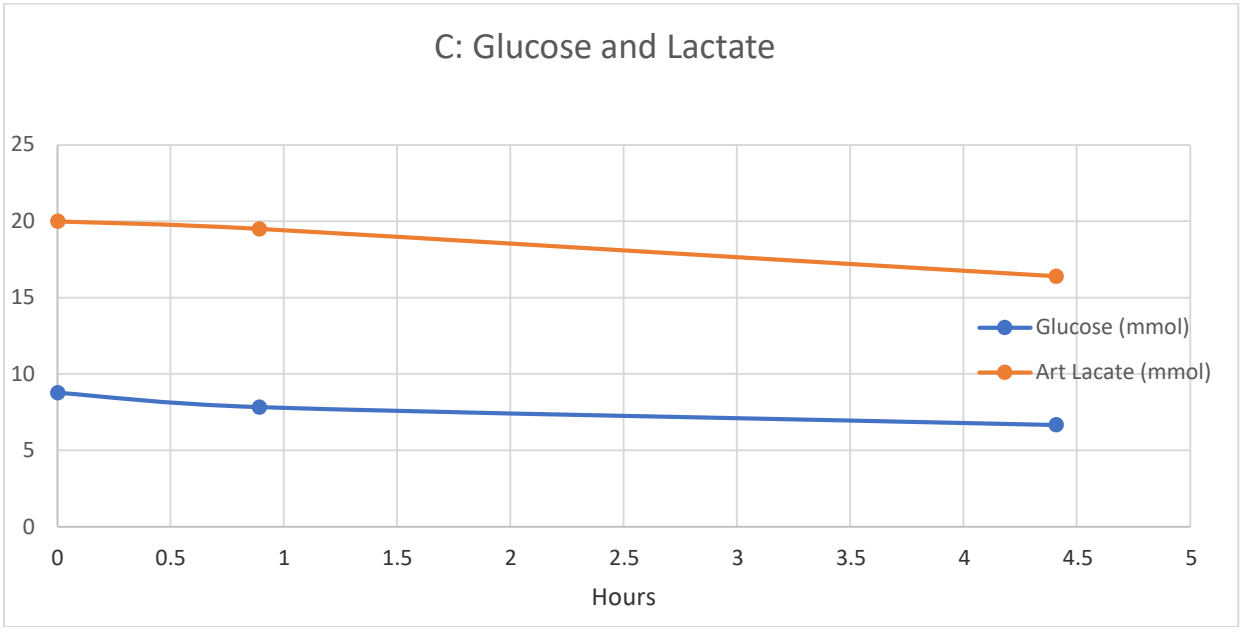
Results A single preliminary test was performed. After 4.4 hours, reduction of lactate concentration was observed in the perfusate while other perfusate chemistry was well-maintained. The following table summarizes the characteristic values obtained and indicates the relevant graph(s) for each column:

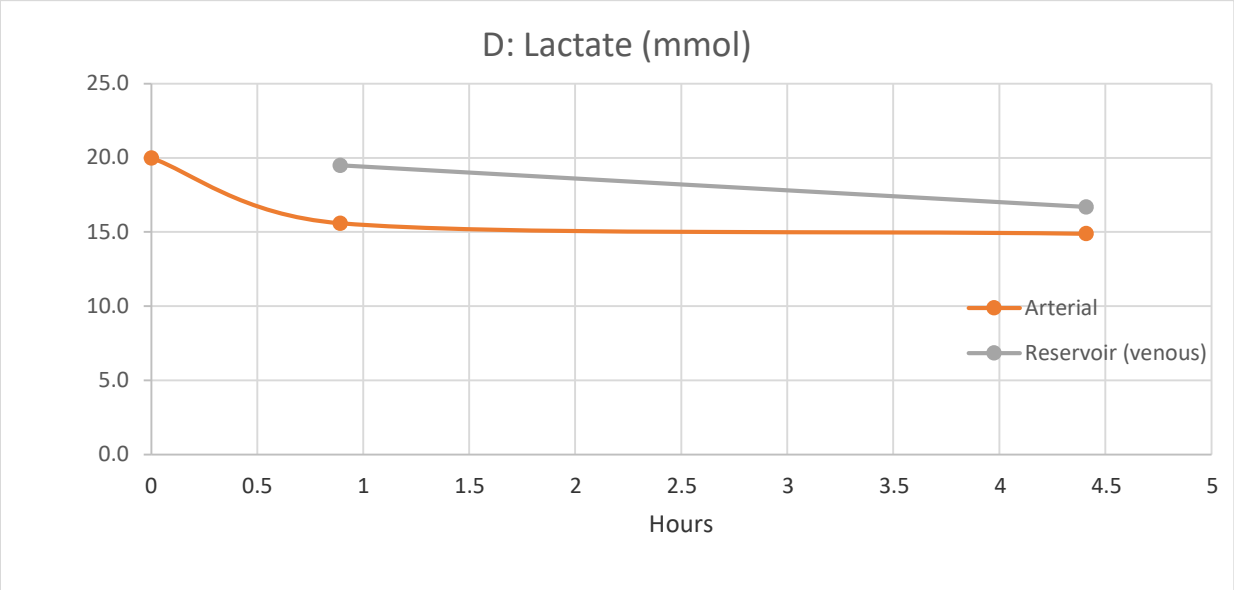
Hours	MAP	Temp	Glucose	Lactate Art.	Lactate Ven.	Sodium	Chloride
	(mmHg)	(^{0}C)	(mmol)	(mmol)	(mmol)	(mmol)	(mmol)
0			8.8	20.0		146	107
0.9	21.7	33	7.8	15.6	19.5	142	113
4.4	40.0	36.2	6.7	14.9	16.7	139	120
Graph	A	B	C	C, D	D	E	E

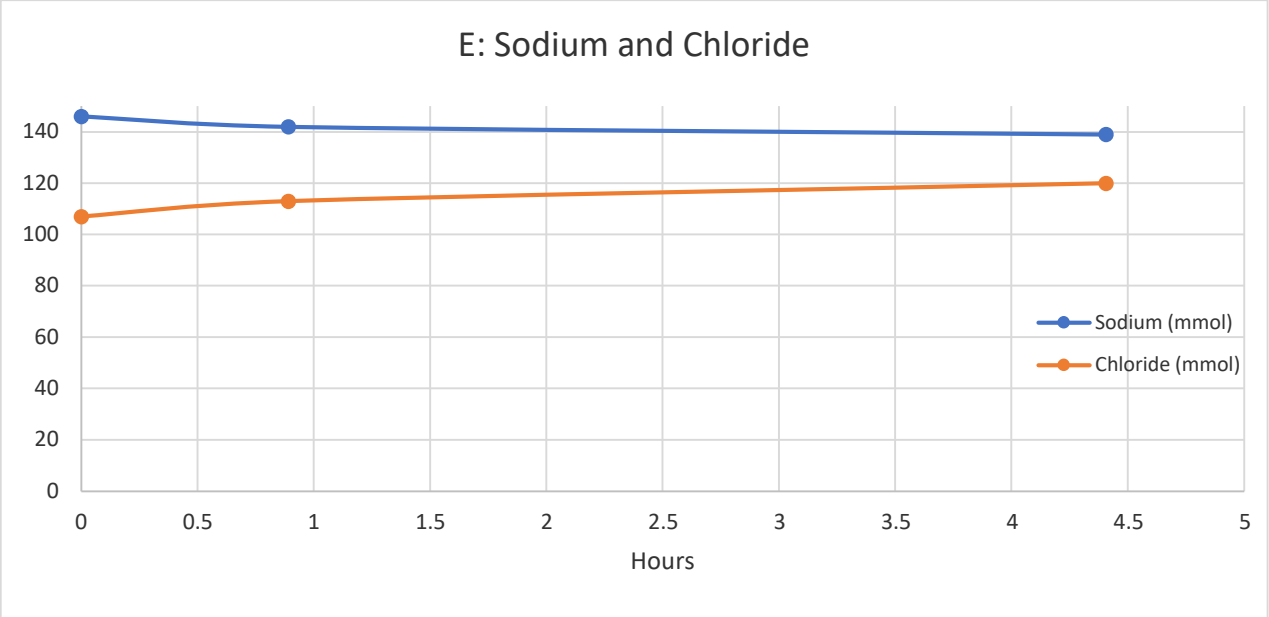
Graphs:


A: MAP (mmHg)
45.0
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
Hours


B: Temperature (⁰C)
36.5
36
35.5
35
34.5
34
33.5
33
32.5
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
Hours


C: Glucose and Lactate
25
20
15
10
5
0
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
Hours
Glucose (mmol)
Art Lacate (mmol)





Discussion

This preliminary experiment shows the initial feasibility using a dialysis circuit on the Model 30 system to remove lactate from the typical recirculating perfusate. Lactate was reduced from 20 millimoles in a 1-liter volume down to 15 millimoles within less than 5 hours. The RS-I, although expensive as a dialysate, did serve that role in a way that preserved the electrolyte chemistry (Graph E). The pattern of lactate removal showed a rapid drop within the first hour to the 15 millimolar level for the arterial perfusate and a more gradual drop in the reservoir lactate, as would be expected, because the dialyzer was in the arterial line which received its direct effect. Dilution effects in the venous reservoir resulted in its lactate levels taking longer to drop (Graph D). The reducing glucose levels throughout the dialysis duration suggest its metabolic consumption by the cellular components of the blood. Since red blood cell metabolism is mainly glycolytic, it is interesting that lactate continued to drop in the perfusate while the red blood cells would have been expectedly adding lactate to it. This suggests that the lactate removing power of the dialysis may have been greater than the measured 5 millimoles in 5 hours, incorporating the additional clearance of lactate coming from the metabolic red blood cells.

Ideally, the dialysis of this study would have cleared a more lactate than it did. Since typical lactate levels in blood are 1 millimolar or less; an additional 14 millimoles or more of lactate would been preferably cleared by the system. This could be accomplished by increasing the dialyzer flow, which must be done judiciously as the portable system of SA3 will be sensitive to weight, such as the potential for additional liters (= kilograms) of dialysate. Managing this tradeoff would be an optimization that would necessarily follow the next step of increasing the dialysis flow in

a similar experiment. Additionally, the application of a low-flux dialyzer may be also indicated as a way to preserve the larger molecular weight components of the blood, such as albumin, and also to maintain the hematocrit, which has been indicated in the Clavien paper (2020). This line of inquiry into dialysis of perfusate is indicated in the project narrative and will be performed in the upcoming project period.