Hypothermic Machine Perfusion of Composite Tissues

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ABSTRACT

Organ perfusion systems have successfully been applied to solid organ preservation and subsequent transplantation. However, their use in limb preservation for Vascularized Composite Tissue Allotransplantation (VCA) has yet to be thoroughly investigated. This thesis explores the potential for hypothermic machine perfusion in prolonging limb graft viability in a swine forelimb amputation model. The experiment was designed with the right and left forelimbs from the same pig randomly assigned to the treatment and control groups. Eighteen (18) limbs were procured from a local abattoir, vessels cannulated, and an initial flush of a modified phosphate buffered saline (PBS) solution was performed. Half of those limbs, assigned to the treatment group, were then preserved with continuous hypothermic machine perfusion for 12 hours. The perfusate was a PBS solution supplemented with 5% w/v dextrose. The remaining nine limbs, assigned to the control group, were placed into a plastic bag and kept at room temperature (ca. 20^{o}C) for the entire duration of the experiment. Methylene blue was used to verify perfusion throughout limbs. Histopathological analysis revealed the presence of significantly greater deterioration of the perfused limbs compared to control. I concluded that PBS solution is not suitable for extended limb preservation. Inadequate perfusate volume and lack of solution replenishment resulted in metabolic waste build up, accelerating total organ damage. Continued research is needed in order to develop clinically relevant hypothermic machine perfusion devices capable of prolonged limb preservation.

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GENERAL AUDIENCE ABSTRACT

Artificial circulatory systems, capable of maintaining organ viability outside the body, have successfully been used for kidney preservation prior to transplantation. However, this has not been thoroughly investigated for limb preservation. This thesis explores the potential for an artificial circulatory system to prolong the life of pig front limbs following amputation. Nine (9) pairs of limbs were used. From each pair, one limb was randomly assigned to the treatment group and the other to the control group. The limbs assigned to the treatment group were attached to a machine that continuously circulated a cold preservation solution for twelve (12) hours. A blue dye was used to verify that the solution was being circulated throughout the whole limb. The remaining nine (9) limbs were in the control group, placed in individual plastic bags and left at room temperature for the entire duration of the experiment. After the experiment, microscopic examination of the tissue revealed that there was greater deterioration of the treatment group limbs versus the control group. I concluded that the study's preservation solution and its use, was not beneficial for limb preservation. Without a greater volume and replenishment of the solution, toxins were able to build up in the solution causing damage to the limbs. Continued research is needed to develop a circulatory system capable of extending the viability of a limb for transplantation.

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Chapter 1 – Introduction

Approximately seven million people in the United States need composite tissue reconstruction each year [1]. Of those, two million are amputees [2] with more than 500 losing a limb each day [3]. Trauma accounts for almost half of these amputations [2]. This demand for Vascularized Composite Tissue Allotransplantation (VCA) is more than double that of solid organs [4]. Solid organ transplantation (kidney. liver, heart, lung) is nonetheless of primary importance, as it is life saving in the majority of instances. However, VCA is still life changing because it aims to replace functional loss and is known to significantly improve the quality of life. To quote Jean-Michel Dubernard, the first surgeon to perform a successful hand transplant: "Most of these grafts are visible and are not vital. They are life-enhancing rather than life-sustaining, but, in the words of the first transplant patients, they are truly life-giving, and not just life-saving."[5].

There is a real need for VCA to become a routine surgical procedure. One of the major barriers to successful preservation and subsequent implantation currently is the allowable ischemia time of limbs [6]. Muscle, which comprises most of the volume of limbs, has an allowable ischemia time of only 4-6 hours[7]. Other tissues are considerably more tolerant of ischemic preservation conditions. Hypothermic machine perfusion has been proven to significantly extend kidney preservation time (24-36 hours) while still maintaining viability. The purpose of my research is to determine the feasibility of hypothermic machine perfusion to extend preservation time for limb tissues. For this purpose, this thesis will start by reviewing the literature on organ preservation and how it applies to composite tissues, to then describe my study design and results obtained, and finish with a discussion on the significance of my work.

Chapter 2 – Literature review

2.1 Defining composite tissue

A composite tissue is a body structure, made of several differing types of tissue, that potentially can be transplanted to an appropriate recipient. A hand, being composed of skin, muscle, bone, tendon, nerves and vessels is an example of a composite tissue. Each component has distinct metabolic requirements and may elicit an immune response of varying character and intensity following transplantation. The allograft will be unsuccessful if rejection occurs in any one of those tissues [8] or if the metabolic needs of each and every tissue component are not met. Proper procurement, preservation and surgical techniques are of paramount importance in preventing rejection [9]. It is problematic to employ a single mode of preservation capable of satisfying the diverse needs of individual tissues found within a composite graft. As a consequence, VCA presents more challenges than kidney transplantation (for example) which involves preservation and implantation of an organ with a relatively homogeneous structure [8,10].

2.2 History of VCA

***Figure 1**: Limb Transplantation by Saints Cosmos and Damian, Swabian c. 1500 (Stuttgart)*

The concept of VCA is not new. Legend has it that twin brothers, Saints Cosmos and Damien, replaced the diseased leg of a man with one from a cadaver in the year 348 AD. Figure 1 is a painting illustrating this first historical reference to VCA. Later, injuries sustained by combatants in World War II renewed interest in the field of VCA, stimulating research and experimentation. However, the continuous challenges of circumventing graft rejection due to the lack of adequate immunosuppressive agents and vascular complications, stifled progress. In 1964, the World's first-hand transplant was attempted in Ecuador. Severe rejection, resulting in re-amputation, occurred two weeks following the procedure, despite the use of both immunosuppressive agents available at the time. More than two decades later, during the 1990s, the development of better immunosuppressive agents (cyclosporine) once again renewed interest in VCA. The first successful hand transplant occurred in Lyon, France in 1998 [4,10]. Since then, over 200 different types of VCAs have successfully been performed around the world, including transplantation of upper extremities, facial tissue, abdominal wall, larynx, trachea, vascularized bone and joint, tongue, uterus, and penis. The procedure is relatively safe, with a 4 to 11% mortality rate, varying

based on the type of composite tissue transplanted and the complexity of the procedure [11]. The chances of successful engraftment are high, with an estimated 81.82% overall graft survival following upper extremity transplantation [12]. Success is defined by functional outcomes and enhancement in quality of life:

- Over 51% of hand transplant patients were able to become independent in daily activities and/or have returned to employment. To date, there is only one report of a patient being unsatisfied with the achieved functional result [11].
- All patients achieved protective sensitivity allowing them to feel pain.
- 90% of them developed tactile sensibility (sensation of touch) while 82.3% also developed partial discriminative sensibility (texture recognition) [12].

Despite general success, VCA is not routinely performed, due to the high morbidity associated with the chronic use of immunosuppressive therapy. Careful consideration must be taken in patient selection, tissue preservation, surgical technique, and follow-up care. All transplant recipients often face health complications associated with immunosuppression such as opportunistic infections, increased risk of cancer development, and metabolic disorders. Composite tissue transplant recipients must also cope with the psychological effects associated with having a foreign body part attached to themselves [11]. The most common causes of graft failure are infection following surgery and non-compliance with lifelong immunosuppressive therapy. Yet failure is still a rare occurrence with loss of only 5 of 57 hand grafts being reported by the international registry on Hand and Composite Tissue Transplantation (IRHCTT)[12].

2.3 Rejection as a cause of failure in VCA

Eighty-five percent (85%) of composite tissue transplant recipients experience at least one episode of acute rejection within the first year of transplantation [12]. Acute rejection is a T cell-mediated event that is characterized by infiltration of T cells and macrophages within the composite tissue allograft [13]. Acute rejection is often caused by a change in immunosuppressant dosage and can be remedied by re-evaluating the immunosuppressive paradigm. [14]. There are two other types of rejection that can occur with

organ transplantation, which transpire rarely with VCA: Antibody-mediated rejection and chronic rejection.

Antibody-mediated rejection occurs when the recipient has prior sensitization to tissues similar to that of the donor through a previous blood transfusion or pregnancy. Cytotoxic antibodies interact with the antigens expressed on the surface of donor cells resulting in rapid and total death of the graft. Antibody-mediated rejection is rare today, due to donor specific cross matching and panel reactive antibody tests that are performed prior to transplantation. To date, no incidence of antibody-mediated rejection has been recorded in VCA [13].

Chronic rejection is characterized by slow progressive injury to an allograft stemming from continuous damage from immunological reactions, including deleterious effects of cytokine-mediated inflammation and vasculitis, as well as direct cell-mediated cell cytotoxicity. Possible symptoms of chronic rejection in hand grafts include narrowing of the vasculature, muscle and skin atrophy, loss of hair, and nail deterioration [13]. To date, there has been one suspected case of chronic rejection documented in the medical literature. This patient experienced progressive severe graft ischemia eventually necessitating the removal of the allograft. Post amputation investigations showed a thickening of the vascular wall that prevented sufficient amount of oxygen and nutrients to reach the tissues [11].

2.4 Donor types

The source of organ procurement, as well as adequate preservation technique, greatly influence the incidence of rejection [9]. Organs for transplantation can come from either living donors or deceased donors. Ninety percent (90%) of deceased donors are brain dead and consent was provided either prior to death or was provided by their families. This type of donation is termed donation after brain death (DBD) and is further classified into two types based on donor's age and cause of death: 1) Standard Criteria Donors (SCD) are donors under 50 years of age, who suffered brain death from any cause and 2) Expanded Criteria Donors (ECD) are donors over 60 years of age, having died of any cause or a donor

over 50 years of age who had at least two life threatening conditions (such as stroke or myocardial infarction) when alive [15].

The remainders of donations come from deceased donors whose organs and tissues are procured after cardiac death (DCD). A DCD donor is a person near death who has suffered irreversible brain damage yet does not meet formal brain death criteria. In this case, supportive care, including cardiorespiratory resuscitation, has been withdrawn and organs are procured only after the heart has stopped beating and death is formally declared. Consequently, organs collected from DCD donors experience some degree of warm ischemia during organ procurement[15].

The classification scheme and nomenclature described in the preceding paragraphs was developed for kidneys and is meant to describe donor characteristics that could potentially affect the quality of the organ. Any organ or set of tissues, whether it is a kidney or an arm, is considered a marginal donation if obtained from an ECD, meaning that the donor was elderly or diseased, or obtained from high-risk DCD donors. Marginal organs have a more limited ability to weather the devastating effects of ischemia and are therefore at higher risk of acute rejection, delayed graft function, and graft loss [16]. ECD kidneys, for example, have a 70% higher likelihood of failure and a projected graft lifespan of 5.1 years compared with 10 years for a kidney received from an SCD source. DCD transplantation was found to have a 42% to 52% risk of delayed graft function compared with 24% in SCD transplantation [17]. Advanced preservation techniques have an even greater importance with marginal organs in preventing rejection and sustaining function.

2.5 Organ preservation

The stages of transplantation - explantation, preservation, and implantation - are accompanied by a myriad of changes as the organ is taken out of its homeostatic state in the donor. Organ retrieval follows circulatory arrest, marking the beginning of warm (near body temperature) ischemia. During this period, ATP levels in all cells drop rapidly in the absence of oxygen, which in turn leads to a decrease in biosynthetic reactions and increased electrolyte imbalance across cell membranes. Most organs can only

tolerate warm ischemia for 30-60 minutes before complete loss of function. Organ preservation techniques must be employed immediately in order to maintain function of the organ during storage and to minimize damage thus increasing the chances of successful transplantation [18,19].

Organ preservation can be categorized into two main approaches, based on the fluid movement within the organ. The first and most commonly used method is static cold (4-10ºC) storage (SCS), where fluid within the organ stays static throughout the preservation period. The second approach is dynamic or machine perfusion (MP) where an organ is continuously and actively perfused during preservation. The only two methods of organ preservation currently approved for clinical transplantation are static cold storage and hypothermic machine perfusion [19].

2.5.1 Static preservation method (SCS)

SCS is the only preservation methodology currently approved for all organ types [20]. Explanted organs are first gravity flushed with a cold organ preservation solution (~ 4 °C) to remove the majority of blood within the organ, rapidly reduce the organ's temperature, provide a limited supply of metabolites, and to provide tissue-preserving anti-oxidants and colloids. Organs are then stored within a rigid plastic container filled with the same fluid, which is, in turn, placed within a Styrofoam cooler filled with ice slush, where the organ will remain until implantation [19]. A SCS container is seen in Figure 2 below.

***Figure 2**: SCS organ transplantation cooler*

The preservation of organs using SCS relies primarily on metabolic suppression due to hypothermic conditions and the use of different preservation solutions tailored to the known metabolic demands of each organ [19]. The main advantages of static cold storage are the low cost and its simplicity. However, with SCS, there are no means to predict post-transplant outcomes affected by storage conditions since there are no assays that can be used to evaluate organ viability and ischemic damage during preservation [20].

2.5.2 Dynamic preservation method

Dynamic preservation is characterized by the active circulation of perfusate within the organ, either through a constant flow generated with a centrifugal pump or a pulsatile flow as generated with a peristaltic pump. Generally, the methods for dynamic preservation vary based on the temperature at which the organ is preserved. Hypothermic machine perfusion (HMP) of kidney and livers is currently the only dynamic preservation method approved for clinical use [19].

For HMP, the explanted organ is first gravity flushed with a cold organ preservation solution to wash out residual blood and achieve moderate cooling. The organ is then continuously perfused with perfusion solution at approximately 4°C [19]. The advantages of machine perfusion are:

- The ability to provide a continuous supply of nutrients with or without oxygen

- The removal of metabolic by-products, toxins and free radicals formed during cold storage
- The maintenance of vascular patency
- The availability of devices and metrics (ie flow, resistance and fluid biopsy) to evaluate organ viability
- The possibility for early therapy implementation (immunomodulatory drugs) to improve organ quality
- The most notable advantage is improved outcomes as demonstrated with reduced rate of delayed graft function (discussed later) [20]

HMP has disadvantages, such as the logistics of pump availability and portability, (most HMP systems are large – 1-2 cubic meters) as well as the upfront cost of the technology, preservation solution and need for trained operators [21]. Groen et al. performed an economic evaluation of a large paired kidney study in order to evaluate the cost effectiveness of HMP (Lifeport Kidney Transporter) compared to SCS. The results of engraftment (graft survival, recipient survival) from the first year after transplantation were combined with long term survival and cost data derived from the literature, using a Markov model. This model revealed cost saving of $86,750 per life year gained in favor of MP, suggesting that MP is the more cost effective of the two methods (HMP vs SCS) [22].

2.5.3 Hypothermia

Both clinically approved preservation methods utilize hypothermia as defense against hypoxic injury and as a means to suppress opportunistic bacterial growth. To quote an article from the journal Cryobiology, “There is no single drug, or cocktail of drugs, that can so safely and effectively suppress metabolism and provide ischemic protection for multiple tissues and organs as the application of hypothermia can” [23]. Hypothermia slows down normal metabolic and degenerative processes occurring within cells that eventually lead to loss of organ viability. Metabolic activity of animals can be decreased

two-fold for every 10°C decrease in temperature [24]. This effect follows van't Hoff's rule, summarized in equation 1 below.

$$Q_{10} = (\frac{k_2}{k_1})^{\frac{10}{t_2 - t_1}}$$

Equation 1: van't Hoff law. Q_{10} is the van't Hoff coefficient for a 10°C change in temperature, k_x is the reaction rate and t_x is the temperature.

Most enzymes involved in metabolic activity vary between a Q_{10} of 1.5-2.0 [24]. This relation shows that a reaction with a Q_{10} of 2.0 would be reduced to a tenth of its original reaction rate when the temperature is decreased from 37°C to 4°C. When applying this concept to organ preservation, an organ cooled from 37°C to 4°C, experiences a decrease in metabolic rate by a factor of 10. Hence, hypothermic storage should extend preservation time by a factor of 10, compared to storage at physiologic temperatures [24]. However, hypothermia, in combination with ischemia, may cause damage to organs during preservation which greatly influences function post-transplantation [18].

2.6 Damage occurring during hypoxia and hypothermia

2.6.1 ATP depletion

During hypothermia, ATP is utilized to meet residual energy requirements of the cell. During hypoxia, anaerobic metabolism prevails, requiring nineteen times more glucose to generate ATP than aerobic metabolism [25]. Consequently, ATP depletion still occurs with hypothermia but is simply delayed[24]. Organs sustain injury during preservation in large part from the inability of cells and enzymes to function without ATP.

2.6.2 Hypothermic-induced cell swelling

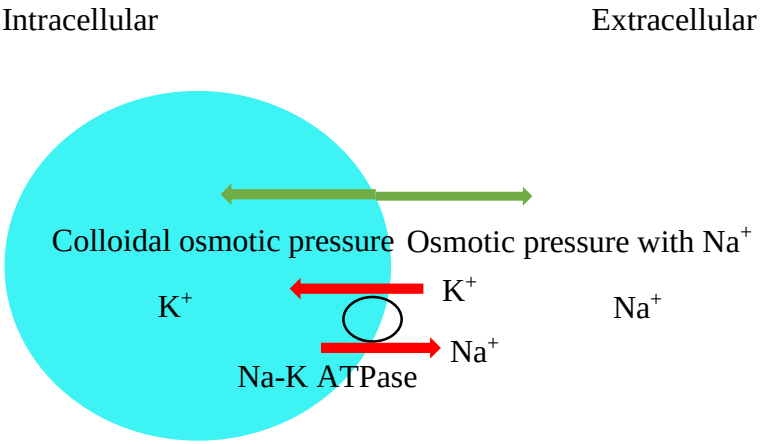

***Figure 3**: Illustrates the electrolyte balance across the cellular membrane. Red lines arrows show cation movement, green arrows show osmotic forces*

Normally, the extracellular environment is high in Na^+ and low in K^+. This balance is maintained by the NA-K ATPase pump and intact cell membranes. Intracellular proteins and impermeable anions allow for a colloidal osmotic pressure within the cell of 110-140 mOsm/kg. This pressure is counteracted by the osmotic pressure produced by extracellular Na^+. Therefore, the ATP-driven pump is vital in maintaining normal cell size and structure.

As ATP production and utilization falls due to hypothermia, Na-K ATPase pump activity is suppressed. As a result, Na^+ enters the cell following a concentration gradient. This may later be exacerbated by cell membrane damage that allows more Na^+ influx. Without the counteractive osmotic pressure of Na^+ outside the cell and the driving intracellular Na^+ load, water accumulates within the intracellular space causing cell swelling, disruption and rapid cell death [24].

2.6.3 The role of increased intracellular calcium

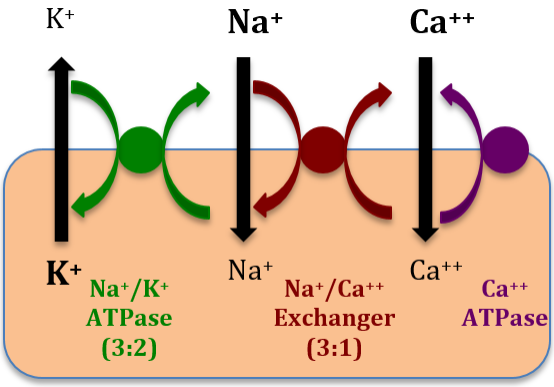

***Figure 4**: Illustrates the mechanisms by which calcium homeostasis is maintained within the cell* [26]

With the increase in Na^+ within the cell, the Na^+/Ca^{++} exchanger (see Figure 4 above) tries to pump Na^+ out of the cell while accumulating and physiologically sequestering Ca^{++} within the cell. Due to the lack of ATP, the Ca^{++} ATPase pump does not function properly, thus allowing for a rise in intracellular calcium levels. An increase in intracellular calcium activates enzymes (lipases, proteases, lysozyme, nucleases) that ultimately lead to membrane injury and protein degradation within the cell [18].

2.6.4 Intracellular acidosis

During ischemia, organs undergo anaerobic metabolism resulting in production of high levels of lactic acid. The resulting decrease in pH causes the release and activation of acidic pH dependent lysosomal enzymes within the cell, thus resulting in irreversible mitochondrial and overall cell damage [24].

2.6.5 Expansion of interstitial space

During perfusion, components of the perfusion solution rapidly diffuses into the interstitial space causing tissue edema. Such interstitial expansion can compress delicate, thin-walled capillaries, resulting in impaired perfusion and pockets of more severe tissue degradation [24].

2.6.6 Free radicals

Hypoxia and hypothermia promote the production of oxygen reactive species that will lead to mitochondrial damage and accentuated apoptosis once reperfusion is established [18,24].

For all these reasons, hypothermia alone is not sufficient for preservation. Hypothermia needs to be coupled with the use of an appropriate preservation solution that: 1) minimizes hypothermic-induced cell swelling, 2) prevents intracellular acidosis, 3) prevents the expansion of the interstitial space, 4) prevents injury from oxygen free radicals (especially during reperfusion), and 5) provides substrates for regenerating ATP [24]. The numerous requirements for effective preservation, combined with the interdependent nature of these needs, constitute a multifaceted, challenging, biomedical engineering problem.

2.7 New preservation modalities

New dynamic preservation modalities that may circumvent some of the damage associated with the current clinically accepted methods are being studied. Several clinical trials of these novel methods are underway [19].

2.7.1 Normothermic machine perfusion (NMP)

The goal of NMP is to emulate the conditions an organ experiences *in vivo*, as accurately as possible, in order to minimize the stresses associated with the explantation and chilling of an organ and therefore lessening tissue injury, while maximizing preservation. In theory, perfect emulation of the physiologic environment would preserve an organ indefinitely. Organs being preserved through NMP would not undergo a forced slowing of function or stasis as they do in SCS and HMP methods, thus allowing real time assessment of organ viability prior to transplantation into the patient [20]. A study conducted with porcine livers, perfused under normothermic conditions (37ºC) for 10h, found improved liver function and preserved histologic architecture when compared to SCS [27]. By perfusing organs at physiologic temperature (37ºC), the organ's metabolism is not suppressed, thus necessitating a continuous, physiologically appropriate supply of nutrients and oxygen to prevent rapid organ failure. Maintaining such an environment is an enormous technical challenge and is currently prohibitively expensive in a clinical setting [20].

2.7.2 Subnormothermic machine perfusion

Subnormothermic machine perfusion (SNMP) is the newest method of dynamic preservation. Organs are perfused and kept at temperatures between 20 and 30°C. This method aims to incorporate the benefits of both HMP and NMP while limiting the disadvantages. With SNMP, the metabolic demands of the organ are reduced but not to the same extent as with hypothermia, thus allowing for lesser depletion in ATP (than normothermic preservation) and decreased damage from accompanying electrolyte imbalances. SNMP does require continuous supply of nutrients and oxygen but again at a significantly lower magnitude compared to NMP, thus allowing for the usage of a less complex solution and machinery [28].

2.8 SCS vs MP

Since 1974, there has been a myriad of clinical studies comparing the effectiveness of MP and SCS for organ preservation. These early studies found little to no difference in graft survival rate or DGF between the two methods [29,30]. SCS, being the simplest and least expensive modality, was therefore adopted as the common standard of care. SCS dominated the marketplace until the last decade, when significant advances in MP technology led to a renewed interest in the preservation method. The transplantation community is once again debating which preservation method is more effective and there have been several robust comparative studies performed to attempt to answer this question.

Two such studies were published by Kwiatowski *et al.* in 2007 and Poyak *et al in 2000.* Kwiatowski *et al.* compared 227 MP kidneys with 188 SCS kidneys and found MP had little effect on the incidence of DGF (32.0% vs 32.3%) but was associated with a slightly increased graft survival rate (87.7% vs 85% at 15 months). Poyak *et al.* (2000) compared 402 MP kidneys with 248 SCS kidneys and found a decreased incidence of DGF (9% vs 24% in non-ECD kidneys) and increased graft survival with MP (96% vs 89%). While these studies did contain data from a large number of transplants in their analysis, the retrospective nature of such evaluation limits the statistical relevance thus possibly explaining the differences in their findings.

In contrast with these studies, Moers *et al.* published the first high volume, prospective, international, randomized controlled trial evaluating the effectiveness of SCS and HMP. This study included 336 consecutive pairs of kidneys from donors. In each pair, one kidney was randomly assigned to HMP (LifePort Kidney Transporter™ machines) and the other to SCS. Recipients were then followed one-year post transplant. Delayed graft function occurred in 70 recipients within the HMP group and in 89 recipients within the SCS group (20.8% and 26.5% respectively). In addition, the HMP group averaged 3 fewer days (10 vs 13 P = .04) of DGF. The one-year graft survival in the HMP group was also significantly higher (94% to 90%, P=.04). This study shows that HMP preservation significantly reduces the incidence of DGF, while increasing the one-year graft survival rate for SCD, ECD and DCD organs [31].

2.9 Challenges of composite tissue preservation

As previously noted, there is a high occurrence of acute rejection within the first year of composite tissue transplantation. Another major challenge is the need to keep ischemia time brief. Among the various tissue types, skeletal muscle is the most vulnerable to ischemia with irreversible cell damage starting after 3 hours that is nearly complete after 6 hours [7]. Currently, to minimize ischemic injury, procured hand allographs are flushed with a cold University of Wisconsin solution and stored at 4°C until transplantation [14]. This method seems to allow for 6 to12 hours of tolerable cold ischemia time (varying due to muscle quantity in the graft) while maintaining a viable organ for transplantation [32,33].

Machine perfusion may provide a substantial expansion of the ischemia time for composite tissue transplantation and therefore buy time for transportation and preparation for the procedure. As demonstrated with kidneys, hypothermic machine perfusion may also decrease the incidence of acute rejection and delayed graft function seen with composite tissue transplantation. Establishing a more reliable and consistent preservation method would allow for composite tissue transplantation to become a routine procedure thus improving the quality of life of many around the world.

Hypothesis: Hypothermic machine perfusion of isolated composite tissue (limbs) improves tissue preservation and tissue quality, as assessed by gross appearance and histopathologic evaluation.

Chapter 3 - Materials and method

3.1 Pilot Study

A pilot study was performed to determine the efficacy of machine perfusion as a preservation method for porcine limbs. Six pigs were used for this experiment. From each pig, both forelimbs and a single rear limb were collected at the time of slaughter. One limb from a set was randomly assigned to the control group and kept at room temperature (ca. 20°C) while the other two were preserved using hypothermic machine perfusion for 12 hours at 10°C. Of the two dynamically preserved limbs, one was perfused with a peristaltic pump while the other with a centrifugal pump. These pumps were chosen to determine if there was a significant difference in preservation effectiveness between a sinusoidal pressure waveform or static pressure. Perfusion, in this pilot experiment, was evaluated using heat flux sensors. These sensors apply a small thermal event to a focal point in the tissue and record the dissipation to measure the heat flux. This heat flux measurement can be used to determine the flow rate within the regional vasculature [34]. Despite being able to visually see perfusion, these sensors were unable to detect a flow rate within the organ. Biopsies were taken post perfusion and histologically evaluated for viability. This experiment found no statistically significant difference between control, and dynamically perfused limbs. However, limbs perfused with the peristaltic pump had marginally less damage than those perfused with a centrifugal pump. These inconclusive results were attributed to the development of significant tissue edema obstructing adequate tissue perfusion.

To overcome problems and deficiencies experienced in the pilot study, adjustments were made and a new experimental protocol followed. First, the preservation solution (PBS) was fortified with a colloid, glucose, to control edema. Methylene blue dye was used to evaluate perfusion by assessing the extent of dye uptake within the entire limb. Finally, a single pump type, peristaltic, was used for hypothermic machine perfusion. A peristaltic pump can more accurately emulate the pressure waveform an organ would experience *in vivo* and was found, from the pilot study, to be the same or slightly better than a centrifugal pump. A new series of nine experiments were performed, aiming again to test the

feasibility of preserving limbs, a composite tissue, with machine perfusion. All experiments followed the same protocol: limb procurement, limb preparation, and limb perfusion coupled with data collection.

3.2 Limb Procurement and Preparation

Figure 5*: Eric in required USDA gear for limb procurement*

All limbs were procured from culled 2+ year-old sows, weighing 225kg to 250kg, at a commercial abattoir in Bedford, Virginia in early to mid-morning. Electronarcosis was first performed with a homemade electric stunner delivering 600 volts of electricity to the sow's head for instantaneous insensibility. Both front limbs were removed by sharp dissection at the level of the radio-humeral joint, immediately prior to whole body exsanguination. The limbs weighed, on average, between 8 kg and 10 kg. Each limb was randomly assigned as either the treatment or control limb.

One of the limbs was chosen at random, in order of procurement, to be the control. The control limb was placed into a plastic bag and kept at ambient temperature (25^{o}C) for the duration of the experiment.

The other limb from the same sow was, by default, allocated to be the treatment limb of the experiment. The main blood vessel of the forearm, the brachial artery, was identified on the medial aspect of the limb *(Figures 6 and 7)*. The brachial artery was cannulated using a Luer lock adapter *(Figure 8)*. Suture or zip ties were used to secure the adapter in place.

***Figure 6**: Blunt dissection performed to identify the brachial artery*

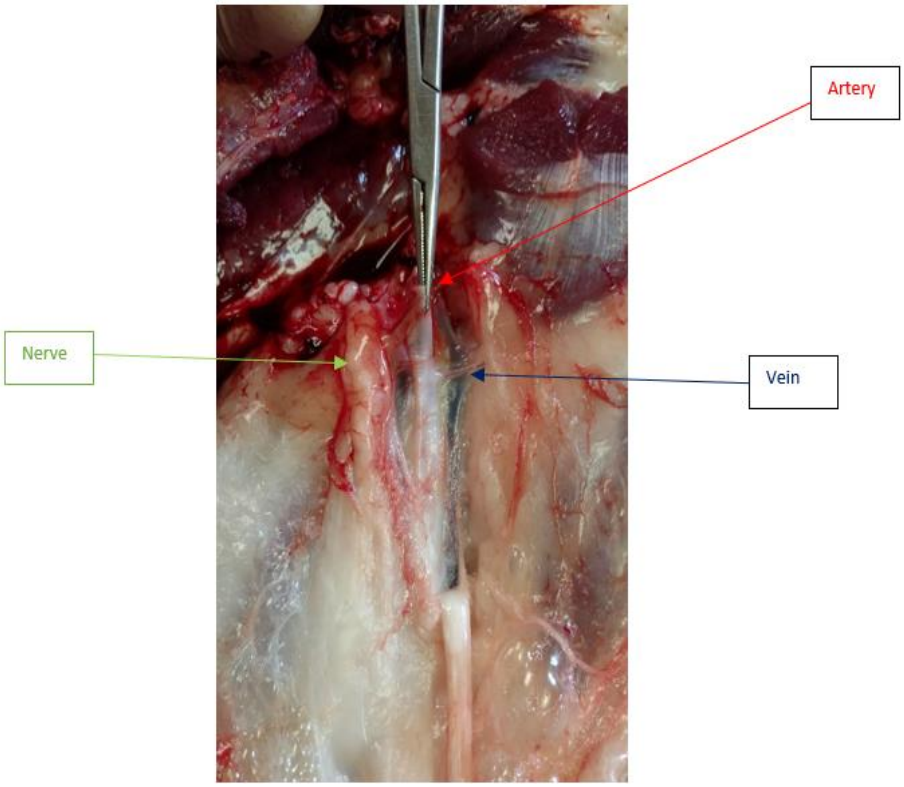

***Figure 7**: Identification of brachial artery*

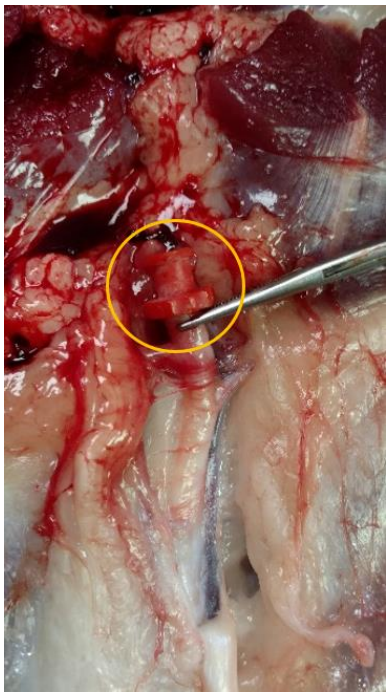

Figure 8*: Brachial artery catheterized with* Luer Lock adapter

After cannulation, the treatment limb was gravity-flushed (see below) with 1 L of cold (~4°C) phosphate-buffered solution (PBS) *(Figure 9)*. The formulation of the PBS solution is shown in Table 1. The cold flush had two main purposes: 1) to remove residual blood from the vasculature (as much as possible), thus preventing post-mortem blood clot formation, and 2) for rapid cooling to reduce the metabolic activity and energy demands of the tissues.

The flush was performed at a pressure of 90 mm Hg, created by elevating the flush container to a height of 1.25 meters over the limb. The standard practice for organ procurement is a low pressure (12 mmHg) initial flush. However, literature showed a higher pressure (100 mmHg) to be more effective for optimizing removal of residual blood [35].

To prevent occlusion of vessels from the weight of the organ, the limbs were periodically re-positioned in a physiologic manner during the flush. During the 90-minute transport interval to the laboratory at Virginia tech, the treatment limb was stored in a plastic bag and submerged in a cooler filled with ice *(Figure 10)*.

Material	Concentration
NaCl	137 mM
KCl	2.7 mM
Na2HPO4	10 mM
KH2PO4	1.8 mM
MgCl2	0.5 mM
CaCl22H2O	1 mM

***Table 1**: PBS recipe*

***Figure 9**: Treatment limb gravity-flush*

***Figure 10**: Treatment limb placed in cooler filled with ice for transport*

3.3 Limb Perfusion

In the laboratory, the treatment limb was preserved with continuous hypothermic machine perfusion for 12 hours on one of three machines that were configured identically for perfusion (see below and Results).

3.3.1 Perfusate:

For machine perfusion, a PBS solution (see Table 1), supplemented with dextrose to a 5% w/v concentration, was used. As previously mentioned, the purpose of adding 5% dextrose to the perfusate was to prevent cellular edema and to delay cell destruction.

3.3.2 Perfusion circuit set-up

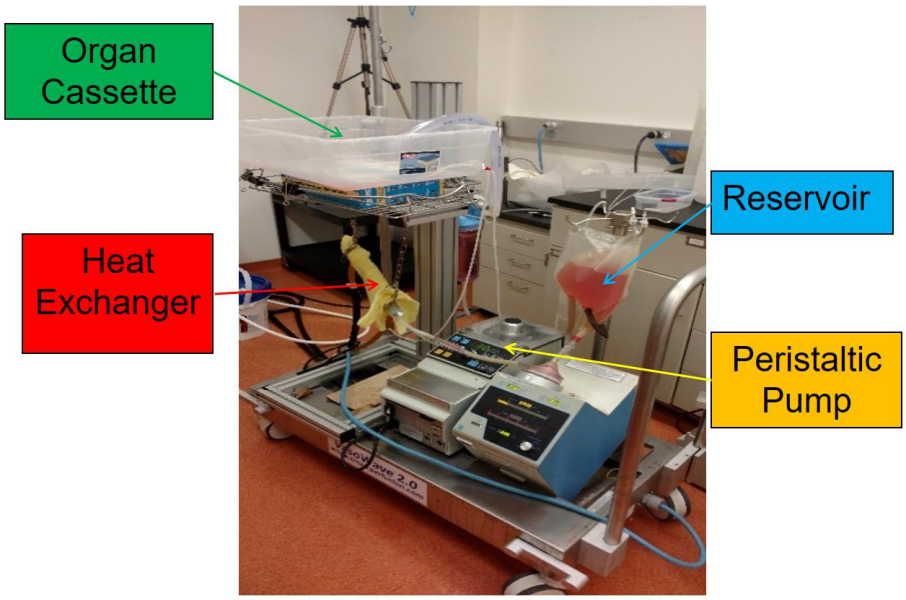

Figure 11*: Perfusion circuit set-up. Components in the perfusion circuit include: heat exchanger, reservoir, cassette to contain the treatment limb and peristaltic pump.*

The limb perfusion system was a basic hypothermic machine perfusion (HMP) device made of four primary components: the peristaltic roller pump, the reservoir, the heat exchanger and the organ cassette *(Figure 11)*. A perfusion system flow diagram is shown in Figure 12 where the perfusate flow is shown in blue. A Hemotherm® heater/cooler (CSZ Medical Systems, Cincinnati, OH) and in-line heat exchange (BIOtherm™, Medtronic, Minneapolis, MN) were used to maintain a perfusate temperature of 4°C. A peristaltic roller pump propelled fluid forward with compression and decompression of tubing. A

roughly sinusoidal waveform was therefore generated by deforming the flexible tubing within the pump, which, as previously discussed, accurately emulated the pulsatile nature of corporal vasculature [36]. The peristaltic pump (Sarns™ 8000 Roller Pump, 3M, Somerset, NJ) RPM was adjusted to generate a target sinusoidal pressure wave form of 80/100 mm Hg to mimic normal swine physiologic circulation. The perfusion line pressure was measured with a manometer (Differential Pressure Manometer, Extech® Instruments, Nashua, NH) placed after the pump and prior to the brachial artery.

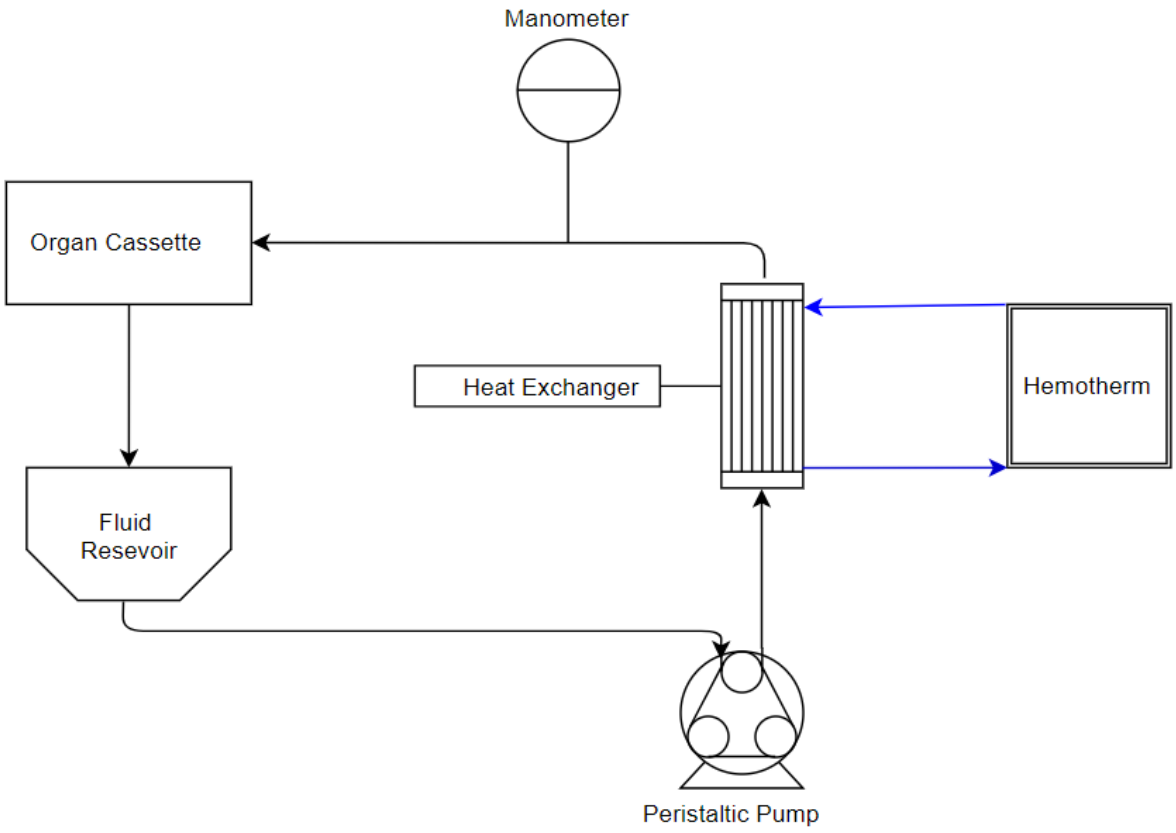

***Figure 12**: Closed-circuit perfusion diagram*

3.4 Data collection

3.4.1 Limb Circumference

Limb circumference was measured with a tape measure, 2 cm anterior to the radiocarpal joint, at time point zero and then at 6 and 12 hours, to gain a rough approximation of tissue swelling. A dye mark was made using an indelible marker to ensure measurements were taken at the same location each time.

3.4.2 Methylene blue

Twelve hours after the beginning of perfusion, the treatment limb was removed from the circuit. In order to assess the extent of perfusion, the limb was further perfused in an open circuit using peristaltic pump and flexible bag containing 1 L of a 1/50 dilution of methylene blue solution (Methylene Blue Indicator: 1% w/v, Fisher Science Education). The simple circuit consisted of a reservoir and the same pump used in the original 12-hour perfusion, as seen in Figure 13.

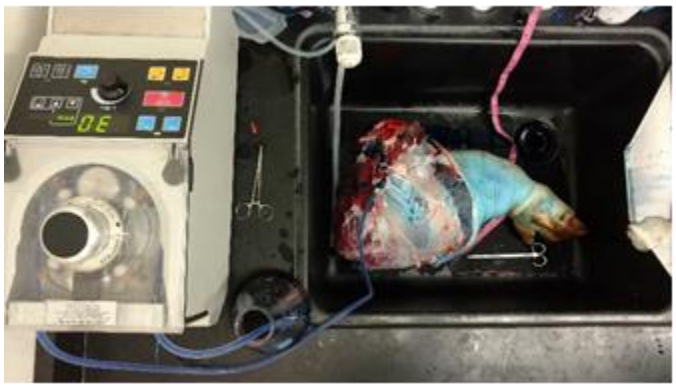

Figure 13*: Limb perfusion with 1/50 dilution of methylene blue solution*

Photographs were taken to document the distribution of the dilute dye solution in each limb.

3.4.3 Biopsy

Four tissue biopsies were taken from the treatment and control limbs to assess viability of the tissue. Samples were acquired from the same locations: skin and muscle were taken near the vasculature *(Figures 14 and 15)* and again distal from the artery *(Figure 16)* in order to evaluate the adequacy of perfusion and effects of preservation conditions on tissue morphology.

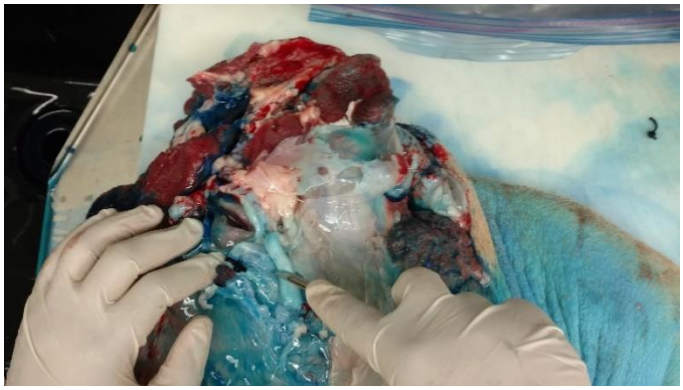

***Figure 14**: Muscle close to artery being sampled*

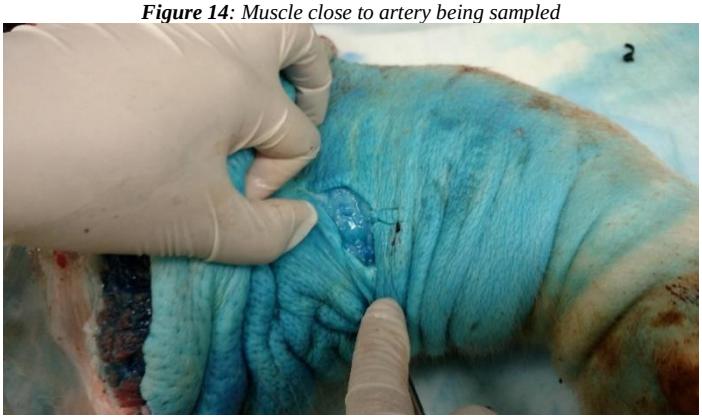

***Figure 15**: Skin close to artery sampled*

***Figure 16**: Skin and muscle far from the artery (at the level of the carpus) being sampled*

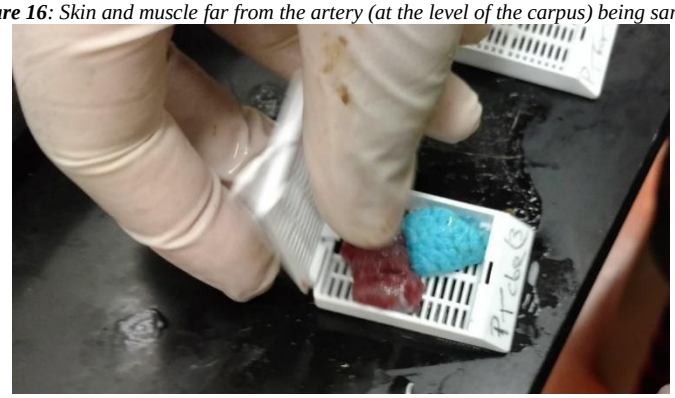

***Figure 17**: Skin and muscle samples from one location placed into the same cassette*

Samples were fixed in 10% neutral-buffered formalin for at least 48 hours. The tissues were trimmed, placed in cassettes and processed for histologic evaluation. Tissues were dehydrated in a graded series of alcohols and xylene, and then infiltrated with paraffin polymer. Three-micron thick sections were cut and stained with hematoxylin-eosin stain, following rehydration. Tissue sections were encoded and given to a pathologist who conducted a blinded evaluation of the samples. Lesions present were scored using a semi-quantitative grading system, with NR= not remarkable (no lesions present), 1= minimal lesions present, 2= mild lesions present, 3= moderately severe lesions present, 4= markedly severe lesions present, and 5= very severe lesions present.

Chapter 4 – Results

It was hypothesized that the use of HMP would improve preservation of composite tissues. Nine pig limbs were perfused using HMP methods and Methylene blue was used to grossly assess the extent of perfusion throughout the limbs. Limb circumference was measured to determine a rough measurement of tissue swelling and evaluate the effectiveness of the addition of glucose to the PBS preservation solution. Finally, histopathology was used to evaluate the viability of the limbs on a cellular level.

4.1 Statistical model

An ANOVA analysis was performed on the data set in order to detect and quantify any significant differences between machines (1, 2 and 3) and sample sites (far and close). A paired T-test was then performed to determine differences between treatment and control limbs. A p (probability) value of 0.05 or less was considered statistically significant. Statistical analysis was performed in NCSS version 11.0.5.

4.2 Statistical findings

4.2.1 Variance Analysis of machines

Using three separate two-way ANOVA models calculated within NCSS, the effect (i.e., similarity/difference in operational efficiency) of individual machines on tissue preservation was examined using an overall α of .05. α represents the probability of rejecting the null hypothesis when the null hypothesis is true for this test, the null hypothesis being that there is statistically significant difference between machines used for perfusion. The lowest p value across all 9 comparisons was p = .036, which occurred between machines 1 and 3 within the muscle samples. The next lowest p value was p = .117 which occurred between machines 2 and 1 also within the muscle samples. This data is depicted below in Tables 2a, 2b and 2c. According to the Bonferroni correction of multiple comparisons, in order to attain an overall α of .05, each individual test must be performed at α = .05/9 or .00555. The Bonferroni correction resulted in a corrected α of .00555 for the individual tests. No comparison resulted in a p value lower than .00555, therefore there was no statistically significant difference between the individual machines used within this experiment.

Machine 3-1 comparison			
Tissue	Difference	SE	p value
Epidermis	0.051	0.854	0.953
Dermis	-1.567	1.04	0.154
Muscle	-2.333	1.008	0.036

Machine 2-1 comparison			
Tissue	Difference	SE	p value
Epidermis	0.234	0.854	0.788
Dermis	-0.658	1.04	0.537
Muscle	-1.683	1.008	0.117

Machine 3-2 comparison			
Tissue	Difference	SE	p value
Epidermis	-0.183	0.854	0.8334
Dermis	-0.909	1.04	0.3983
Muscle	-0.65	1.008	0.5301

Table 2: ***Table 2 a*** *(top left)* ***Table 2 b****(top right) and* ***Table 2 c*** *(bottom) show data from an ANOVA analysis. Table 2a highlights the comparison between Machine 3-1, Table 2b highlights the difference between Machine 2-1, Table 2c highlights the difference between Machine 3-2.*

4.2.2 Difference between sample sites, proximal and distal to the perfusion source (perfused limbs)

The two-way ANOVA models were used to compare the proximal and distal sample sites in each of the individual tissue types, to determine if the perfusion had a uniform effect on each limb. The p values found for epidermis, dermis and muscle were .192, .783 and .653 respectively. As these p values are greater than 0.05, there was no significant difference in the two sample sites, showing that the treatment had an equal effect regardless of the distance from the catheterization site.

Far close comparison			
Tissue	Difference	SE	p value
Epidermis	-0.954	0.686	0.192
Dermis	0.239	0.849	0.783
Muscle	0.378	0.823	0.378

Table 3*: Data from the far and close comparison from the ANOVA analysis.*

4.2.3 Differences in biopsy lesion scores between perfused and control limb tissues

A paired Student's T-test was performed to determine the difference in treatment versus control in all three tissues types: epidermis, dermis, and muscle. A significant difference in the incidence and severity of lesion scores was found in all three tissues, related to the method of preservation (SCS vs. HMP). Muscle had the highest mean difference at -1.544 (p = .00332), followed by epidermis at -1.294 (p = .00121) and finally dermis at -.825 (p=.04757). A summary of relevant statistical data may be seen in Table 4 below with full statistical reports found in Appendix B.

Treatment vs Control paired t-test			
Tissue	Difference	SE	p value
Epidermis	-1.294	0.3297	0.00121
Dermis	-0.825	0.3863	0.04757
Muscle	-1.544	0.4526	0.00332

***Table 4**: Data from the t-test performed in order to determine if there was a significant difference between the treatment and control*

4.3 Limb circumference

Subcutaneous edema was present in all nine perfused limbs, but not in the control (non-perfused) limbs. The circumference, measured 2 cm above the radiocarpal joint did increase in a time-dependent manner after perfusion. This data is summarized in Table 5.

Circumference (Inches)	**Time**	**Limbs**								
		Set 1			**Set 2**			**Set 3**		
		1	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
	Start	9	10	11	10	10.5	12	10	10	10.5
	Half time	12	11.5	13	11	11	14	13	14	13
	End	12	13	13	15	13	16	14	15	14

***Table 5**: Limb circumference*

4.4 Gross evaluation of perfusion, visualized with Methylene Blue staining.

The skin, muscle and subcutaneous tissues in all nine limbs visibly turned bright blue following perfusion with a solution of phosphate-buffered saline containing 1:50 methylene blue. The dye penetrated the entire limb, spanning from the cut extremity at the level of the radio-humeral joint to the digits.

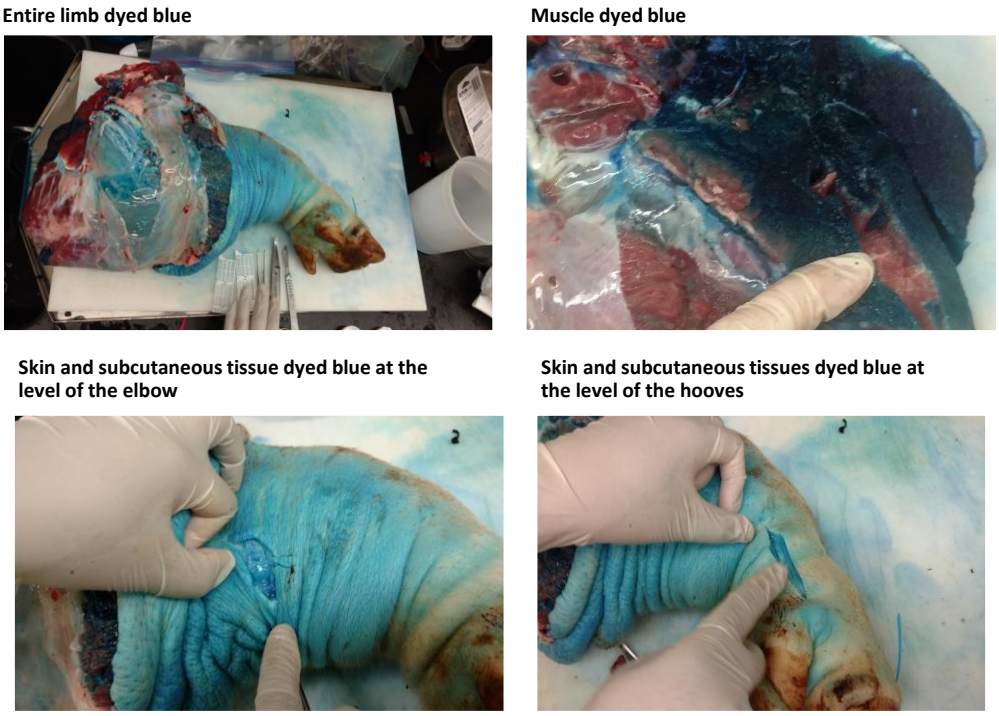

***Figure 18**: Pictures of a limb after perfusion with methylene blue*

4.5 Histological evaluation of biopsies to determine tissue viability

Tissue sections, containing epidermis, dermis and skeletal muscle, taken from control (non-perfused) and perfused limbs were evaluated. Different tissue characteristics were examined with histopathology in order to assess the effects of preservation conditions. Lesions present were scored using a semi-quantitative grading system, with 0 = no lesions present, 1= minimal lesions present, 2= mild lesions present, 3= moderately severe lesions present, 4 = markedly severe lesions present, and 5= very

severe lesions present. Grade 0 and Grade 5 lesions will be illustrated below for epidermis, dermis and muscle.

4.5.1 Epidermis and dermis

Normal porcine epidermis is shown in Figure 19 as an illustration of a grade 0. Composed of epithelial cells and layers of superficial keratin, normal epidermis is 4-6 cells thick, including the mildly vacuolated basal cell layer. The underlying dermal collagen is arranged in regular bundles that intersect which is highlighted with a blue arrow in Figure 19. In contrast, an example of tissue with severe lesions, graded at a 5, is shown in Figure 20. Significant degenerative changes, such as attenuation (thinning) of the epidermis and vacuolation of the basal cell layer (red arrow), can be seen. The dermal collagen is hyperchromic (red-orange), fragmented, and in disarray which are all indicators of significant collagen degeneration (orange arrow).

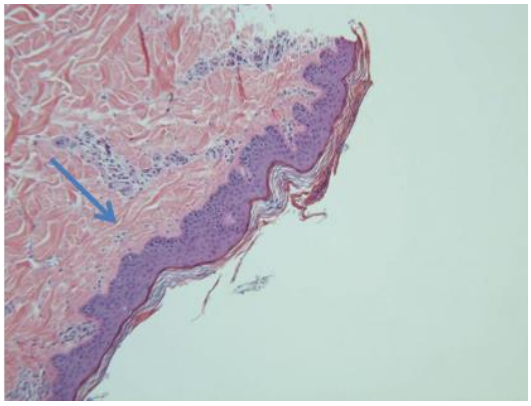

***Figure 19**: Normal porcine epidermis and dermis, hematoxylin-eosin stain, original magnification 10x.*

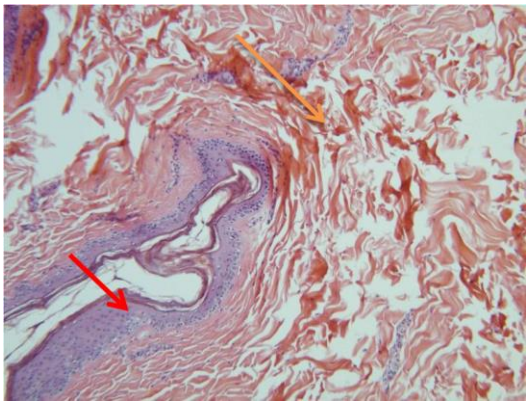

***Figure 20**: Degenerate porcine epidermis and dermis, hematoxylin-eosin stain, original magnification 10x.*

4.5.2 Skeletal muscle

Figure 21 below shows normal porcine skeletal muscle fibers, given a grade of 0, characterized by the packing of robust fibers and the consistent staining of myofibrils within each muscle bundle. This differs significantly from Figure 22, graded at a 5, in which the muscle fibers are shrunken and separated by edematous loose connective tissue. Several fiber bundles are fragmented and hyperchromic indicating poor preservation and deterioration. One such bundle is highlighted with a green arrow in Figure 22.

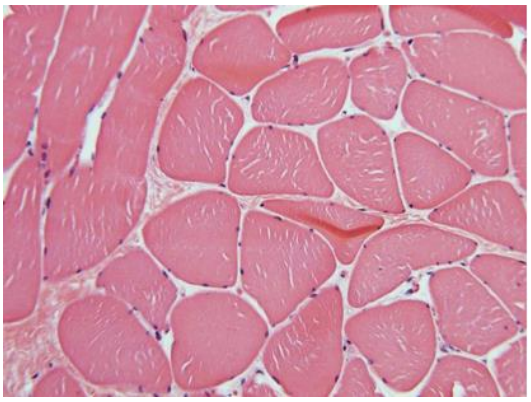

***Figure 21**: Normal porcine skeletal muscle fibers, cut in gross section, hematoxylin-eosin stain, original magnification 25x*

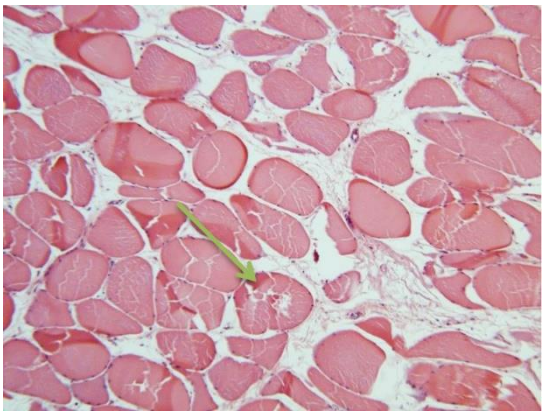

***Figure 22**: Degenerate porcine skeletal muscle, cut in cross section, hematoxylin-eosin stain, original magnification 25x. Green arrow indicates one of several fiber bundles that are fragmented and hyperchromic.*

Tissue scores for all samples may be found in appendix A.

Chapter 5 – Discussion

Experiments were conducted to assess the efficacy of machine perfusion for preservation of VCA. The results indicate MP had a deleterious effect on limb preservation under the condition of these experiments. Perfusion was first assessed with methylene blue. The entire limb, from elbow to hooves, including all the different tissue types (muscle, skin, vessels…), was uniformly dyed blue following perfusion with methylene blue. This initial confirmation of successful perfusion was then reinforced by the histopathology results. According to the ANOVA, no significant difference could be detected between tissue samples obtained near the catheterization site or distally near the hooves. This level of homogeneity implies adequate perfusion throughout the limb. The ANOVA also proved that there was no significant difference between three perfusion systems used implying that all perfused effectively. These results indicate there was extensive and effective tissue penetration, reaching even the small capillary beds, allowing for complete organ perfusion. Drawing from previous experience with livers and kidneys, adequate perfusion should foster tissue preservation. However, across all tissue types, machine perfusion was found to be significantly less effective than the control in preserving tissue. That is to say, a limb chilled at 4ºC and machine perfused with our preservation solution, deteriorated significantly faster than a non-preserved limb, left at room temperature for 12 hours.

Despite the efficiency of the machine in perfusion of the tissues, the treatment, contrary to what was hypothesized, significantly accelerated deterioration. The most plausible explanation for these findings is the inadequate perfusate composition used within the experiment. Commercially prepared preservation solutions are composed of four basic elements aiming to counteract damage caused by ischemia. These elements are electrolytes, colloids, buffers, and antioxidants. Each element will be individually described in the following paragraphs, aiming to

evaluate the strengths and weaknesses of the fortified PBS solution used in this experiment [37]. Table 6 below, presents the composition of several clinically used preservation solutions as they compare with the fortified PBS solution.

	Solutions commonly used for machine perfusion				Solution used in experiment	Solution no longer used
Perfusate	UW	HTK	Celsior	Perfadex	PBS	Euro Collins
Na	25	15	100	138	157	10
K	120	10	15	6	4.5	107
Colloid	Lactobionate, raffinose, hydroxyethyl starch	Mannitol	Lactobionate, mannitol	Dextran	Glucose (277.5)	Glucose (194)
Buffer	Phosphate	Histidine	Histidine	Phosphate	Phosphate	Phosphate, bicarbonate
Antioxidant	Allopurinol, Glutathione	Tryptophan, mannitol	Glutathione, mannitol	None	None	None
Osmolarity (mOsm/L)	330	310	320	292	350	355
Ca	-	0.02	0.25	None	1	None
Mg	5	4	13	0.8	.5	None
Cl	20	32	-	142	142.7	15

***Table 6**: Compares the solutions commonly used for machine perfusion with the solution used for this experiment and Euro Collins. All units are expressed in mmol/L unless otherwise specified* [37,38]

Electrolytes in perfusion media maintain the osmotic balance across cell membranes [37]. Our fortified PBS solution is slightly hypertonic, with an osmolality of 350 mOsm/L. This is similar to the osmolality of clinically used solutions such as HTK, UW and Celsior with values of 330, 310 and 320 mOsm/L respectively (Table 6). Our perfusion solution has a high sodium and low potassium composition, resulting in an equilibrium between the intracellular protein anions and the extracellular sodium preventing cell swelling. Over the last decade, this electrolyte composition and osmolality have been shown to produce effective organ preservation solutions [39].

Colloids are impermeable molecules to the cell that promote water retention within the vasculature, preventing both interstitial and cellular edema [37]. During the pilot study, extreme

interstitial edema, caused by the lack of a colloid within our preservation solution, collapsed vessels, resulting in extremely limited perfusion of the limb. As an adjustment, the PBS solution for this experiment was fortified with glucose (Table 6). Glucose has been used as an impermeant in preservation solutions in the past, most notably within the Euro Collins solution which was considered the gold standard for preservation prior to 1988 [37]. The addition of glucose significantly reduced the swelling of the limbs caused by edema in the current study. An average circumferential increase of 51% observed in the pilot study, was reduced to an average circumferential increase of 33% in this study, with no incidence of cellular edema being found in any histological samples. However, Euro Collins solution has been discredited as the most effective means for organ preservation in part due to having glucose as one of its components. Glucose is enzymatically broken down to lactate which diffuses into the cell resulting in subsequent cellular edema. Consequently, glucose is suboptimal as an impermeant thus favoring the use of newer, more effective preservation solutions containing alternate impermeants such as lactobionate and mannitol [40]. While the addition of glucose did significantly reduce tissue edema in this study, it is likely that the edema would increase over time with continued break down of glucose into lactate.

Buffer molecules are used to prevent metabolic acidosis [37]. Metabolic acidosis occurs because of the increased production of acidic compounds during anaerobic metabolism. The fortified PBS solution used in this experiment employed mono- and dibasic-phosphate, which is a proven buffer that is commonly used in preservation solutions such as UW solution (Table 6). In fact, PBS solution has successfully maintained kidney pH in prior experiments for up to 48 hours. As additional hydrogen ions are produced, hydrogen phosphate converts into dihydrogen phosphate maintaining the pH balance at around 7.4. In our study, the limb's larger metabolic

mass in comparison to the kidney, may have resulted in a significant increase in the acid produced thus requiring more buffering activity. However, the same volume of PBS solution was used for both kidney and limb experiments. While satisfactory for the kidneys, the acid produced by muscle metabolism in the limbs may have eventually overwhelmed the phosphate buffering capacity contained within the solution. Without adequate buffering, metabolic acidosis overwhelms cells causing the release and activation of lysosomal enzymes leading to extensive mitochondrial and overall cell damage.

Antioxidants combat reactive oxygen species within the tissue [37]. Free radicals are produced during ischemia. These unstable molecules promote oxidation reactions within proteins, lipids and even DNA resulting in tissue damage and impaired function [41]. Antioxidants are needed to neutralize free radicals. The muscle contains a small number of endogenous antioxidants but these are insufficient to counteract the free radical build up resulting from ischemia. As a result, preservation solutions need to contain antioxidants, which our PBS solution lacked, allowing free radicals to cascade throughout the tissue.

In summary, the fortified PBS solution used in these experiments, while providing adequate electrolyte composition, could not meet the multifaceted needs of limb preservation. The fortified PBS solution afforded minor oncotic support with glucose as an impermeant and no antioxidants to combat damage caused by free radicals. The phosphate buffer, although suitable in itself, was insufficient to counteract the acidosis of the limbs due to the small total volume of perfusate used.

The shortcomings of this solution were further amplified by the closed nature of the perfusion system. Without regularly replacing the perfusate, toxic metabolites continuously circulate throughout the entire limb resulting in total organ damage. Refreshing the perfusate, by

removing portions of the volume and replacing it with fresh solution, could minimize damage caused by unstable molecules fashioned from acidosis and free radicals. This would be the focus of future studies.

Hypothermic machine perfusion is only recently being considered as a possible preservation method for limbs. One study, performed by Kueckelhaus *et al.*, published in 2016, evaluated the efficacy of a purposefully designed "portable extracorporeal membrane oxygenator device" in swine limb preservation [33]. Approximately 35 kg Yorkshire pigs were used for this study. Four limbs, obtained from these pigs, were allocated to the control group and stored on ice slurry for 4 hours at 4°C. Another 3 limbs were perfused for 12 hours at 10°C. All seven limbs were then reimplanted onto the pigs and monitored for 7 days following the period of preservation. Histopathologic evaluation and clinical observation demonstrated less morbidity and tissue damage within the HMP group despite the extended preservation time. This study differs from ours in many significant ways which will be explored in the upcoming paragraphs emphasizing how these differences account for the disparate results.

Kueckelhaus' s experiment used an acellular Perfadex solution. Similar to the fortified PBS solution we used, Perfadex is low in potassium, high in sodium with a phosphate buffer (Table 6). The main difference between the two, is the use of dextran in the Perfadex solution as a colloid. As previously discussed, glucose, the colloid in the PBS solution, does provide some degree of edema prevention but eventually breaks down to lactate, which exacerbates cellular edema. Dextran, in comparison, is a metabolically inert substrate that has been proven to be an effective impermeant and therefore confers less damage to the tissues [42].

Solution composition as well as overall volume and handling substantially impact preservation outcomes. In the study by Kueckelhaus, et. al, 5.6 liters of Perfadex solution were

used to perfuse limbs obtained from 35 kg pigs compared to 3 liters of PBS used to perfuse limbs obtained from 225 kg pigs. Therefore, 90% more solution was used to perfuse limbs that are notably smaller than the limbs from our experiment. This larger volume of preservation solution likely contained the necessary amount of buffering ability to combat the development of metabolic acidosis. Furthermore, the perfusate was replenished after 7 hours of perfusion allowing for removal of toxic metabolites. This combination of superior preservation solution, larger perfusion volume and fluid refreshing, account for the more successful preservation of Kueckelhaus' s limbs.

Another notable difference between our study and Kueckelhaus's, is the use of a membrane oxygenator. Generally, preservation solutions have to be coupled with either perfluorocarbons, polymerized hemoglobin, or red blood cells to effectively transport and deliver oxygen to tissues [43]. Without these, the Perfadex solution in the Kueckelhaus et.al. study could only deliver a comparatively small amount of oxygen to the tissues. Although the oxygen provided may inhibit, to a certain degree, hypoxic damage, the preservation solution as well as the manner in which it is used most likely account for success of this study.

In conclusion, it is probable that with better formulated preservation solution, as well as adequate volume and solution replenishing, HMP may be an effective means of limb preservation. Other studies were performed which showed successful limb preservation with SNMP and NMP [6,44]. However, these preservation methodologies are not readily portable due to significant continuous nutrient and oxygen requirements. The optimal dynamic preservation system would travel from the site of procurement to the site of transplantation in order to reduce ischemia time. The complexity of SNMP and NMP systems preclude portability with currently available technology. As a consequence, HMP is the most promising in terms of clinical

application of a dynamic preservation modality for limbs. Further research must be performed to understand the requirements of limb preservation in order to design a standardized, effective and portable HMP apparatus.

Chapter 6 – Conclusions

In the work reported here, a hypothermic machine perfusion system, shown to be effective for the preservation of kidneys, was modified for use in porcine limbs. Gross evaluation and statistical analysis, while indicating effective perfusion with the system used, also showed significant degradation of the perfused limbs compared to the non-preserved controls. These results directly contradict the hypothesis stated earlier that HMP of VCA would improve tissue preservation and quality. These findings transpired from inadequate perfusate composition as well as insufficient perfusate to tissue ratio and lack of perfusate replenishing.

Chapter 7 – Recommendations

Additional experiments must be performed in order to determine if HMP is a viable preservation method for VCAs. Ideally organs should be procured in house, flushed and immediately placed on the machine. For this experiment, the limbs were maintained in SCS for the ninety-minute transit time from abattoir to lab. During this time without perfusion, vessels may have collapsed and cell damage initiated in the absence of adequate substrates. Results may have been affected from having to employ both SCS and HMP preservation modalities.

A commercially available perfusate, providing electrolytes, a buffering system, antioxidant support and a colloid to prevent interstitial edema, should be used. Individual tissue types have different preservation requirements, which are met by specific preservation solutions. Therefore, finding one solution to meet the needs of each and every tissue type within a composite tissue is challenging. The University of Wisconsin (UW) solution is currently used for composite tissue preservation. As previously mentioned however, not a lot of research has been done on VCA. There may be another preservation solution better suited for composite tissue preservation. Further research is again needed, not only to find the right one but also to establish the adequate volume needed to satisfy tissue requirements. The solution must be regularly replenished to remove toxic metabolites from the circuit.

For future experimentation with an adequate perfusate, further data streams are needed in order to better understand the limbs state throughout preservation. In line real time sensors tracking changes in pH, conductivity, dissolved oxygen and glucose levels would greatly contribute to understanding the exact degradation pathways occurring within the perfused limbs. While methylene blue is an effective indicator of gross perfusion, there are other more technical means of evaluating local perfusion. Incorporating infrared (IR) technologies to observe changes

in surface temperature associated with perfusion, or heat flux sensors to record local perfusion values, would better characterize any change in flow rates experienced throughout the preservation. We would then obtain a more accurate picture of what is occurring throughout the experiment and be able to make adjustments to achieve better preservation.

The best indicator of limb health is histopathological observation of cells. While time intensive, biopsies taken throughout the experiment would give a greater idea of how the cells are changing throughout the preservation. Biopsies may negatively affect the vasculature impacting the preservation of the limb as a whole. Care must be taken to reduce this effect by taking small biopsies from multiple dispersed locations.

Further optimization of this machine may be performed with the addition of an oxygenator, which would prevent the damage occurring from hypoxia. This addition may confer more complications than benefits for very specific requirements must be met to effectively deliver oxygen to cells. However, if the aforementioned changes fail in composite tissue preservation, oxygen persuflation in combination to HMP may be considered.

In conclusion, these experiments found the composition, volume and handling of our modified PBS solution to be ineffective in the preservation of limbs. However, HMP may still be an effective means of limb preservation, provided that a better-formulated preservation solution is used and regularly replenished. Additional data streams are needed to further isolate any causes of tissue degradation with the HMP system. Other studies have shown success in the preservation of limbs using SNMP and NMP. However, these technologies are not readily portable due to the heightened metabolic demands of a limb in this state. HMP remains promising as a clinical dynamic preservation methodology and further research is required to determine its efficacy in VCA.

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Appendix A: Tissue Scores

Below is the raw histological scoring data for the tissue biopsies, rated on a scale from 0-5. 0 = no lesions, 1= minimal lesions, 2 = mild lesions, 3 = moderately severe lesions, 4 = markedly severe lesions, and 5 = very severe lesions present

Section ID #	Limb Pair ID	Far/Close	Cont/Treat	Machine	Epidermis	Dermis	Muscle	C.Epidermis	C.Dermis	C.Muscle
IC CLOSE (2	1	Close	Control	One	0	3.3	0	0	3	0
IT CLOSE (2	1	Close	Treatment	One	0	5	3.2	0	5	3
ICFOR (2	1	Far	Control	One	0	0	0	0	0	0
IT FOR (2	1	Far	Treatment	One	0	5	3.5	0	5	3
IIC CLOSE (2	2	Close	Control	Two	0	2.9	0.9	0	3	1
IIT CLOSE (2	2	Close	Treatment	Two	3	4.95	0	3	5	0
IIC FOR (2	2	Far	Control	Two	0	1.9	0	0	2	0
IIT FOR (2	2	Far	Treatment	Two	1.5	3.9	4.1	1	4	4
IIIC CLOSE 2	3	Close	Control	Three	2.5	4	2	2	4	2
IIIT CLOSE (2	3	Close	Treatment	Three	2.5	5	4	2	5	4
IIIC FOR 2	3	Far	Control	Three	0	3.5	2.1	0	3	2
IIIT FOR (2	3	Far	Treatment	Three	0	0	3.3	0	0	3
1C CLOSE (1)	4	Close	Control	One	0	2.2	0	0	2	0
1T CLOSE (1)	4	Close	Treatment	One	2.3	4	4.3	2	4	4
IC FOR (1)	4	Far	Control	One	0	1.2	1	0	1	1
1T FOR (1)	4	Far	Treatment	One	0	1.9	4.5	0	2	4
2C CLOSE (1)	5	Close	Control	Two	0	3.3	0	0	3	0
2T CLOSE (1)	5	Close	Treatment	Two	1	1.2	1	1	1	1
I2C FOR (1)	5	Far	Control	Two	0	0	0	0	0	0
2T FOR (1)	5	Far	Treatment	Two	0	1.7	2	0	2	2
3C CLOSE (1)	6	Close	Control	Three	0	3.2	2	0	3	2
3T CLOSE (1)	6	Close	Treatment	Three	3	3	3.2	3	3	3
3C FOR (1)	6	Far	Control	Three	0	2.3	1	0	2	1
3T FOR (1)	6	Far	Treatment	Three	2.5	3	3.3	2	3	3
BC CLOSE (3	7	Close	Control	One	0	3.5	1.2	0	3	1
BT CLOSE 3	7	Close	Treatment	One	4	4.1	2	4	4	2
BC FOR (3	7	Far	Control	One		3.4	1		3	1
BT FOR 3	7	Far	Treatment	One	2	3	3	2	3	3
PC CLOSE 3	8	Close	Control	Two	4	4	1	3	4	1
PT CLOSE 3	8	Close	Treatment	Two	4.5	4.5	3	4	4	3
PC FOR 3	8	Far	Control	Two	1	3.2	2	1	3	2
PT FOR 3	8	Far	Treatment	Two	3.4	4.5	1	3	4	1
GC CLOSE 3	9	Close	Control	Three	3.2	4	3.4	3	4	3
GT CLOSE 3	9	Close	Treatment	Three	5	5	2	5	5	2
GC FOR 3	9	Far	Control	Three	0	2	3	0	2	3
GT FOR 3	9	Far	Treatment	Three	0	3	1	0	3	1

Appendix B: NCSS statistical reports

Below are the raw statistical reports from the paired t tests performed in NCSS v11.0.5 for each of the individual tissue types, Epidermis Dermis and Muscle.

Epidermis

Paired-Sample Report

Dataset Untitled

Descriptive Statistics

Variable	Count	Mean	Standard Deviation	Standard Error	95.0% LCL of Mean	95.0% UCL of Mean
EC	17	0.6294118	1.293138	0.313632	-0.03545844	1.294282
ET	17	1.923529	1.749117	0.4242233	1.024216	2.822843

T* for Confidence Limits: T* (EC) = 2.1199, T* (ET) = 2.1199

Two-Sided Confidence Interval of the Mean Difference

Variables	Count	Mean Difference	Standard Deviation	Standard Error	T*	d.f.	95.0% C. I. of Mean Diff. Lower Limit	Upper Limit
EC - ET	17	-1.294118	1.359444	0.3297136	2.1199	16	-1.993079	-0.595156

Paired-Sample T-Test

Paired Difference: (EC) - (ET)

Alternative Hypothesis	Mean Difference	Standard Error	T-Statistic	d.f.	Prob Level	Reject H0 at α = 0.050
Mean Diff. ≠ 0	-1.294118	0.3297136	-3.9250	16	0.00121	Yes

Tests of Assumptions

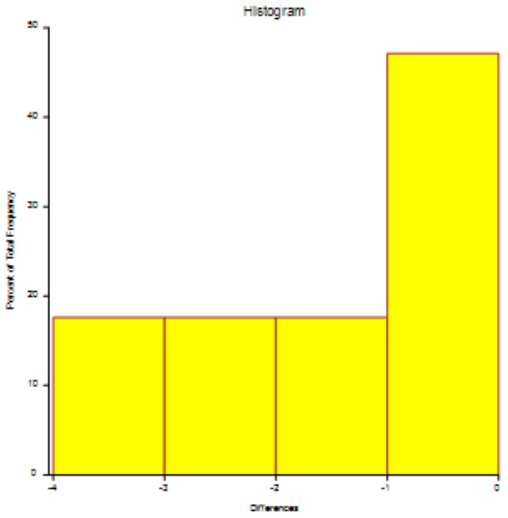
Paired Difference: EC - ET

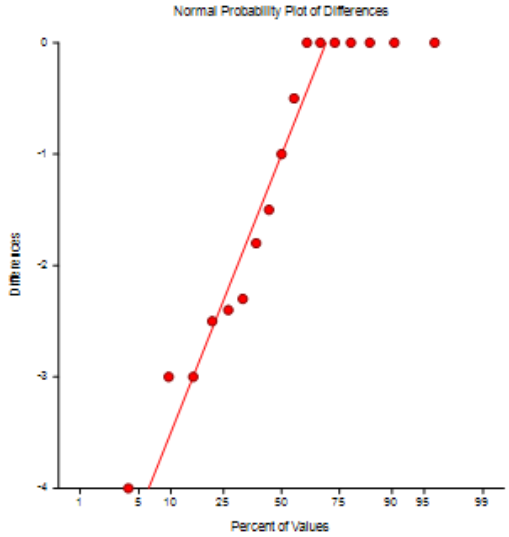
Assumption	Value	Prob Level	Decision (α = 0.050)
Skewness Normality	-0.9777	0.328203	Cannot reject normality
Kurtosis Normality	-1.3353	0.181776	Cannot reject normality
Omnibus Normality	2.7390	0.254231	Cannot reject normality
Correlation Coefficient	0.637427		

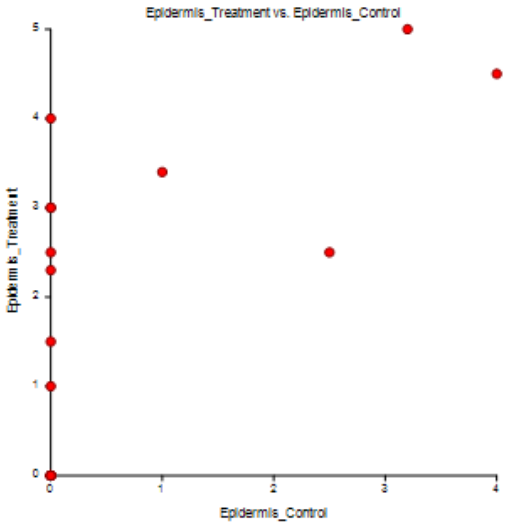
Paired-Sample Report

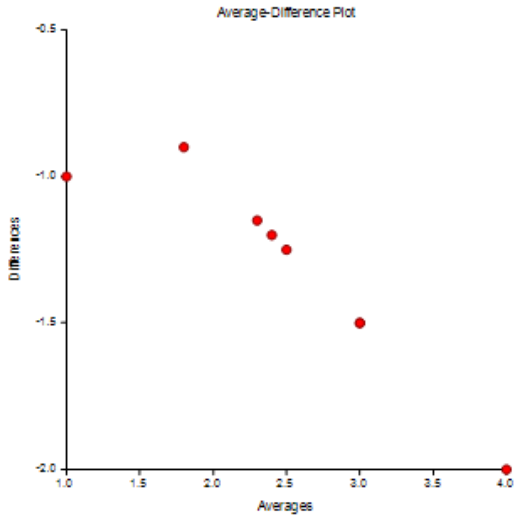
Dataset Untitled

Plots Section









Dermis

Paired-Sample Report

Dataset Untitled

Descriptive Statistics

Variable	Count	Mean	Standard Deviation	Standard Error	95.0% LCL of Mean	95.0% UCL of Mean
DC	18	2.661111	1.24718	0.2939631	2.040903	3.281319
DT	18	3.486111	1.499196	0.353364	2.740578	4.231644

T* for Confidence Limits: T* (DC) = 2.1098, T* (DT) = 2.1098

Two-Sided Confidence Interval of the Mean Difference

Variables	Count	Mean Difference	Standard Deviation	Standard Error	T*	d.f.	95.0% C. I. of Mean Diff. Lower Limit	Upper Limit
DC - DT	18	-0.825	1.639068	0.386332	2.1098	17	-1.640089	-0.009910628

Paired-Sample T-Test

Paired Difference: (DC) - (DT)

Alternative Hypothesis	Mean Difference	Standard Error	T-Statistic	d.f.	Prob Level	Reject H0 at α = 0.050
Mean Diff. ≠ 0	-0.825	0.386332	-2.1355	17	0.04757	Yes

Tests of Assumptions

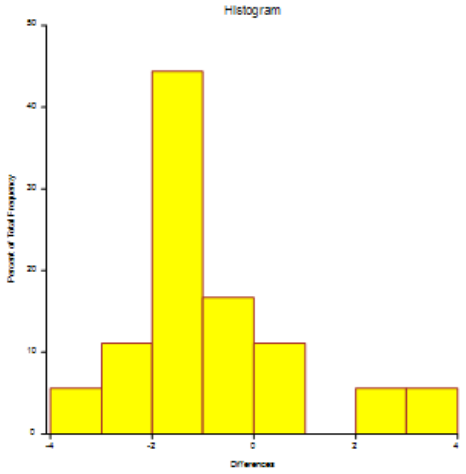
Paired Difference: DC - DT

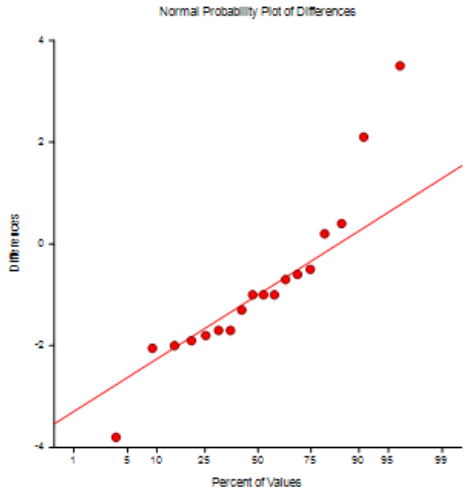
Assumption	Value	Prob Level	Decision (α = 0.050)
Skewness Normality	2.0678	0.038661	Reject normality
Kurtosis Normality	1.8434	0.065265	Cannot reject normality
Omnibus Normality	7.6740	0.021559	Reject normality
Correlation Coefficient	0.298567		

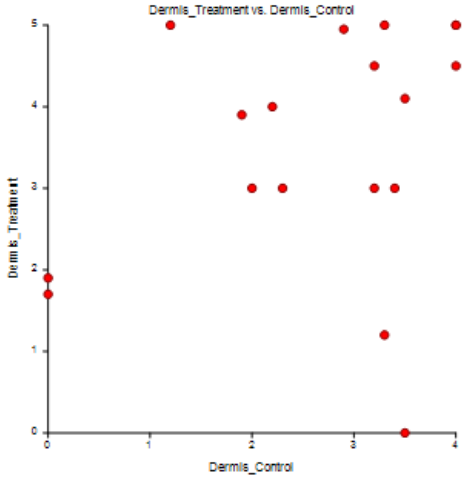
Paired-Sample Report

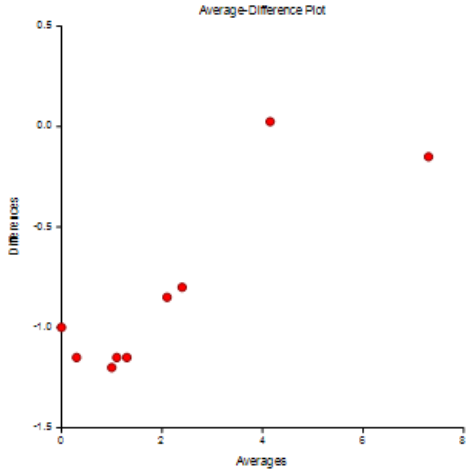
Dataset Untitled

Plots Section









Muscle

Paired-Sample Report

Dataset Untitled

Descriptive Statistics

Variable	Count	Mean	Standard Deviation	Standard Error	95.0% LCL of Mean	95.0% UCL of Mean
MC	18	1.144444	1.074968	0.2533723	0.6098756	1.679013
MT	18	2.688889	1.304241	0.3074127	2.040305	3.337473

T* for Confidence Limits: T* (MC) = 2.1098, T* (MT) = 2.1098

Two-Sided Confidence Interval of the Mean Difference

Variables	Count	Mean Difference	Standard Deviation	Standard Error	T*	d.f.	95.0% C. I. of Mean Diff. Lower Limit	Upper Limit
MC - MT	18	-1.544444	1.92024	0.4526048	2.1098	17	-2.499357	-0.5895317

Paired-Sample T-Test

Paired Difference: (MC) - (MT)

Alternative Hypothesis	Mean Difference	Standard Error	T-Statistic	d.f.	Prob Level	Reject H0 at α = 0.050
Mean Diff. ≠ 0	-1.544444	0.4526048	-3.4123	17	0.00332	Yes

Tests of Assumptions

Paired Difference: MC - MT

Assumption	Value	Prob Level	Decision (α = 0.050)
Skewness Normality	0.5907	0.554691	Cannot reject normality
Kurtosis Normality	-0.4360	0.662826	Cannot reject normality
Omnibus Normality	0.5391	0.763727	Cannot reject normality
Correlation Coefficient	-0.296258		

Paired-Sample Report

Dataset Untitled

Plots Section