Abstract – PYRUVATE- ENRICHED PRESERVATION OF MACHINE PERFUSED KIDNEYS

Purpose: Kidney transplantation remains the gold-standard treatment for patients diagnosed with end-stage renal disease (ESRD). However, the demand for donors currently far outpaces the supply. Kidneys donated after cardiac death (DCD) are a promising source, as cardiac arrest remains the leading cause of death in the United States. Studies have demonstrated the efficacy of hypothermic machine perfusion (HMP), a process by which kidneys are cannulated and perfused with a pulsatile flowing solution under hypothermic conditions, in reducing insult to organs compared to standard static cold storage (SCS) [6-8].

To date, perfusate additives to further optimize the outcomes of HMP preserved kidneys have not been extensively studied. The glycolytic end product, pyruvate, has demonstrated efficacy with protecting the heart from ischemia-reperfusion injury, improving post-ischemic contractile function of the heart through its function as an antioxidant and energy substrate, maintaining the glutathione redox state, and inducing erythropoietin production [15, 16]. We aim to investigate the impact of pyruvate on DCD kidneys maintained under HMP conditions.

Methods: Four pilot experiments were performed in a large animal model, the domestic pig. After cardiac arrest 60 min of warm ischemia, the kidneys were accessed by laparotomy, explanted and flushed with cold saline. Biopsies of cortex and medulla were collected from one kidney to serve as a pre-perfusion control. The other kidney was cannulated via the renal artery and installed in an organ perfusion system for HMP with control or pyruvate-enriched University of Wisconsin preservation solution. Perfusate was sampled periodically for measurements of cytokine concentrations. At 72 h HMP the kidney was removed, and cortical and medullary biopsies were taken. The tissue samples from the pre-perfused and 72-hour perfused kidneys were evaluated for renal tissue integrity via histological assessment and abundances of mRNA encoding pro- and anti-inflammatory cytokines.

Results: In pyruvate treated DCD kidneys, both renal cortex and renal medulla samples demonstrated better preserved tissue integrity compared to control. Additionally, markers for pro-inflammatory response markedly increased in the control group compared to the treatment group and vice versa for anti-inflammatory markers, including erythropoietin.

Conclusions: This pilot study supports the hypothesis that pyruvate-enriched machine perfusion will improve preservation of DCD kidneys, at least in part by dampening the rate formation of pro-inflammatory cytokines and increasing the formation of anti-inflammatory cytokines.

SIGNATURE PAGE

PYRUVATE-ENRICHED PRESERVATION OF

MACHINE PERFUSED PORCINE KIDNEYS

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TITLE PAGE

PYRUVATE- ENRICHED PRESERVATION OF MACHINE PERFUSED KIDNEYS

INTERNSHIP PRACTICUM REPORT

Presented to the Graduate Council of the Graduate School of Biomedical Sciences University of North Texas Health Science Center at Fort Worth in Partial Fulfillment of the Requirements

For the Degree of

Master of Science

By

Salma Omar, B.S.
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I also thank Arthur Williams for assisting with the surgeries and ensuring all the necessary materials arrived on time, Dr. Sarika Chaudhari for teaching me how to section and stain tissue samples for histology, Dr. Logan Konty for instructing me on how to operate the LifePort® kidney transporter, and Jessica Morales for showing me how to run successful ELISA assays. I am emphatically grateful for their time, patience, advice, and kindness through this process.

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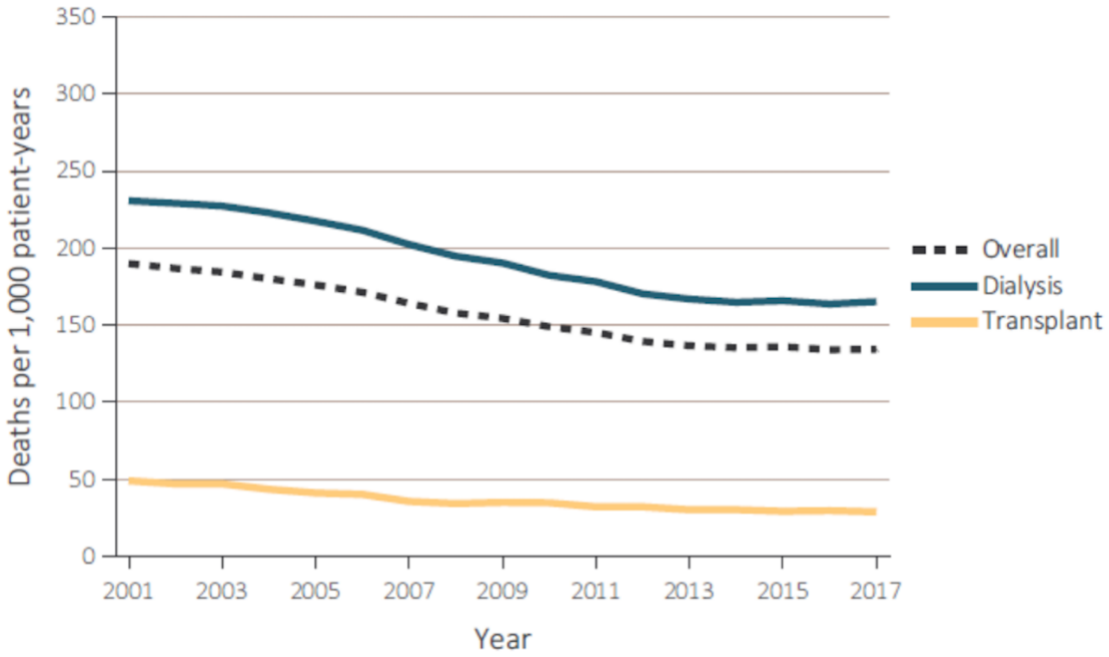

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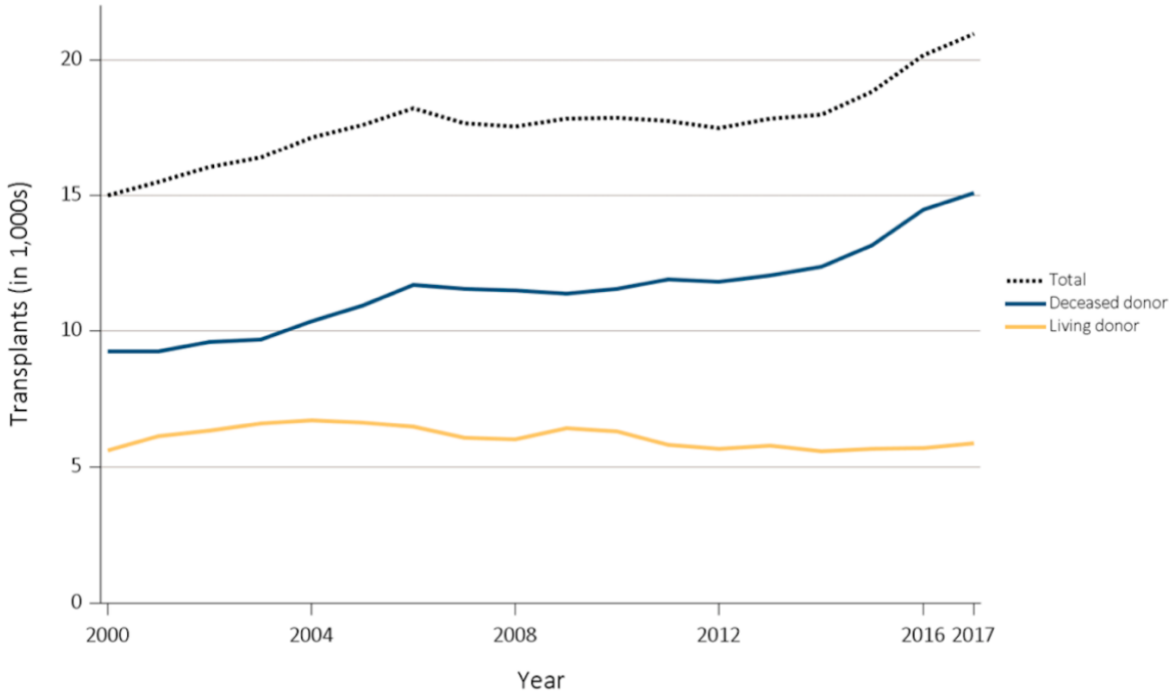

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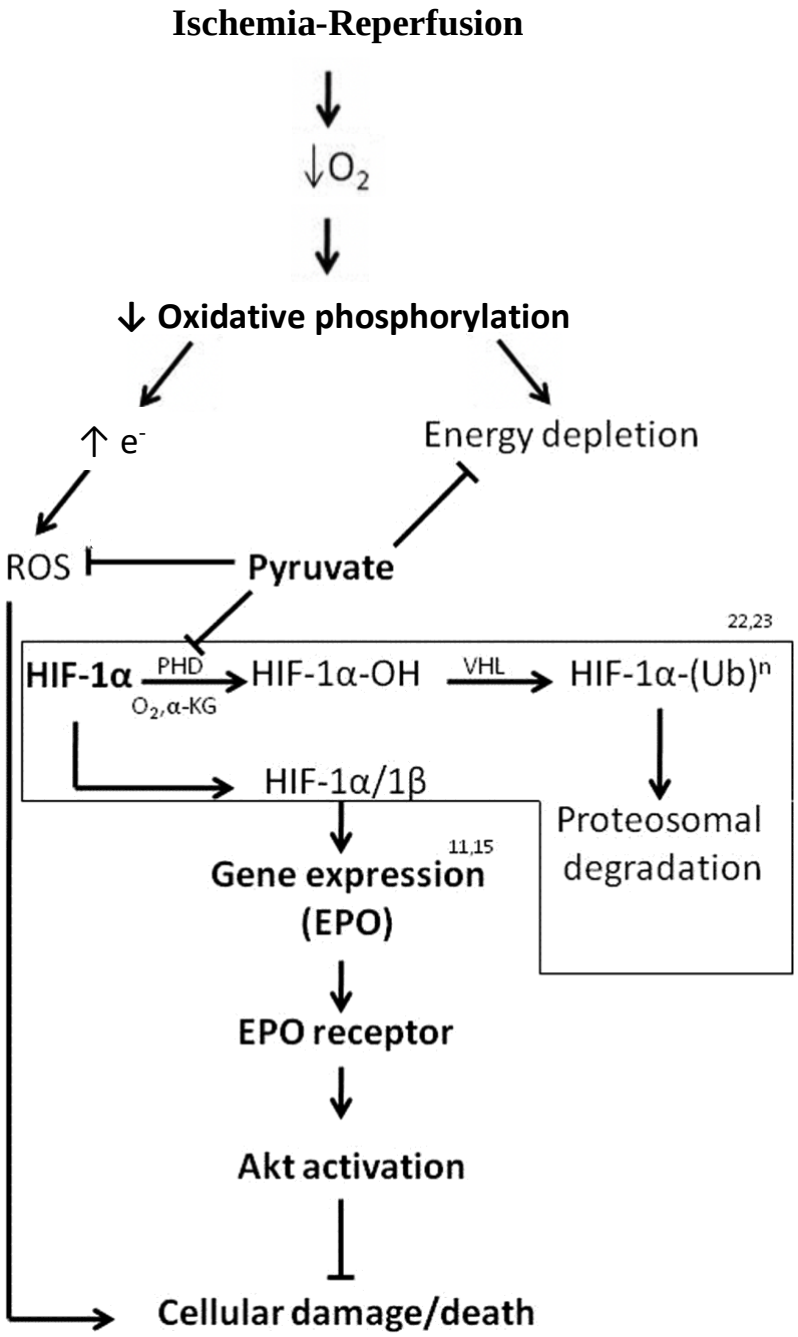

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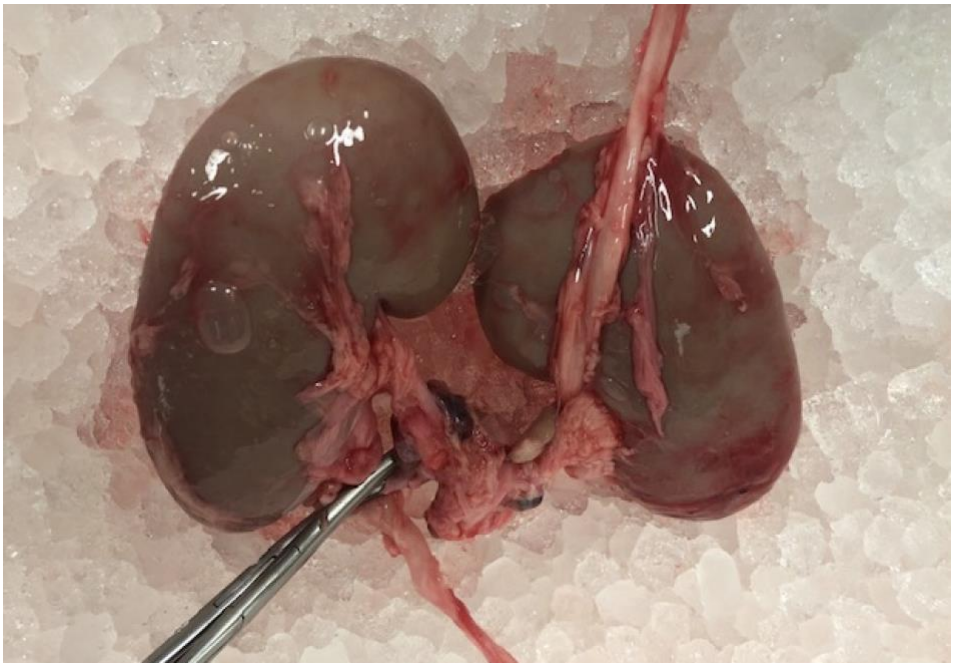
(Modified from Mallet and Ryou, 2012)



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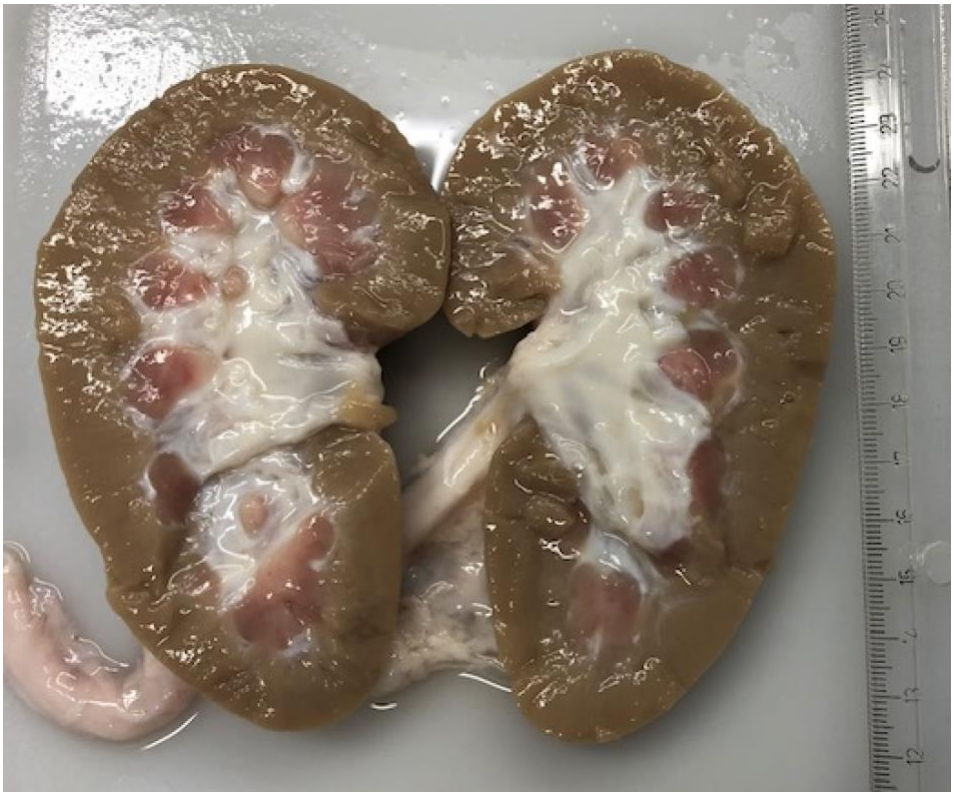

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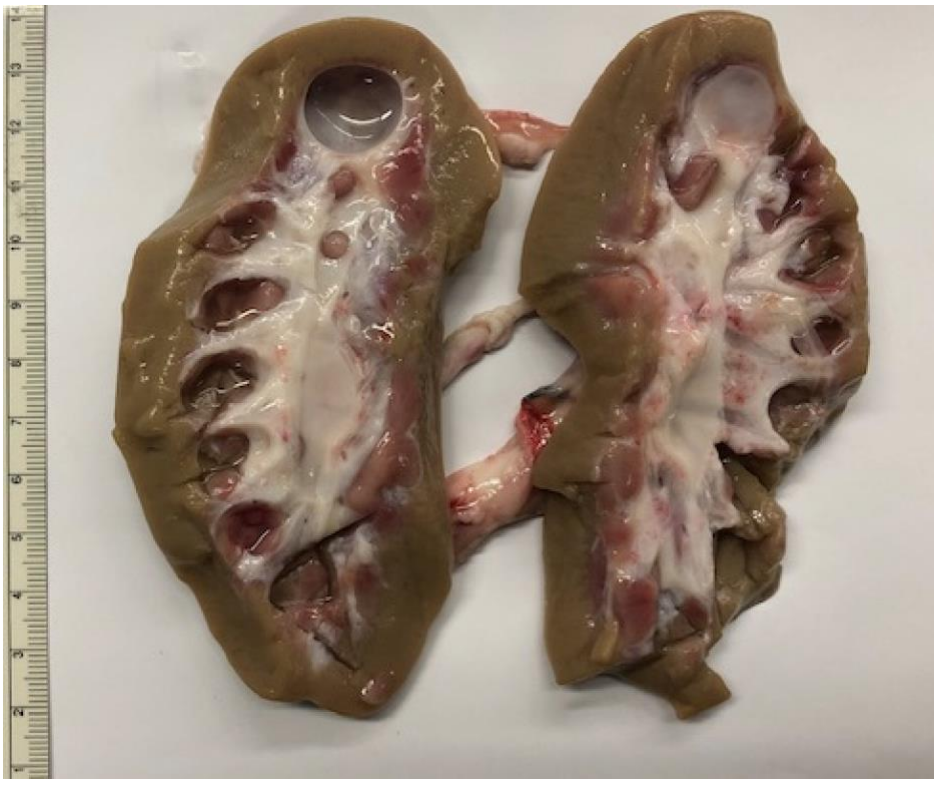

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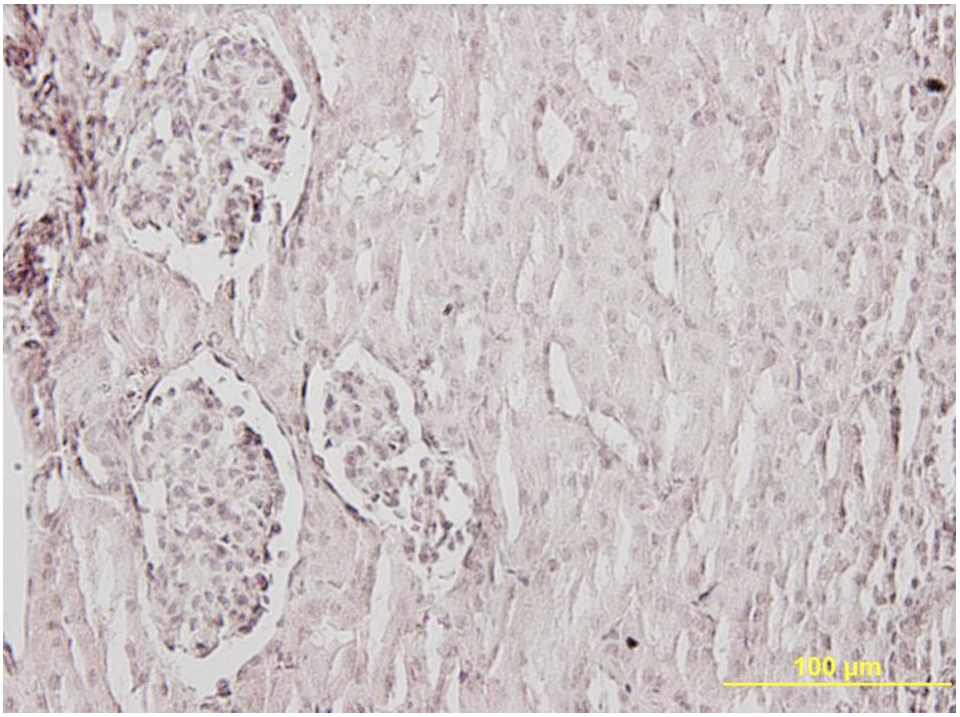

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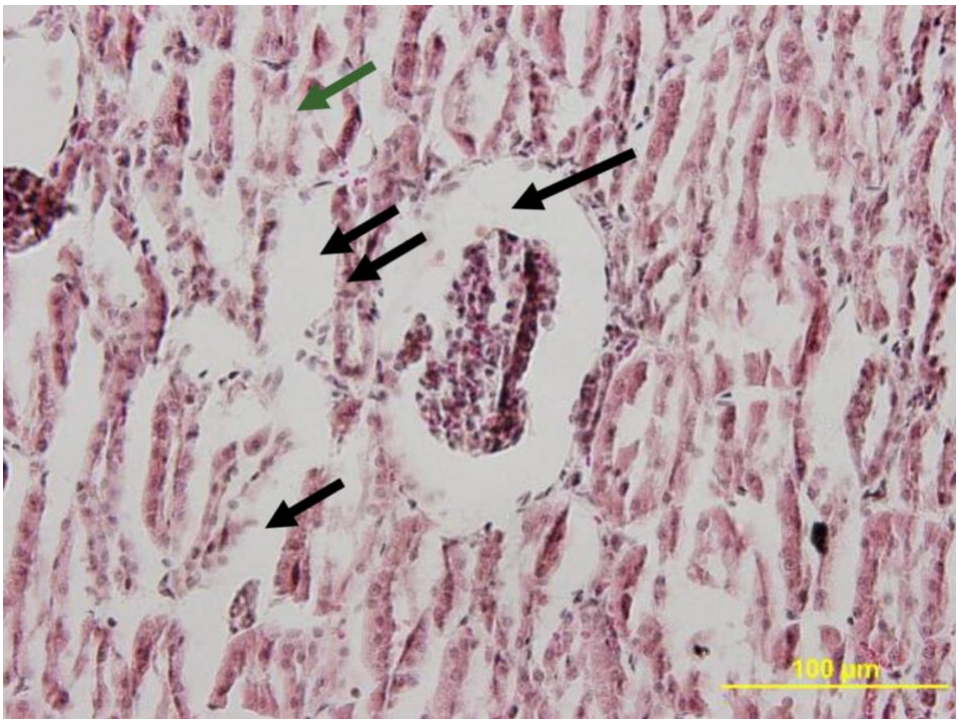

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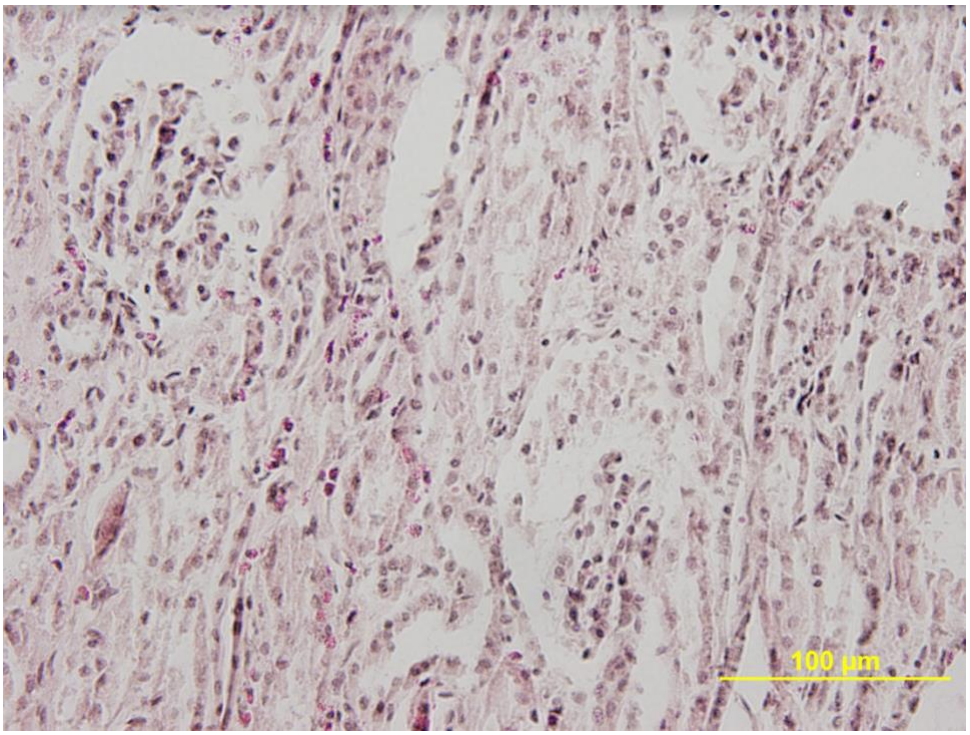

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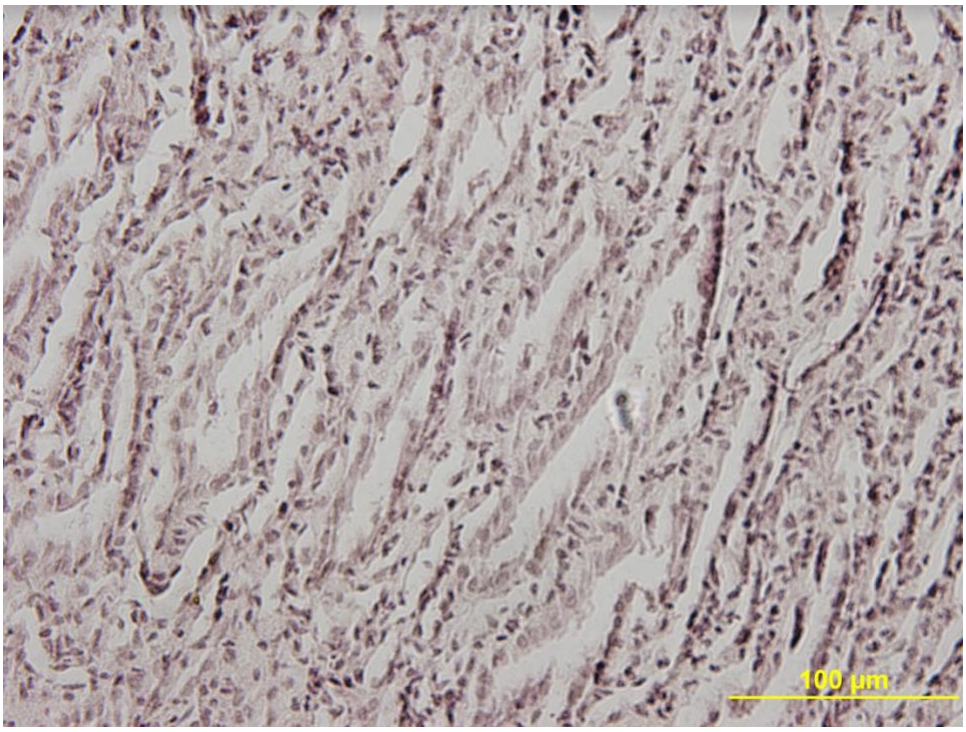

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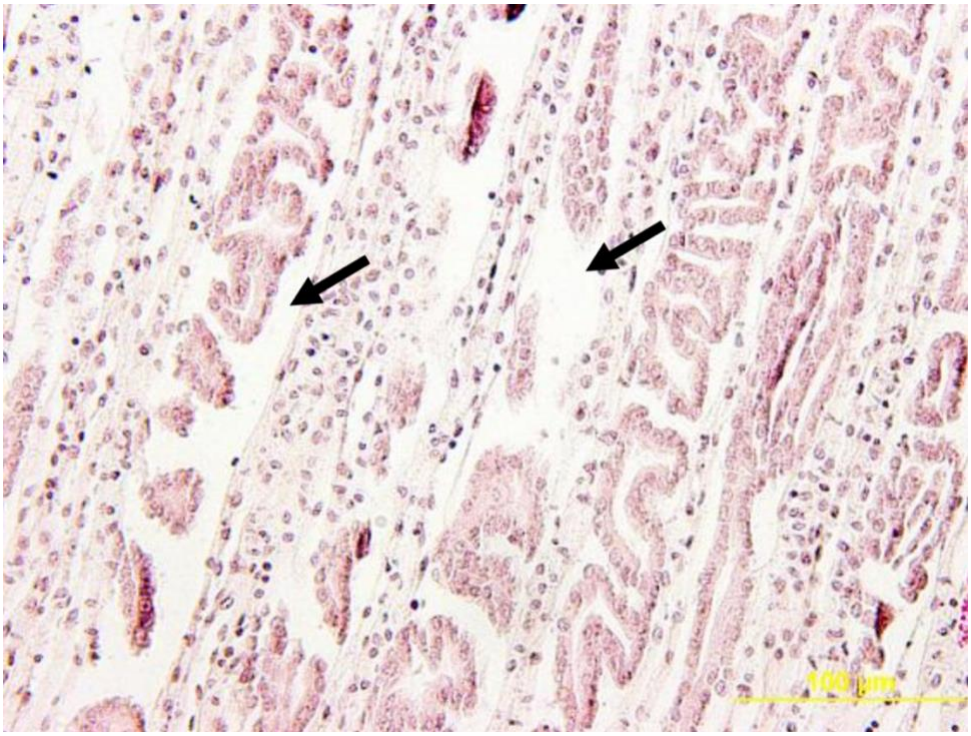

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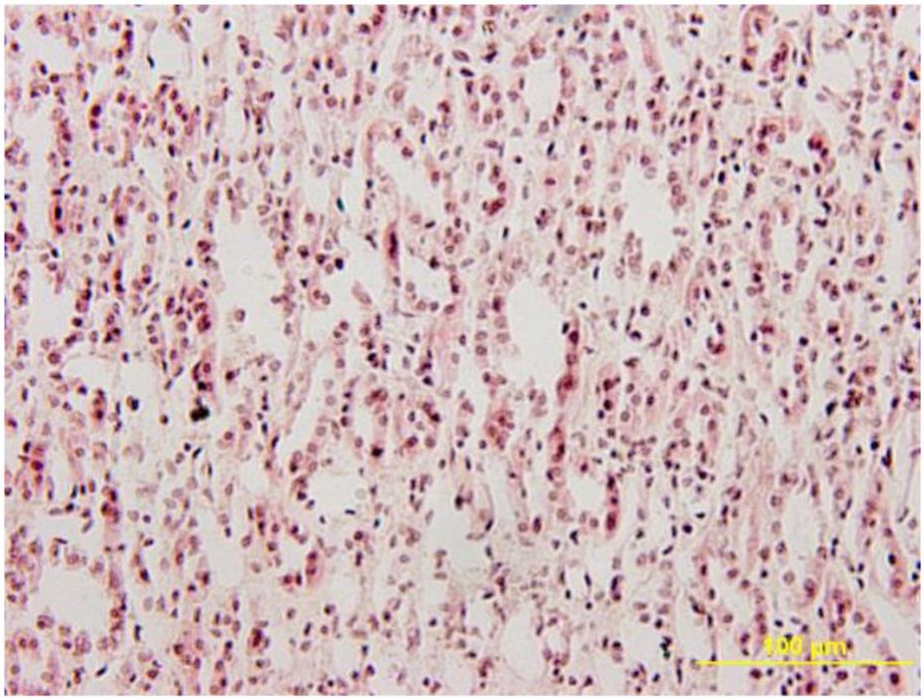

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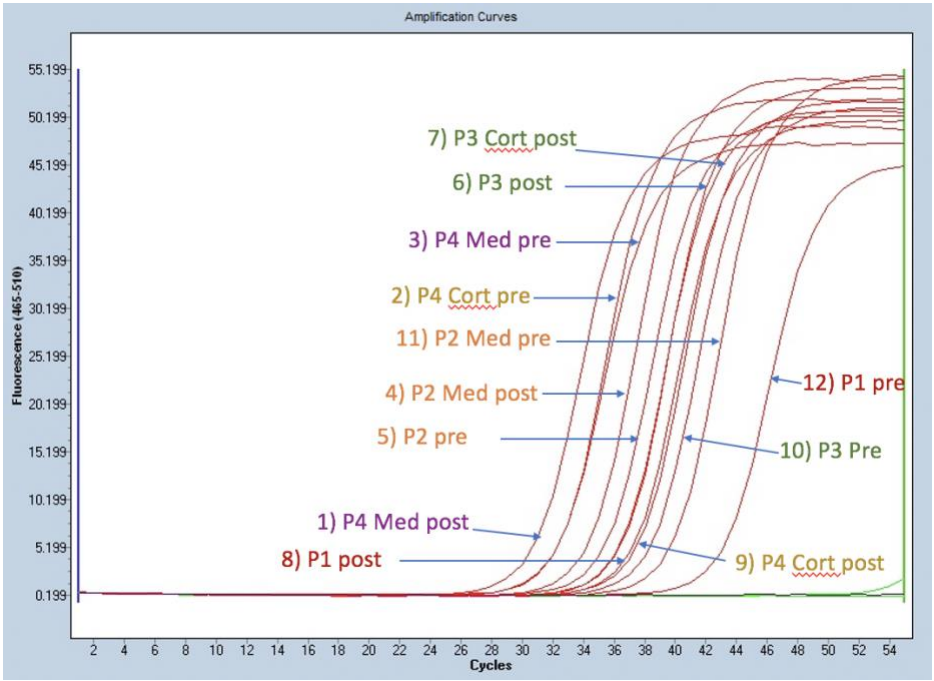

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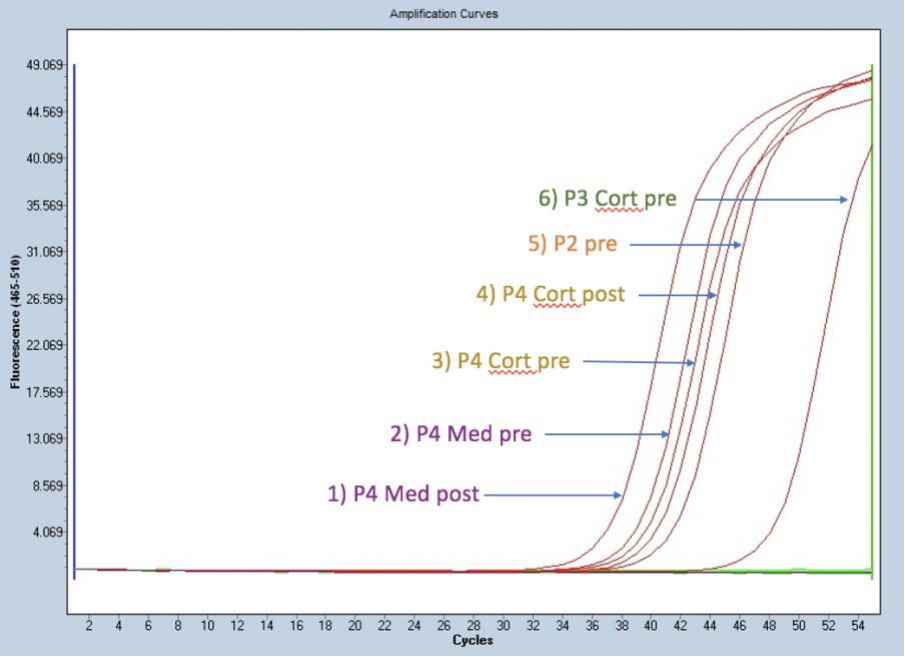

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Compound	Mol Wt (g/mol)	Concentration	Grams of solute			
			1 liter	2 liters	4 liters	6 liters
NaCl *	58.44	100 mEq/l	5.844	11.688	23.376	35.064
NaCl†	58.44	120 mEq/l	7.013	14.026	28.051	42.077
KCl	74.6	5 mEq/l	0.373	0.746	1.492	2.238
Na_2HPO_4	141.96	2 mEq/l	0.284	0.568	1.136	1.704
NaH_2PO_4	137.99	0.5 mEq/l	0.069	0.138	0.276	0.414
Glucose	180.2	20 mM	3.604	7.208	14.416	21.624
$MgCl_2$	203.3	10 mEq/l	1.0165	2.033	4.066	6.099
Na Pyruvate	110.04	20 mM	2.201	4.402	8.803	13.205
Hetastarch	200 kDa	5 g/dl	50	100	200	300

Table 1. *Compositions of kidney preservation solutions.* * Amounts of NaCl for pyruvate-enriched preservation solution. † Amounts of NaCl for non-pyruvate preservation solution, where an additional 20 mM NaCl is included to compensate for the absence of the sodium and total osmols contributed by Na pyruvate.

Chapter 1: Background and Rationale

In 1954, Dr. Joseph Murray and Dr. David Hume successfully performed the first kidney transplant on identical twins, for which they won the Nobel Prize in Medicine in 1990 [1]. Kidney transplant remains the only definitive treatment capable of curing end-stage renal disease (ESRD). Diseases that may result in ESRD include diabetes, hypertension, and autoimmune diseases. The massive increases in hypertension and diabetes have engendered a mounting epidemic of end-stage renal disease, in which renal function is no longer enough to sustain the patient. Although hemodialysis is a temporary measure, it is not a long-term solution, and it cannot completely replace the function of healthy kidneys. As the average time frame for receiving a transplant ranges from 3-5 years, patients are often maintained on dialysis for a period of time, which is not ideal (Figure 1). The longer the patients receive dialysis, the greater the risk for post-transplant morbidity, mortality, and graft loss [2]. Long-term dialysis can also result in vascular calcification and a fourfold increased risk for kidney cancer.

The demand for transplantable kidneys continues to mount, but the supply of living donor kidneys has plateaued (Figure 2). In 2018, 21,167 kidney transplants were performed, yet the waiting list still included 94,944 patients [3]. Of the transplants performed, 70% of the organs came from deceased donors. As the number of living donors has remained stagnant over the past ten years, expanding the deceased donor pool to include "extended criterion" donors are becoming increasingly critical. Specifically, kidneys donated after cardiac death (DCD), are a promising source, as cardiac arrest remains the leading cause of death in the United States. These organs are considerably underused largely due to their exposure to a period of warm ischemia (WI), in which the absence of blood perfusion post-mortem coupled with the cadaveric warm body temperature results in ischemia reperfusion injury (IRI). Additionally, the incidence of delayed graft function (DGF), a measurement of the time it takes a grafted kidney to resume sufficient function to sustain the recipient independently of dialysis, is markedly high in these organs – 25% compared to 1.6% in living donor grafts [4, 5]. In order to increase the utilization availability of transplant-quality DCD kidneys, measures need to be implemented to better preserve and/or restore renal tissue integrity before transplantation and improve graft survival.

The current standard for transport of kidney grafts is static cold storage (SCS), whereby the explanted organ is double bagged in ice prior to transplant in the recipient. Studies have

demonstrated the efficacy of hypothermic machine perfusion (HMP), a process by which explanted kidneys are cannulated and perfused with a pulsatile flowing solution under hypothermic conditions, in reducing the incidence of delayed graft function (DGF) compared to standard static cold storage (SCS) [6-8]. However, even kidneys preserved by HMP show appreciable rates of acute failure and DGF. To date, perfusate additives to further optimize the outcomes of HMP preserved kidneys have not been extensively studied. Although SCS is by and large more often used due to the low cost and ease of use, a large, international, randomized controlled trial demonstrated the superiority of hypothermic machine perfusion to SCS for one-year graft survival [9].

The main disadvantage of HMP is the limited supply of oxygen for metabolism. Oxygen is essential to generate ATP by oxidative phosphorylation, so the kidney undergoing HMP suffers ATP depletion that compromises the various ATP-consuming processes essential for renal function, of which the sodium-potassium ATPase is a prime example. Indeed, decreased Na^+,K^+ ATPase activity leads to increased intracellular sodium concentration, osmotic cell swelling, and cell death [10]. Also, when oxygen supply is limited, electrons cannot flow freely through the electron transport chain, since there isn't enough oxygen available to take on the electrons via the conversion of oxygen to water. Consequently, electrons build up within the electron transport chain. These built-up electrons react with the small amounts of oxygen still present, but in an uncoordinated fashion that generates superoxide, not water (Figure 3). Superoxide, itself a free radical compound, is the precursor of many other, more highly reactive compounds, including peroxynitrite, hydrogen peroxide and hydroxyl radicals [11]. The diminished oxygen supply leads to oxidative stress [12, 13]. In response to free radical-induced injury, resident fibroblasts in the kidney differentiate into cytokine-secreting myofibroblasts [14].

The glycolytic end product, pyruvate, has demonstrated efficacy with protecting the heart from ischemia-reperfusion injury, improving post-ischemic contractile function of the heart through its function as an antioxidant and energy substrate, and maintaining the glutathione redox state [15, 16]. In a porcine model of cardiopulmonary bypass, pyruvate-enriched cardioplegia augmented myocardial free energy of ATP hydrolysis [17], maintained redox state of the intracellular antioxidant, glutathione [18], suppressed activity of pro-inflammatory matrix metalloproteinase [19] and activated mRNA expression and synthesis of the anti-inflammatory cytokine, erythropoietin [15]. The anti-inflammatory and anti-apoptotic properties of

erythropoietin are potentially protective to tissues and organs threatened by ischemia-reperfusion.

The LifePort® Kidney Transporter machine perfusion is the most widely used machine perfusion device in North America. Machine perfusion has shown evidence of decreasing delayed graft function and primary nonfunction in kidneys donated after cardiac death [20-22].

Pyruvate has been shown to protect both the brain and heart and activate the production of erythropoietin in the brain and heart [15, 16]. Erythropoietin may mediate pyruvate's protective effects in post-ischemic organs, as erythropoietin has been shown to provide anti-apoptotic and anti-inflammatory benefits [23]. As the kidneys are by far the primary endogenous producers of erythropoietin, we are examining the impact of pyruvate-enriched preservation on erythropoietin expression and production by the machine-perfused kidney.

Chapter 2: Research Project

I. Specific Aims

Kidneys obtained from extended-criterion donors, including cardiac arrest victims, are susceptible to delayed graft function, ischemia/reperfusion injury, and primary nonfunction. The use of additives in preservation solutions have not been extensively investigated. Pyruvate has anti-inflammatory properties and induces erythropoietin production in the brain and heart. Erythropoietin is well understood to induce red blood cell production and has also demonstrated anti-apoptotic and anti-inflammatory effects. As kidneys are the primary endogenous producers of erythropoietin, we propose the addition of pyruvate will augment erythropoietin production by the machine-perfused kidney. Additionally, erythropoietin has been found to enhance tubular epithelial regeneration, exert cytoprotective effects, and promote renal functional recovery [24]. The extensive use of erythropoietin in large animal models has demonstrated benefits toward reducing renal ischemia-reperfusion injury and in a controlled non-heart-beating donor kidney model [25]. We propose the use of pyruvate-enriched perfusate will preserve renal tissue integrity, decrease cellular pro-inflammatory response, and increase renal anti-inflammatory response. Accordingly, this pilot project was conducted to lay the groundwork for testing the following specific aims:

Specific Aim 1: To test the hypothesis that pyruvate-fortified preservation solution preserves the tissue integrity of kidneys undergoing protracted machine perfusion

Specific Aim 2: To test the hypothesis that pyruvate-enriched machine perfusion suppresses pro-inflammatory cytokine production and augments renal production of the anti-inflammatory cytokine, erythropoietin.

II. Significance and Innovation

In 2016, the prevalence of end-stage renal disease (ESRD) rose to 726,331 cases in the United States, yet only 20,161 kidney transplants were performed [26] – a ratio of 36:1. It is estimated only 20% of patients in need of a transplant will ever receive one, and the median time to transplant is four years. The health disparities of kidney disease are staggering, as African Americans are three times more likely to develop ESRD than non-Hispanic whites. In order to increase the donor pool, research now focuses on optimizing the function of traditionally underused organs. These so-called "marginal organs" are obtained from extended criteria donors (ECD) with comorbidities including cardiovascular disease, hypertension, or diabetes. Kidneys from ECDs are particularly susceptible to delayed graft function (DGF) [26]. Evidence demonstrates that, when a grafted kidney takes longer than a week to resume function, the likelihood that it will remain functional after five years is only 50% [27]. On the other hand, kidneys donated after cardiac death (DCD) potentially could vastly increase the donor pool, as cardiac arrest remains the leading cause of death in the United States. However, cardiac arrest interrupts blood flow to the kidneys, subjecting them to a period of warm ischemia (WI) before their removal from the deceased donor, in which the absence of blood perfusion after cardiovascular death coupled with warm body temperature results in ischemia reperfusion injury (IRI). This project aims to develop a novel perfusion medium to better preserve these organs.

III. Materials and Methods

Animals

The studies of kidney preservation are being conducted in a large animal model, Yorkshire pigs, an excellent animal model of the human kidney. The porcine kidney is almost as

large as human and can be installed and perfused in the same organ preservation system. Like the human kidney, the porcine kidney has multiple lobes [28]. In general, porcine proteins have excellent homology with their human counterparts, so analytical kits for human proteins will likely be suitable for analyzing pig proteins [28]. Unlike human kidneys, in which configuration of the renal arteries vary considerably, over 95% of porcine kidneys have a single renal artery [28], which will simplify their cannulation for machine perfusion.

All procedures involving animals were approved by the Institutional Animal Care and Use Committee of the University of North Texas Health Science Center (protocol no. IACUC-2017-029). Kidneys were obtained from juvenile (ages 5-7 months) Yorkshire pigs weighing 40-60 kg Prior to surgery, pigs were fasted overnight and sedated with TELAZOL (100 mg/kg) and xylazine (100 mL/kg). Pigs were intubated and mechanically ventilated with 2–3% isoflurane. To mimic DCD, pigs were subjected to cardiac arrest induced by rapid electrical pacing, and kidneys were explanted after a 60-minute period of warm ischemia. The renal arteries were then cannulated and flushed with 3-4 liters of either the control or the pyruvate-enriched organ preservation solution (Table 1) until the IVC drainage was clear of blood.

Hypothermic Machine Perfusion

The explanted left kidney served as a pre-perfusion control for the effects of hypothermic machine perfusion. The right kidney was cannulated, installed in the LifePort® organ perfusion system (Organ Recovery Systems) and continuously perfused with pyruvate-enriched or control University of Wisconsin solution for 72 h. The right kidney was chosen because its longer renal artery allows for better cannulation. The LifePort® system maintains a constant pressure of 30 mmHg by adjusting the flow rate and resistance. Perfusate temperature was maintained between 2-3°C. The contents of the control and pyruvate-enriched preservation solutions are listed in **Table 1**. During machine perfusion, the perfusate was sampled periodically via a sampling port, for measurements of pro-and anti-inflammatory cytokines and energy metabolites.

Histology

Renal cortex and medulla samples were fixed in 10% formaldehyde, paraffin-embedded, sectioned into 5 μm sections using a microtome, and then stained with hematoxylin and eosin Sigma-Aldrich (St. Louis, MO). The histological sections were examined by a light microscopy.

Reverse Transcriptase PCR

Reverse transcriptase polymerase chain reaction (RT-PCR) was utilized to measure the mRNA abundance of pro- and anti-inflammatory cytokines. RNA extraction followed by the addition of reverse transcriptase will convert RNA samples to cDNA, which will be analyzed using an ABI Prism 7000 (Thermo Fisher) sequence detection system.

Limitations

The use of juvenile Yorkshire pigs with no known comorbidities as a source of deceased donors is in contrast to the real-world quality of deceased donor kidneys, as these grafts generally come from much older, extended criteria donors with comorbidities including hypertension, diabetes, and heart disease.

Additionally, the LifePort machine is limited in that we don't know the time-course by which the concentration or partial pressure of oxygen declines over the course of the 72-hour perfusion. This may introduce reactive oxygen species and consequently oxidative damage to the cells. In future studies, the addition of free radical scavengers such as reduced glutathione and/or allopurinol could likely minimize the incidence of cellular damage.

IV. Results

Kidneys perfused without pyruvate-enriched solution demonstrated notable swelling and edema 72 hours after machine perfusion (Figures 4-6). Additionally, the flow rates reported by the hypothermic machine perfusion apparatus on average was lower for the control perfusate group compared to the pyruvate-enriched group. This edema experienced by the control kidneys likely resulted in the observed dampened flow rate.

Histology

Cortex and medulla renal samples prior to perfusion show no edema, epithelial sloughing, or tubular dilation (Figures 2-7). Cortex from the kidney maintained by HMP without pyruvate for 72h showed extensive interstitial edema surrounding the glomerulus and kidney tubules (black arrows), sloughing (green arrow), and loss of cellular definition. Cortex from the kidney preserved for 72 h with pyruvate-enriched HMP shows less edema, although sloughing is

present. The medulla of the 72h HMP + pyruvate kidney shows less epithelial sloughing and more organized tubule architecture compared to the medulla from the kidney maintained by 72h HMP without pyruvate.

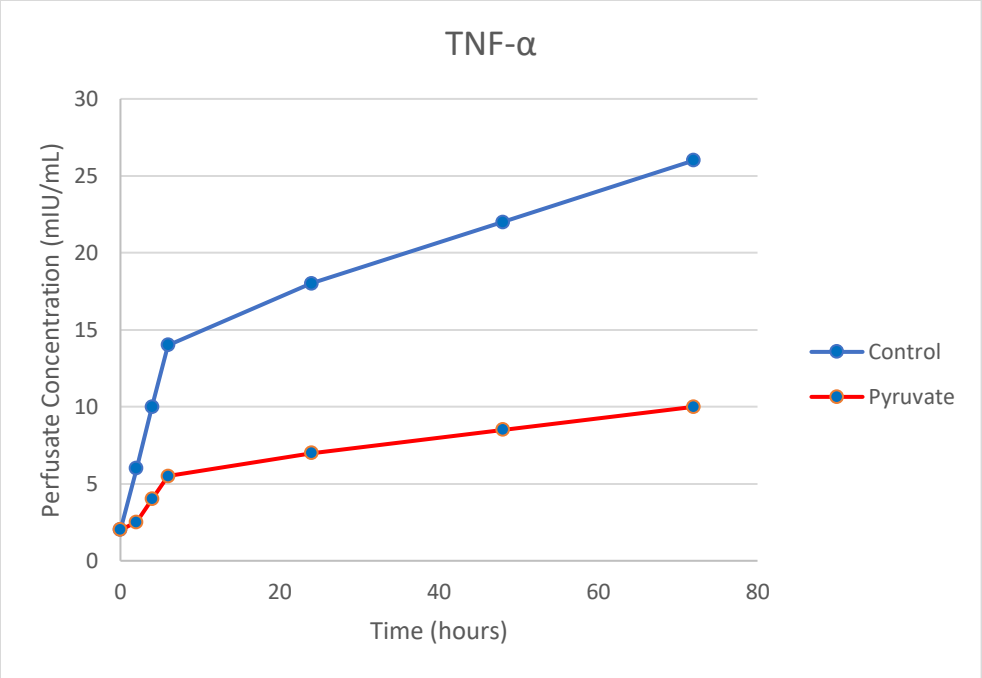
Reverse Transcriptase PCR

The abundances of mRNA encoding TNF- α in renal cortex and medulla were noticeably increased in the kidneys perfused with control preservation solution compared to renal cortex and medulla of the kidney perfused with pyruvate-enriched solution (Figures 11). There was no noted differences between renal cortex and medulla samples, either pre- or post-perfusion. Erythropoietin (EPO) mRNA abundance in the medulla was increased after pyruvate-enriched perfusion. Interestingly, the abundance of EPO mRNA slightly decreased after pyruvate treatment in the renal cortex.

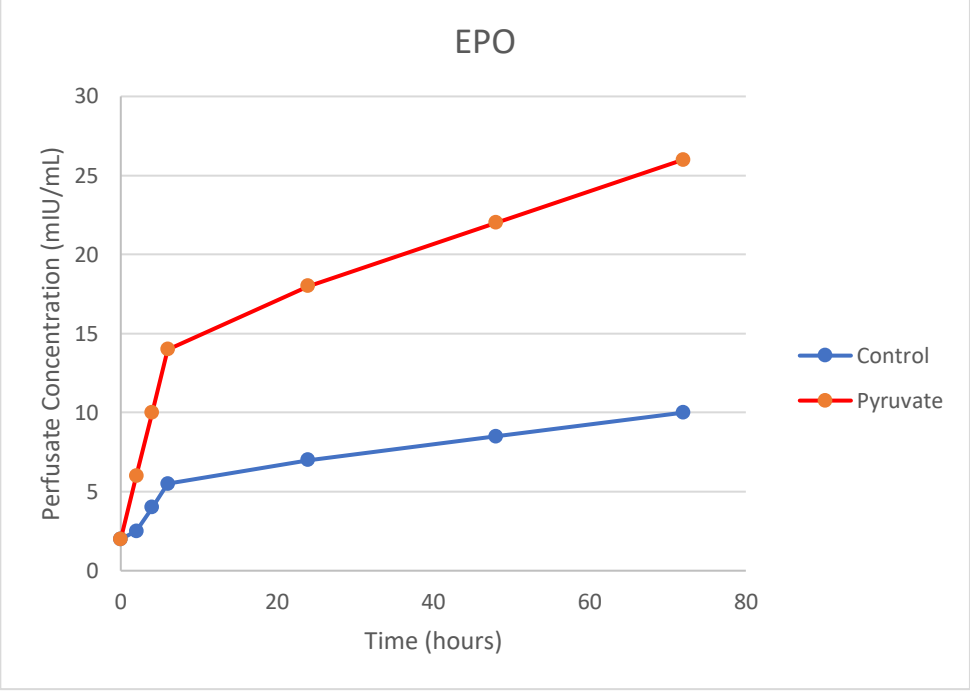
V. Discussion

Our preliminary analysis show that enrichment of pyruvate may be beneficial in HMP. Pyruvate-enriched HMP kidneys displayed less edema, decreased epithelial sloughing, and improved retention of tubule architecture. Additionally, the dampened increase in pro-inflammatory cytokine concentration and more pronounced rate of increase of erythropoietin concentration lend further evidence to the protective effects of pyruvate. This pilot study supports the hypothesis that pyruvate-enriched HMP may afford robust renoprotection to improve function and reduce renal allograft failure.

Next steps in the investigation include running the ELISA assays. We expected the concentrations of pro-inflammatory cytokine TNF-α to be greater in the control samples compared to the pyruvate-enriched samples (Figure 8). Conversely, we expected anti-inflammatory cytokine IL-10 and erythropoietin to be greater in pyruvate-enriched samples (Figures 9 and 10). The expected trends we hypothesize are summarized in the figures below.



Example 1. TNF-alpha concentration from perfusate samples over a 72-hours of hypothermic machine perfusion.



Example 2. Erythropoietin (EPO) concentration from perfusate samples over a 72-hours of hypothermic machine perfusion.

In future studies, we would also like to analyze energy metabolism (ATP, ADP and inorganic phosphate) and glutathione redox state (i.e., [GSH]/[GSSG]) in the renal cortex and

medulla. The long-term objective of this work, if pyruvate-enriched preservation solution is indeed superior to conventional solution, is to test the post-transplant performance of kidneys which had been perfused with pyruvate-enriched vs. control solution.

VI. Summary and Conclusions

In these experiments we investigated the effects of pyruvate-enriched preservation solution on porcine kidneys exposed to a period of warm ischemia prior to organ explant. Renal tissue integrity was assessed through histological evaluation, and pro- and anti-inflammatory cytokine expression were evaluated through ELISA assays and reverse transcriptase PCR. The results of this pilot study permits the following conclusions:

Porcine kidneys are an excellent preparation for examining the impact of hypothermic machine preservation and of additives like pyruvate on tissue architecture, cytokine expression and release, and other measures of inflammatory damage to the isolated organ.

For the first time, pyruvate was tested as an additive to machine preservation solution. Although preliminary, these first experiments suggest pyruvate may afford better preservation of renal structure, possibly by suppressing pro-inflammatory cytokines.

Even after completing the Medical Sciences program, this year of research has been one of the most challenging and rewarding experiences. My level of growth and understanding of the work required to conduct quality research and advance the body of knowledge in any given field has exceeded my expectations prior to beginning this research year. It's been a thrilling experience to delve into the scientific literature, begin to understand the questions that need answering, and to work with scholars and experts in the field to execute a research experiment. I've learned how to be a good manager through this process, as I found it essential for organizing when and how each experiment would be conducted. The wonderful team of colleagues and researchers I had the opportunity to work with was an invaluable experience as well. I'm thrilled to be attending medical school next year at this institution and hope I am able to participate in future studies with this amazing group of individuals.

VII. **REFERENCES**

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