ENHANCING METABOLISM OF DONOR HEARTS FOR CARDIAC TRANSPLANTATION

BY

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Presented to the Faculty of the Graduate School of

The University of Texas Southwestern Medical Center at Dallas in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY IN BIOMEDICAL ENGINEERING

THE UNIVERSITY OF TEXAS SOUTHWESTERN MEDICAL CENTER AT DALLAS

MAY 2012

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ACKNOWLEDGEMENTS

I would like to acknowledge the Biomedical Engineering Department at the University of Texas at Arlington and UT Southwestern Medical Center, and the faculty and staff of the Department of Cardiovascular and Thoracic Surgery at UT Southwestern Medical Center for the pursuit of improvement of treatments and quality of life. Without research grants and funding from South Central Affiliate of the American Heart Association, this project would not be possible. Furthermore, the Organ Transport Systems, Frisco, TX, instrumental in the development and advancement of the device, is acknowledged.

I would personally like to thank Dr. Michael Jessen, MD for adding me to his stellar team of researchers and residents, Matthias Peltz, MD and LaShondra West, MS. I would also like to acknowledge the research contributors of Organ Transport Systems, Tom Franklin, Howell Warner, Ken Merte, and the rest of able staff that are truly shifting the way we think about organ transplantation. I would also like to recognize the engineering team from the RealTime Group, Marshall Wenrich, Gerald Miles, Don Hurd, Mike Polcari, and Rob Davidson, in addition to the contributing companies that have developed this device. This dissertation has also received considerable contributions from the late Dr. Julian Peterson, a renowned biochemist at UT Southwestern. Finally, I would like to thank my family, Lindsay, Mom, Dad, Andrew, and Emily, you all are my inspiration and without your love and support I would not be the man I am today.

March 8, 2012

ABSTRACT

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Congestive heart failure is a major health problem that affects millions of patients and represents a major cost to the health care system. Heart transplantation remains the most effective treatment for end-stage heart failure. However, transplantation can only be offered to a small fraction of patients with this disease due to the inadequate supply of available organs. A major factor limiting the donor pool relates to the poor ischemic tolerance of hearts removed and stored prior to implantation. Currently used heart preservation techniques involve storage of the organ near 0ºC to minimize donor organ metabolism and limit ongoing deterioration of the organ during storage prior to implantation (static storage).

A new technique (machine perfusion preservation) is under development and works by perfusing the organ with oxygenated preservation solution. This method may extend the donor ischemic interval and may permit utilization of less-than-optimal donor hearts - thus expanding the donor pool for cardiac transplantation. This strategy has been used successfully for kidney transplantation in the clinical arena and initial experimental evidence suggests that it appears promising for preserving hearts as well. However, optimal myocardial perfusion conditions and techniques have not yet been defined. In fact, available preservation solutions do not contain substrates that are metabolized by the heart and available perfusion devices do not reliably deliver oxygen and substrates to the capillary bed. We believe that optimizing machine perfusion conditions in a manner designed to increase cardiac metabolism will improve the support of cellular processes, improve donor heart preservation, and potentially reverse

myocardial injury sustained during the events leading to brain death. We have examined methods to optimize metabolism in two ways.

First, we identified substrates which, when added to the circulating preservation solution, maximize myocardial metabolism. Our initial experiments were conducted in a rat model of *ex vivo* perfusion preservation. Hearts were perfused with cold, oxygenated organ preservation solution (University of Wisconsin Machine Perfusion Solution) supplemented with carefully-selected candidate carbon-13 (^{13}C) labeled myocardial substrates. Magnetic resonance spectroscopy (MRS) was used to define the substrate or substrate combination that maximized oxidative metabolism during the storage interval.

Second, conventional strategies of heart machine perfusion preservation have to date utilized antegrade coronary perfusion whereby the preservation solution is delivered into the ascending aorta. Prior studies have demonstrated that certain perfusion conditions allow the aortic valve to become incompetent, leading to reduced perfusate delivery and poor myocardial preservation. We proposed an alternate strategy that exploits perfusion through the coronary sinus (as is currently utilized for cardioplegia delivery during cardiac surgery) to overcome this limitation of antegrade delivery. We defined optimal retrograde coronary perfusion parameters in a well established canine model of heart transplantation. Regional perfusate flow delivery and high energy phosphate levels were determined over a range of coronary sinus flow rates. We subsequently compared antegrade and retrograde machine perfusion preservation techniques (and to conventional static storage) in the canine transplantation model to define the optimal preservation strategy. In these experiments myocardial function was quantified using load-independent technique, and metabolic state was measured using magnetic resonance spectroscopy to define substrate utilization parameters.

An inherent limitation to transplanting hearts, especially those stored for long intervals, is predicting the suitability of the organ prior to implantation. In our human heart experiments,

we applied a magic angle spinning (MAS) magnetic resonance spectroscopy technique to quantify the metabolic state of the heart prior to implantation. In these studies, after completion of the storage interval, MAS was performed on micro-biopsies of left ventricular tissue. Data from these experiments have allowed us to define optimal machine perfusion techniques to allow safe application of this technique into the clinical arena. This offers the potential to significantly expand the pool of useable donor hearts for transplantation. Findings in the proposed experiments may have wider applications in preserving myocardium during other (non-transplant) forms of cardiac surgery and in improving machine perfusion preservation of other organs

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PEER REVIEWED PUBLICATIONS

1. Peltz M, Cobert ML, Rosenbaum DH, West LM, Jessen ME. Myocardial perfusion characteristics during machine perfusion for heart transplantation. *Surgery* 2008;144(2):225-32.

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5. Cobert ML, Peltz M, West LM, Merritt ME, Jessen ME. Glucose Is an Ineffective Substrate for Machine Perfused Donor Hearts. *Journal of Surgical Research*. In Press.

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1. Cobert ML, Peltz M, West LM, Jessen ME. Maintenance of Human Heart Oxidative Metabolism after 12 hour Perfusion Preservation. *J Heart Lung Transplant* 2009;28:S126-7.

2. Cobert ML, West LM, Jessen ME, , Peltz M. Retrograde Coronary Sinus Perfusion: An Alternate Approach for Machine Perfusion Preservation of Donor Hearts for Transplantation. J Heart Lung Transplantation 2011;30(4), S198.

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CHAPTER 1

INTRODUCTION

1.1 Heart Function and Metabolism

The heart is the body's pumping station, supplying deoxygenated blood to the lungs and oxygenated blood to the body. The anatomy of the heart consists of four chambers, two atria and two ventricles, represented in Figure 1.1. Oxygenated blood returns from the lungs, via the pulmonary veins, into the left atrium then passes to the left ventricle and is ejected through the aorta and the arterial system. Deoxygenated blood returns into the right atrium, via the superior and inferior vena cava, then flows to the right ventricle and then circulates in the lungs for oxygenation.

Heart tissue is comprised of involuntary striated muscle cells known as cardiac myocytes. Like all muscle cells, cardiac myocytes rely on blood to supply oxygen and other nutrients as well as for removal of carbon dioxide, a process that occurs by the coronary arteriovenous system. Cardiac myocytes are highly resistant to fatigue due to their large number of mitochondria, enabling continuous aerobic metabolism. The main energy unit in the cell is adenosine triphosphate (ATP). This phosphorylated nucleotide provides the chemical energy for contractile processes, maintaining ion transport, and other aspects of basal metabolism by hydrolyzing into adenosine diphosphate (ADP) and inorganic phosphate ($P_{i)}$, releasing the free energy that can be utilized for these chemical reactions. The heart is able to utilize a wide variety of fuels to generate ATP. Under basal aerobic conditions, a primary energy source is fat (free fatty acids or triglycerides), followed by carbohydrates and other amino acids and ketone bodies.

In the initial steps of catabolism larger energy sources are broken down into their individual components, ultimately becoming acetyl-CoA. The first steps yield only a few ATP molecules. Most ATP production occurs from further catabolism of acetyl-CoA to CO_2 and H_2O, a process known as the citric acid cycle, which produces energy rich molecules NADH and $FADH_2$. Further catabolism through oxidative phosphorylation oxidizes these molecules by passing electron pairs to generate ATP from ADP

and P_i. In the end, oxygen is terminal electron acceptor, and the molecular basis for oxygen requirements.

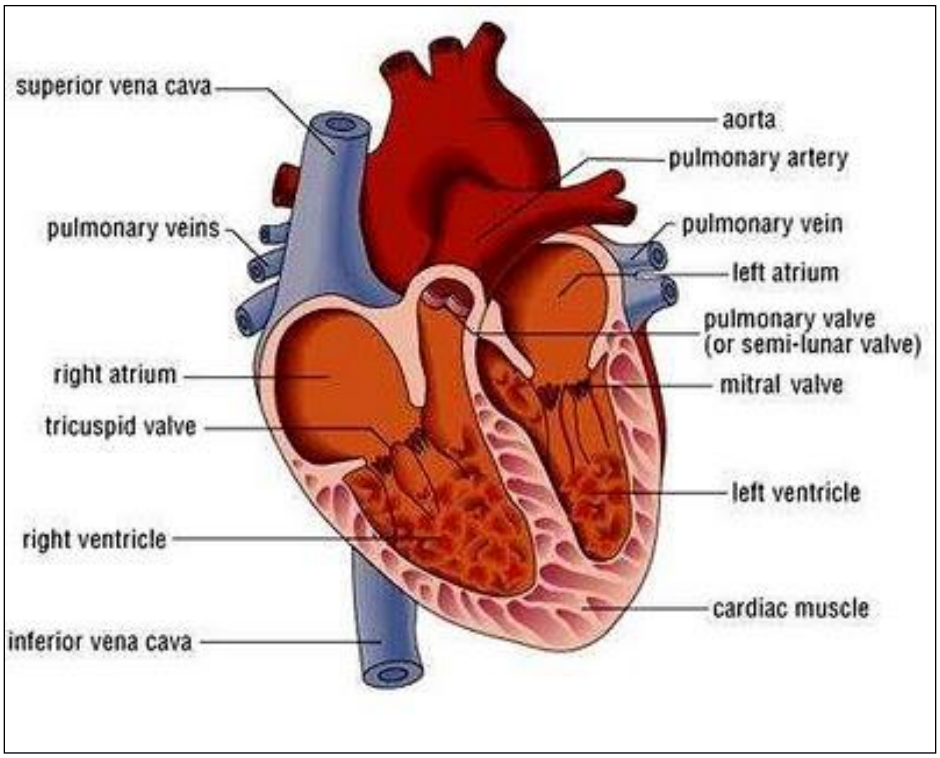


Figure 1.1 The heart consists of four chambers: left and right ventricles (LV, RV) and left and right atria (LA, RA). Desaturated blood enters the right atrium through the inferior and superior vena cava (IVC, SVC) which is then pumped through the right ventricle and into the pulmonary vasculature. Oxygen rich blood then returns to the left atrium and subsequently to the left ventricle which ejects the blood through the aorta to supply the entire body. The heart muscle receives its blood supply from the coronary arterial system.

1.2 The Citric Acid Cycle

The citric acid cycle (TCA cycle) is the main pathway of aerobic energy production. Acetyl-CoA, which is can be generated from pyruvate from the glycolytic pathway or directly from fatty acid degradation or ketone oxidation, is the substrate that begins the cycle. The oxidative carboxylation of pyruvate to form acetyl-CoA is catalyzed by the pyruvate dehydrogenase (PDH) complex. The subsequent steps in the cycle complete the oxidation of acetyl-CoA and contribute to the energy production process by generating reduced electron carriers, see Figure 1.2.

The reactions that comprise the TCA cycle have interactions with other metabolic pathways, and TCA cycle intermediates may be removed to contribute to these pathways. For example, malate may be decarboxylated to pyruvate, which can then be used to synthesize glucose. Processes that replete intermediates lost in this manner are termed *anaplerosis*. The principal anaplerotic pathways in myocardial metabolism include pyruvate carboxylation, forming malate or oxaloacetate from pyruvate, and propionate carboxylation, forming succinate from propionate. Other suggested pathways may involve transamination of the amino acids glutamate and aspartate through α-ketoglutarate and oxaloacetate, respectively. These anaplerotic reactions are believed to maintain TCA cycle intermediates to prevent an eventual decline in TCA cycle activity.

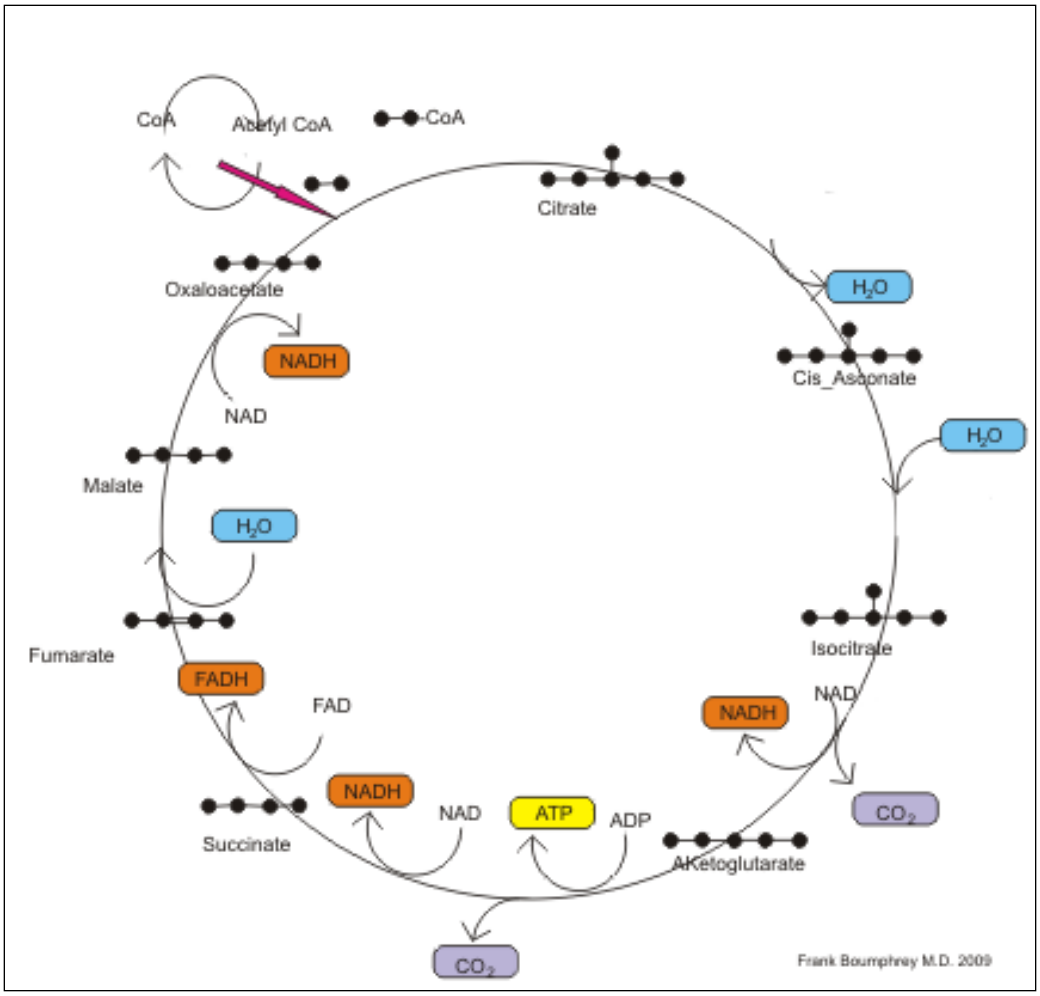


Figure 1.2 Tricarboxylic acid cycle. Energy production from glucose catabolism through glycolysis to pyruvate, from pyruvate decarboxylation to acetyl-CoA, and from oxidation of acetyl-CoA within the TCA cycle. Each glucose molecule yields two acetyl-CoA units. Therefore (2*ATP + 4*NADH) are produced outside the TCA cycle, whereas (6*NADH + 2*$FADH_2$ + 2*GTP) are produced within the TCA cycle.

1.3 Heart Transplantation Overview

Over the past half century organ transplantation has gained much attention due to positive clinical results. However, the fact remains that the vital organs, the heart, lungs, liver, and kidneys, have a relatively short allowable period of ischemic time in which to complete the transplant operation. In general, the acceptable time for organ transport by simple immersion method is less than 6 hours. The United Network for Organ Sharing (UNOS) standard for heart transport is a 500 mile radius from the donor site.[1] Heart transplantation dates back to 1967, when Dr. Christaan Barnard of Cape Town, South Africa successfully transplanted a heart from a deceased 25 year old woman into a 55 year old man at the same institution. The recipient only lived for 18 days after the transplant, but the surgery quickly gained notoriety and inspired much developmental work. Due to limited knowledge about immunocompatability, early patients were only able to survive a limited amount of time. Research at Columbia University Medical Center into immunosuppressive drugs, cyclosporine in particular, increased donor acceptance from days to years.[2] Furthermore, in 1984 the first successful pediatric heart transplant was performed at Columbia. The patient subsequently received a second transplant, in 1989, and with the aid of immunosuppression, is currently able to function normally. However, immunocompatability issues remain and require careful clinical attention.

The donor pool is markedly inadequate due to the ever increasing recipient demand. Current statistics from UNOS indicate that there are approximately 4000 registered recipients awaiting transplantation yet fewer than 2500 heart transplants are performed yearly due to the limited donor supply.[1] This disparity only increases, due to the time needed for graft assessment and tissue typing, and the limitations of transport time with existing procurement and storage techniques. For this and other reasons an improved method of long term heart preservation during transport from donor to recipient is required.

1.4 Limitations of Current Technology

The current approach for heart preparation for transport and transplant is similar to that of other organ preservation methods. The heart is arrested with a cardioplegic solution, and then immersed into a container filled with cold saline solution. The container is then placed into a cooler filled with ice, and allowed to cool down to approximately 0º C. As stated above this method of simple immersion greatly limits the time a usable organ can be transported. The primary concern with this treatment is the damage incurred by the myocardium, caused first by a lack of oxygen, which leads to the buildup of metabolic end products and ultimately, apoptotic death of myocytes. Once the tissue is set into an ischemic state, aerobic metabolism slows to a halt and anaerobic metabolism predominates. Compared to aerobic metabolism, which yields 36 ATP per glucose molecule, the net yield of ATP from of anaerobic metabolism in the form of ATP, is only two molecules.[3,4] Without a constant oxygen supply, tissue ATP production diminishes, and lactate levels increase dramatically due to catabolism of intracellular glycogen stores to provide more glucose for anaerobic metabolism. The tissue lactate buildup causes a condition known as lactic acidosis and the cells quickly fatigue, see Figures 1.3 and 1.4.

At the time of heart arrival at the recipient site the recipient has been placed on cardiopulmonary bypass and his/her diseased heart has been removed. The donor heart is then removed from the cooler and brought into the sterile operating field. The heart is then reattached (transplanted) by anastomoses of the left atrium, right atrium (or both vena cavae separately), pulmonary artery, and aorta, Figure 1.5. Removal of the cross clamp then restores normothermic blood to the transplanted heart to not only rewarm the heart, but to remove the metabolite burden.

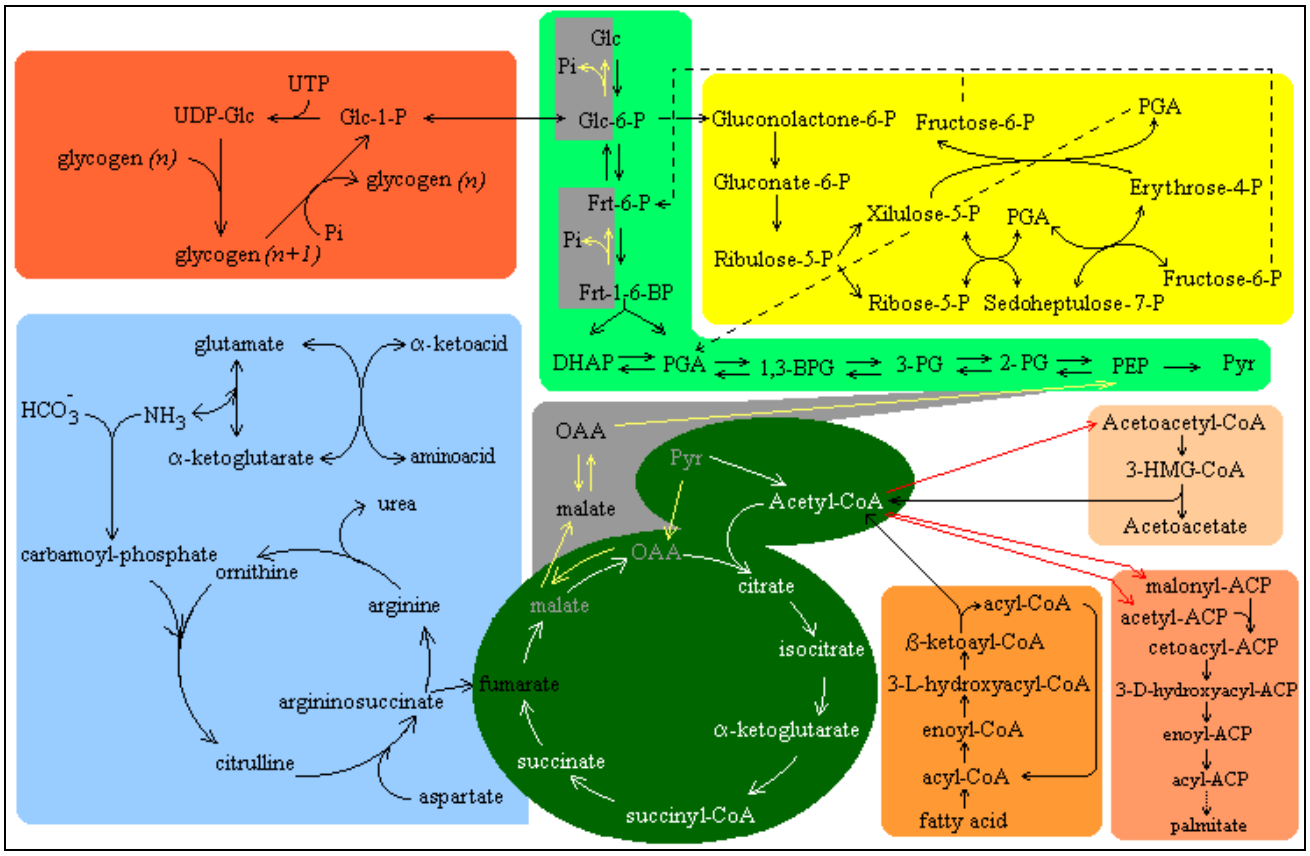


Figure 1.3 Aerobic metabolic pathways[3]. Aerobic metabolism utilizes oxygen to convert nutrients (carbohydrates, fats, protein) into ATP. There are three primary processes in aerobic metabolism, the TCA cycle (dark green), electron transport (not pictured), and oxidative phosphorylation (not pictured). Other pathways seen here contribute to the TCA cycle

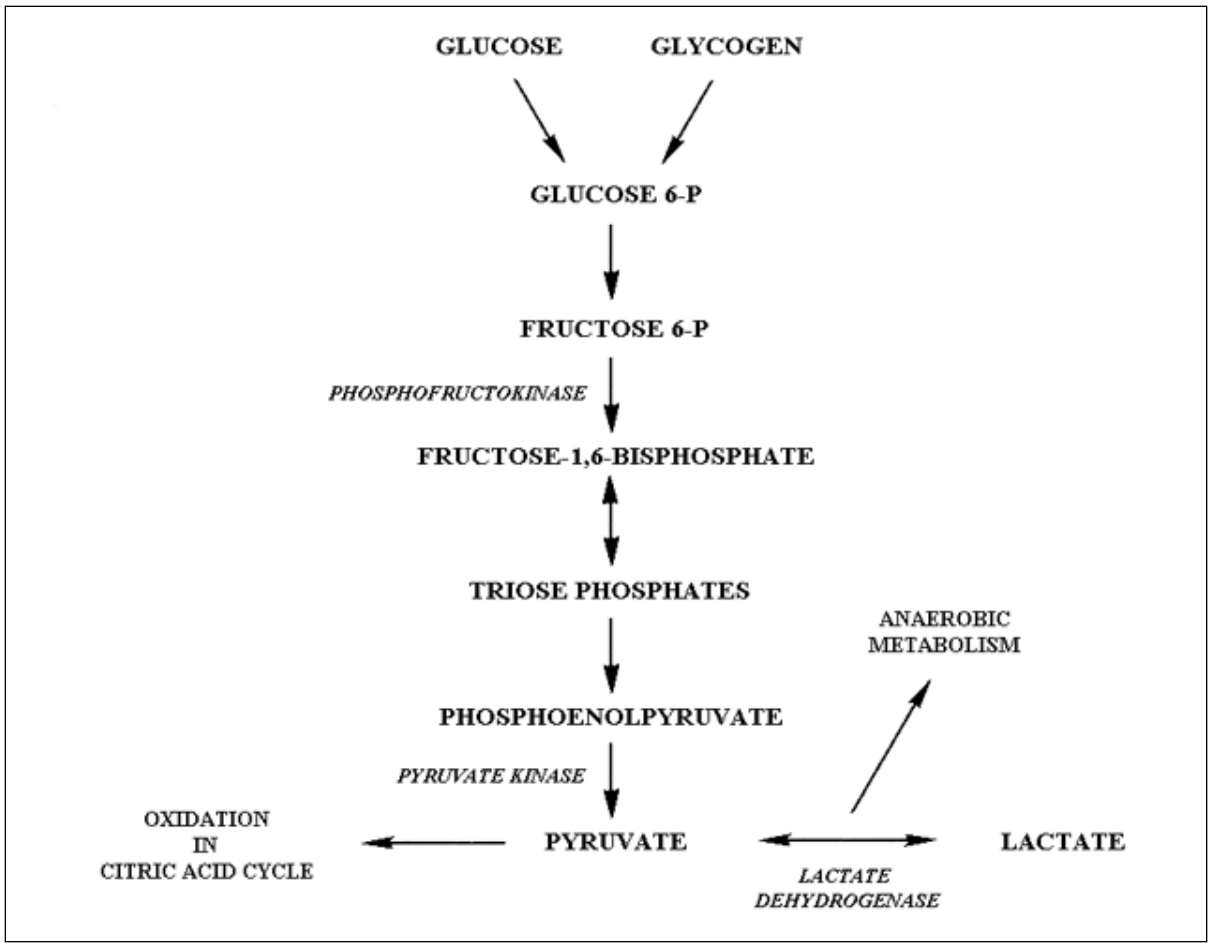


Figure 1.4 Anaerobic metabolism[4]. The anaerobic pathway also called anaerobic glycolysis creates ATP exclusively from carbohydrates with lactate being the end-product.

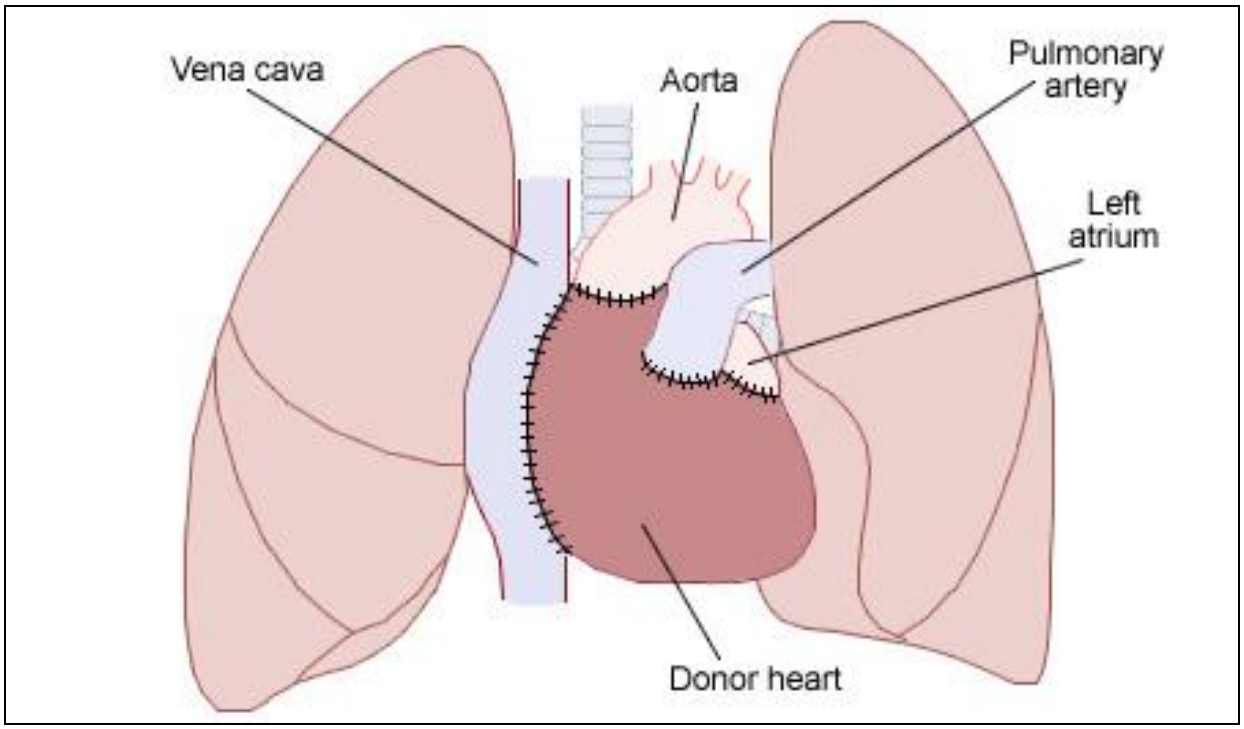


Figure 1.5 Heart transplantation: Anastomoses performed in the following order – Left atrium, right atrium, pulmonary artery, and aorta.[5]

While this approach is logical, the cold anaerobic donor tissue must come into contact with the warm oxygenated recipient blood, with an increased risk for reperfusion injury. As it is now well known, the direct effects of reperfusion injury are inflammation and oxidative stress.[5] In prolonged ischemia, hypoxanthine is formed as breakdown product of ATP metabolism. The enzyme xanthine dehydrogenase acts in reverse, characteristically like xanthine oxidase, as a result of the higher availability of oxygen. This oxidation results in molecular oxygen being converted into highly reactive superoxide and hydroxyl radicals. Xanthine oxidase also produces uric acid, which may act as both a pro-oxidant and as a scavenger of reactive species such as peroxinitrite. Excessive nitric oxide produced during reperfusion reacts with superoxide to produce the potent reactive species peroxynitrite. Such radicals and reactive oxygen species attack cell membrane lipids, proteins, and glycosaminoglycans, causing further damage. They may also initiate specific biological processes by redox signaling.[6] The net effect is a significant risk for damage to the donor heart, particularly if storage intervals are prolonged. If damage is too great the donor heart may fail. Primary graft failure remains a significant problem in transplant centers today.

1.5 Potential Benefits of Machine Perfusion

The theoretical benefits of a machine perfusion approach to heart transport for transplant include: increasing the donor-recipient transport time (thus the transport radius), and improved early graft function, potentially reducing ICU time, and enhancing long term outcome. Quantitatively, a reduced ischemic burden will reduce myocardial damage and the potential for reperfusion injury. In addition, the potential for increased aerobic metabolism and washout of metabolites during perfusion should aid in improved graft function.

By increasing the donor-recipient radius, more transport time is allowed which in turn allows a potentially higher priority patient to receive the heart. Pumping the heart with an oxygenated solution mimics the body's natural perfusate, blood. If the tissue is able to maintain aerobic metabolism during machine perfused transport, the likelihood of myocardial damage is reduced.[36] Another potential benefit to this method may be to increase the donor pool through the inclusion of marginal and non-heart beating

donors. The ability to provide nutrients to these hearts en route has been noted, with the potential to further expand the transport and storage interval.[7]

1.6 Previous Experimental Investigations

There have been several studies exploring the benefits of machine perfusion devices; however, there has not been a consensus on an ideal approach. There has been much debate over which preservation solution (intracellular or extracellular composition) works best, and which modifications or additives should be implemented. The optimal heart preservation solution remains elusive with a recent survey of transplant programs in the United States revealing the use of 167 different solutions in 147 transplant centers.[8] A review of literature of experimental studies of machine perfusion similarly reveals little consistency in the approach, as there appears to be a wide array of solutions used, a wide variation in devices and perfusion conditions, and even a lack of consensus on animal models. Different studies may or may not include transplantation or reperfusion of the stored heart, and have reported a wide range of outcome variables, including early reperfusion graft function and markers of myocardial metabolism. The most discussed issue in the reviewed studies was tissue water buildup (myocardial edema) with varying results based on perfusion conditions and preservation solution selection.

The nearly 50 studies available in the literature can be subdivided into three general categories:

1. Functional/Metabolic effects of perfusion preservation of animal hearts
2. Perfusion preservation and resuscitation of marginal hearts
3. Model development using various *ex vivo* studies, such as air persufflation

A review of these studies is provided in Appendix A.[10-72]

There has also been discussion as to which direction of perfusion is most effective.[9] Antegrade perfusion is delivery via the aortic root or via the innominate artery to the coronary arteries, and retrograde perfusion is the delivery to the coronary arteries through the coronary sinus, located on the posterior surface of the heart, Figure 1.6.

Studies by Aizaki et al suggest that a retrograde perfusion allows for better nutrient flow, whereas studies by Ferrera et al argue for antegrade flow under microperfusion conditions. Though varied in design, one theme remains consistent: all articles demonstrated beneficial effects on long term preservation with perfusion preservation.[10-14]

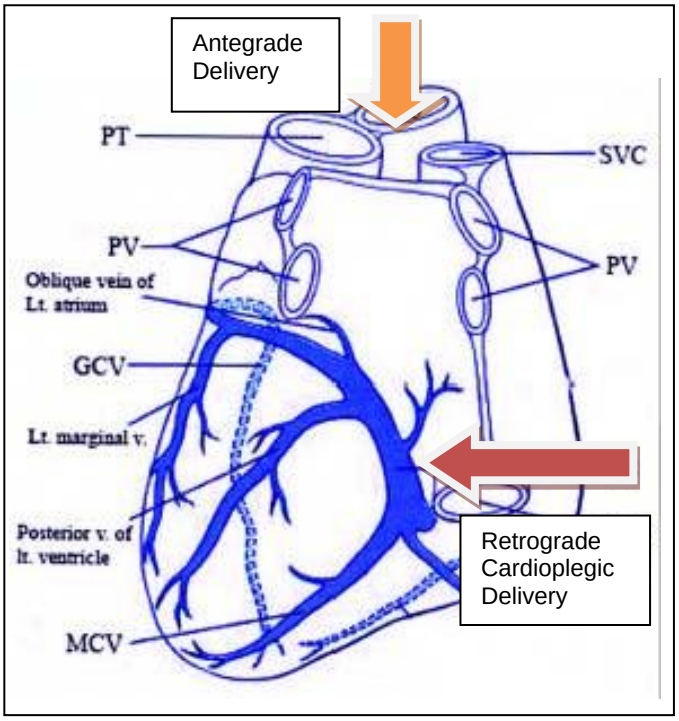


Figure 1.6 Diagram of heart perfusion. Antegrade aortic perfusion (Orange) through the aorta and Retrograde cardioplegic delivery (Red) through the coronary sinus.[9]

1.7 LifeCradle Development and Engineering

The LifeCradle™ is a new technology designed to overcome limitations of the current paradigm of organ donation and procurement. The parent company, Organ Transport Systems, Frisco, TX, acquired patent rights and developed the technological concept almost a decade ago. The company has since grown and matured, gaining the resources necessary to make the transition from concept to product.

The basic concept of the device is to provide a more physiological environment for organ transport between donor and recipient site. The first prototype consisted of a heat and mass exchanger, fed by bulky oxygen tanks and a large fluid pump attached to the organ container [Appendix B, Figure B1]. The next prototype was designed around large igloo coolers which were packed with ice around the organ container, with a roller pump and membrane oxygenator to allow oxygenated fluid pumping through the organ [American Pneumatic Tools, Gardena, CA]. The third model incorporated externally mounted temperature and pressure sensors. While the methods embodied in these prototypes incorporated the idea of pumping fluid across the organ, they were still cumbersome and difficult to transport [Appendix B, Figures B2 and B3]. The next generation of design benefited from the inclusion of an experienced engineering prototype company to increase functionality and transportability [The RealTime Group, Plano, TX]. The prototype LifeCradle, as used in the swine and canine experiments described below, shifts from a large cooler containing the components to a small isolated cooler for the organ only with the other components in a surrounding shell [Appendix B, Figure B4]. The major change to this more portable unit is the incorporation of a dual Peltier cooling method, in which the temperature can be controlled by manually adjusting a dial, with a range from 5° to 12°C. In addition to manual temperature control the roller pump can also be dialed into the desired flow rate from 0 to ~75 ml/min [OEM Series, Watson-Marlow Pumps, Falmouth, England]. Third, a new miniature membrane oxygenator was included in the fluid path, with a pore size of 20 microns, replacing the bulkier oxygenator [Membrana GmbH, Obernburg, Germany]. A final incorporation to this new portable design is the ability to run off of AC power in addition to battery power [Inspired Energy, Inc., Newberry, FL]. Both power sources are approved for medical device usage, and the battery power life of the two batteries is approximately 3 hours when fully charged.

The transition from prototype into a clinical/commercial model has once again required an overhaul of the design. In order to facilitate user control, the unit has been redesigned to incorporate a touch screen computer [Appendix B, Figure B5]. As mentioned above, the current standard for heart transport is a simplistic sterile method involving a three layer sterile barrier. Keeping this in mind, the device evolved into two components, the durable unit and the disposable circuit [Appendix B, Figure B5]. The durable unit contains the computer, which runs a Visual C++ program to allow user interface, one OEM 102R low flow roller pump, four Inspired Energy 14.4V Li-Ion batteries that are supplemented with a medical grade AC power connector, and locations for the single use oxygen tank and disposable circuit. The disposable circuit contains the chiller, Membrana Mini-Module membrane oxygenator, and organ container interface and bag [Appendix B, Figure B6]. The portability of the device has also been effectively addressed with an ergonomic design to the durable shell and the inclusion of light weight components; the final fully loaded unit weighs a mere 35 pounds. The clinical unit also addresses the issues of the sterile field inside the operating room by allowing the user to set up the disposable circuit and all of its components on a sterile back table. Once complete, the same technician then activates and initiates the device on a separate non-sterile table. All priming and device activation takes approximately 10 minutes, almost the same time it would take to lay out and organize the components for a simple storage transplant.

1.8 Prior Canine and Swine Studies

A comparison study was performed with a group of eight pigs to determine the efficacy of machine perfusion compared to static storage. Hearts were arrested and stored with glucose modified Celsior solution for durations of four hours. For the machine perfused hearts a normalized flow rate of 10mL/100g/min was used. Left ventricular (LV) function was evaluated using a load-independent analysis of ventricular performance from dimension data acquired by sonomicrometry crystals and pressure data acquired from an intraventricular micromanometer-tipped catheter. Metabolic activity was determined by magnetic resonance spectroscopy of excised left atrial appendage samples. After

preservation, the hearts were then transplanted into recipient pigs and reperfused for six additional hours, at which point left ventricular water content and serum creatine kinase-MB isoenzyme levels were measured. Results of the experiments revealed that LV function and water content were similar in both groups. Tissue lactate levels were lower in continuously perfused hearts and serum creatine kinase-MB levels were significantly higher in statically preserved hearts. This study suggested that continuous perfusion of donor hearts reduces the production of undesirable metabolites and has the potential to improve graft function.[15]

A similar study was performed using a canine model. A total of twelve dogs were studied using a four hour preservation interval with a six hour recipient reperfusion. In addition to measuring post-reperfusion LV water content and serum creatine kinase-MB isoenzyme levels, myocardial apoptosis was evaluated using a TUNEL test. Results of this study again showed that tissue lactate levels were reduced in the machine perfused hearts and LV water content remained similar. The CK-MB levels however, were similar in both groups. The apoptotic cell count although low, was markedly higher in the statically preserved hearts, further suggesting that continuous perfusion offers significant benefits for tissue viability.[16]

1.9 Antegrade Perfusion Study Using the LifeCradle Prototype

There were several unanswered questions from these initial machine perfusion studies. First, objective measures of myocardial perfusate delivery were not provided, and up to that point had not been reported in literature. Second, there was a strong suspicion that some canine hearts may have received limited nutrient flow as the lactate:alanine ratios were reduced but to a lesser degree than we had observed in swine. Unlike perfusion preservation in kidneys where all flow to the renal artery must directly perfuse the renal parenchyma, perfusate that is delivered to the ascending aorta may have other fates. For example, some flow could enter the coronary circulation but shunt past the capillaries through the Thebesian channels. Alternatively any incomplete closure of the aortic valve during perfusion may result in aortic valve insufficiency with loss of nutrient flow to the myocardium. Another phenomenon

might be of greater importance in a system that features non-pulsatile flow at low flow rates and low aortic root pressure. In these experiments, any flow that entered the ascending aorta but passed into the collection chamber (i.e. did not enter the coronary capillary system) was considered non-nutrient flow.

The fraction of flow (in %) that was nutrient flow was calculated as:

*[(inflow bead count - bead count in collection chamber)/ (inflow bead count)] * 100*

where

inflow bead count = (concentration of beads in bead container x flow rate x 2 minutes), and

bead count in collection chamber = (concentration of beads in sampling container x final volume of sampling container)

The fraction of flow (in %) that was non-nutrient flow was calculated as:

*[(bead count in collection chamber)/ (inflow bead count)] * 100*

where

inflow bead count = (concentration of beads in bead container x flow rate x 2 minutes), and

bead count in collection chamber = (concentration of beads in sampling container x final volume of sampling container)

Tissue flow rate (in mL/100 gram/min) was calculated from bead counts measured in the tissue samples and bead containers as follows:

myocardial blood flow = [(*bead count in tissue/ tissue weight*) * *concentration of beads in bead container*] *100/ *2 minutes*

The effects of flow conditions on regional myocardial perfusate delivery were previously not known.

A series of experiments was used to examine four specific hypotheses. The first hypothesis was that device flow rate is a primary determinant of tissue perfusion. Secondly it was hypothesized that

aortic attachment conditions affect tissue perfusion. Thirdly it was hypothesized that adequate tissue perfusion would permit a better tissue metabolic profile and that higher tissue perfusion would increase the risk of myocardial edema development. All of these questions remain important to the development of perfusion techniques for clinical transplantation.

1.9.1 Effect of Device Flow Rate on Tissue Perfusion

For this group of six dog hearts, the selected machine perfusion solution was a modified Celsior solution. Our hypothesis was that the lower flow rates may lead to lower pressure in the ascending aorta, resulting in a less competent aortic valve and thus leading to a diminished nutrient flow via coronary circulation. To test this theory, the perfusion device was modified to include a side-branch from the recirculating tubing. This side-branch led to a Y-connector that enabled the device to infuse one of two alternate solutions for a limited time. One of these solutions consisted of modified Celsior solution cooled to 5°C and supplemented with 15 micron colored microspheres [IMT Laboratories, Irvine, CA] at a final concentration of 15,000 beads/mL and 0.01% TWEEN 80. The other solution consisted of cold modified Celsior solution without beads. A sampling container the same size as the perfusion device chamber containing one liter of cold modified Celsior solution was also prepared.

Each dog heart was initially established in the device at one of six flow rates (5, 10, 15, 20, 25 or 30 mL/100 g/min) in random order. This flow rate was continued with the recirculating preservation solution for 20 minutes (Figure 1.7); and pressure in the ascending aorta and fluid temperature were continuously recorded.

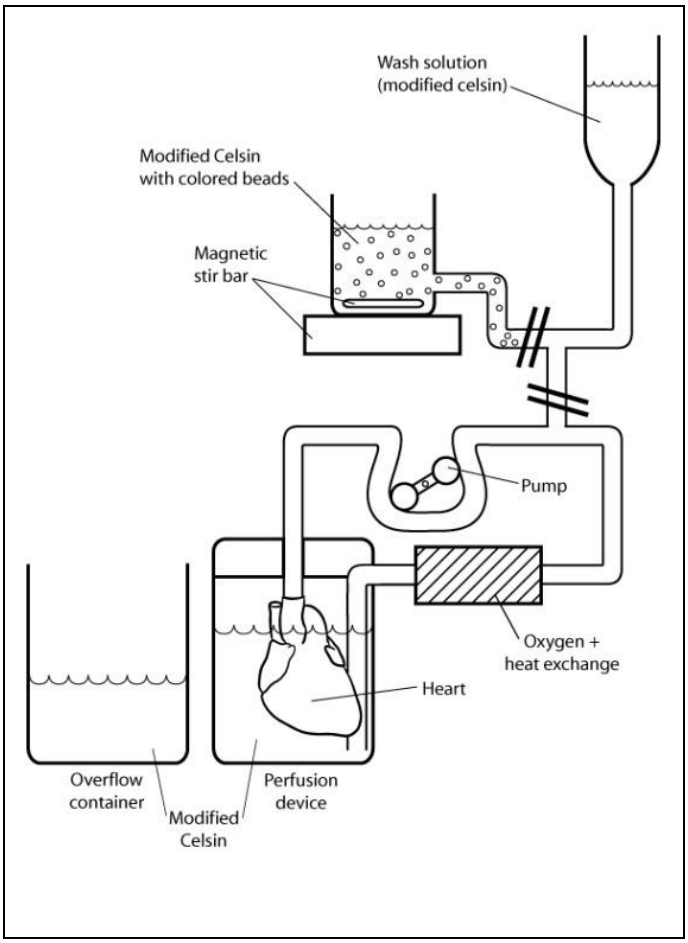


Figure 1.7 Standard perfusion setup of device

Myocardial oxygen consumption (MVO_2) was calculated, based on pO_2 levels measured in the aortic inflow line and from a small catheter placed in the coronary sinus. At this time the heart was gently repositioned from the perfusion chamber to the sampling container, the side-branch was opened and the recirculating line was clamped to allow delivery of the bead-containing solution into the device through the inflow cannula for 2 minutes at the same flow rate without recirculation, Figure 1.8.

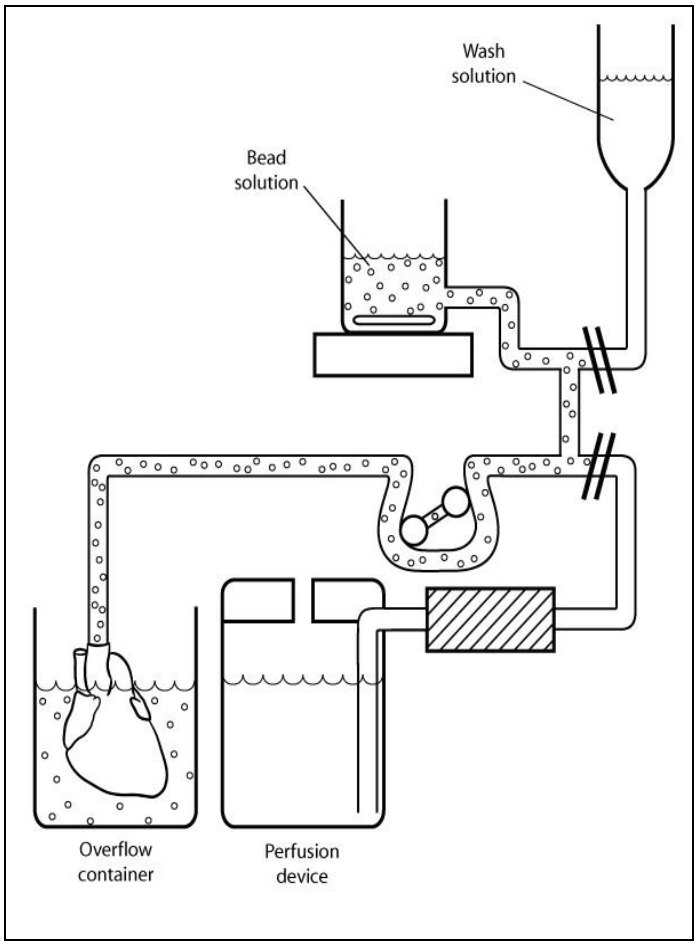


Figure 1.8 Bead solution delivery with organ in overflow container

This enabled measurement of tissue perfusion. A sample of the bead solution was collected for control measurement of bead concentration in perfusate, necessary for quantification of total inflow bead count, a component of the tissue perfusion calculation. After 2 minutes, the inflow was switched to the washout solution to clear any beads from the tubing by flowing at the same rate for a time calculated to deliver 120 mL of solution from the chamber, Figure 1.9.

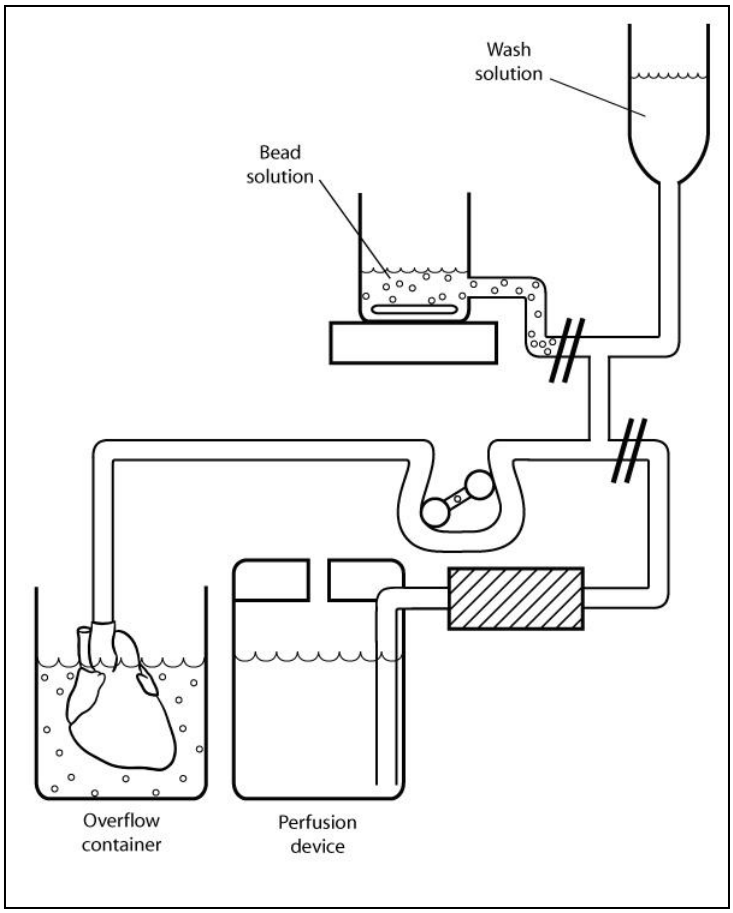


Figure 1.9 Bead washout solution flowing through the heart

At this point the heart was returned to the initial perfusion chamber and the second randomly selected flow rate was applied. The microsphere solution was then changed to one containing one of the other five bead color preparations. After 20 minutes of perfusion with this solution, the sequence was repeated until all six flow rates with separate bead labels had been completed.

After the final washout, the heart was removed from the sampling chamber. The heart was sliced and a 1 cm slice at the mid-ventricular level was selected for analysis. Tissue sections, approximately 2-3g each, comprising samples of endocardial and epicardial tissue taken from the anterior, lateral and posterior LV, the left and right ventricular septa, the RV free wall, and the left atrial appendage were harvested, labeled, bagged and stored in a -80°C freezer until further use. Furthermore, a fluid sample from the overflow chamber of the perfusion circuit was collected and the final volume of the overflow

chamber was measured. Tissue and fluid samples were sent to IMT Laboratories in Irvine, CA, where all measurements of tissue and fluid bead counts were performed. Myocardial tissue perfusion rates and the fraction of non-nutrient flow were calculated from these data, as described in the analytical methods section.

1.9.2 Effect of Aortic Attachment Configuration on Tissue Perfusion

In this study, a group of four dog hearts were harvested as described above and the ascending aorta was attached to an adaptor allowing connection to the perfusion device, as shown above. Each heart was then attached to the device in one of six different conditions described in Table 1.1.

The conditions varied in that the heart was either first immersed in the solution prior to attachment or attached to the device out of the solution and with a starting flow rate that was high (60 mL/min) or low (5 mL/min). For the final two conditions, the connector was removed and the aortic valve was first sutured closed for the perfusion period and then excised for the final duration, the connector was re-attached and reconnected to the device for both. For every set of conditions, after the connector was attached, the flow rate was gradually altered, to arrive at a final rate of 10 mL/100g/min over the course of 2 minutes at a temperature of 5°C; perfusion was then maintained at this rate for 20 minutes. A measurement of myocardial perfusion and non-nutrient flow was then made by a technique similar to the one described above; in this instance the heart was not moved to a sampling container. After completion of the bead infusion and washout steps, the heart was detached from the device and re-attached to a second, identical device for the next loading condition. This was repeated for each heart until all 6 conditions were studied. At this point the heart was removed, tissue was harvested and fluid samples were collected for microsphere analysis as described previously.

Table 1.1 Conditions under which the heart was attached to the perfusion device

Condition	Heart Position when Connector Attached	Initial Flow Rate when Attached	Final Flow Rate	Status of Aortic Valve
1	Immersed in solution	60 mL/min	10 mL/100g/min	No manipulation
2	Immersed in solution	5 mL/min	10 mL/100g/min	No manipulation
3	Suspended above solution, then lowered	60 mL/min	10 mL/100g/min	No manipulation
4	Suspended above solution, then lowered	5 mL/min	10 mL/100g/min	No manipulation
5	Immersed in solution	60 mL/min	10 mL/100g/min	Valve sewn closed
6	Immersed in solution	60 mL/min	10 mL/100g/min	Valve excised

1.9.3 Effect of Heart Inclination on Tissue Perfusion

Two hearts were harvested as described above and the ascending aorta of each was attached to an adaptor. The heart was then attached to the device in one of six different inclinations, Table 1.2.

Two sets of inclinations, maintained a similar control angle of 0°, but differed in angles of tilt in relation to the coronary sinus, Figure 1.10.

The heart was first immersed in the perfusate solution with a high starting flow rate, 60 mL/min, and over the course of 2 minutes, the flow rate was gradually brought to a tissue perfusion rate of 10 mL/100g/min, while maintaining a temperature of 5 ± 2°C; perfusion was then maintained at this rate for 20 minutes. A measurement of myocardial perfusion and non-nutrient flow was then made by a similar technique to the one described above; again, the heart was not moved to a sampling container. After completion of the bead infusion step and washout the heart was detached from the device and re-attached to a second identical device for testing of the next loading condition. This process was repeated until all 6 inclinations were studied. At this point the heart was removed, tissue was harvested and fluid samples were collected for microsphere analysis as previously described.

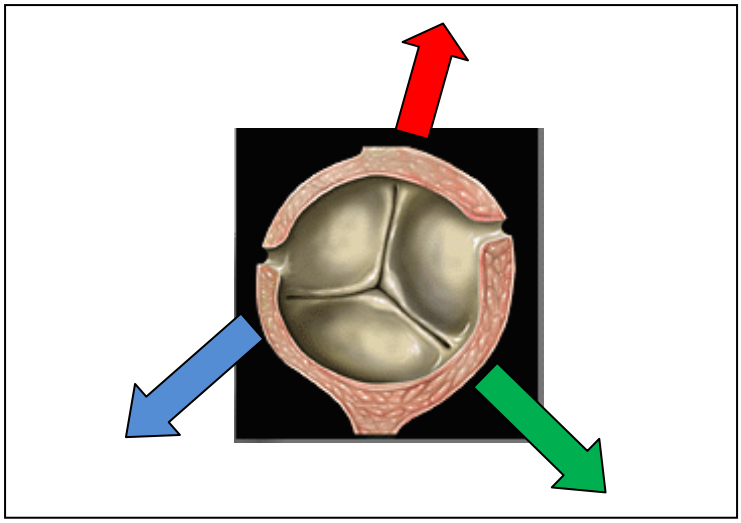


Figure 1.10 Heart inclination study coronary sinus directions. Away from non-coronary sinus (red). Away from left coronary sinus (green). Away from right coronary sinus (blue).

Table 1.2 Conditions under which the heart was inclined in the perfusion device.

Angle	Heart Position	Heart Position when Connector Attached	Initial Flow Rate when	Final Flow Rate
45°	Away from Non-coronary Sinus	Immersed in solution	60 mL/min	10 mL/100g/min
0°	Control	Immersed in solution	60 mL/min	10 mL/100g/min
75°	Away from Non-coronary Sinus	Immersed in solution	60 mL/min	10 mL/100g/min
15°	Away from Non-coronary Sinus	Immersed in solution	60 mL/min	10 mL/100g/min
30°	Away from Non-coronary Sinus	Immersed in solution	60 mL/min	10 mL/100g/min
60°	Away from Non-coronary Sinus	Immersed in solution	60 mL/min	10 mL/100g/min
30°	Away from Right Coronary Sinus	Immersed in solution	60 mL/min	10 mL/100g/min
60°	Away from Left Coronary Sinus	Immersed in solution	60 mL/min	10 mL/100g/min
30°	Away from Left Coronary Sinus	Immersed in solution	60 mL/min	10 mL/100g/min
0°	Control	Immersed in solution	60 mL/min	10 mL/100g/min
60°	Away from Right Coronary Sinus	Immersed in solution	60 mL/min	10 mL/100g/min

1.9.4 Effect of Flow Rate on Myocardial Metabolism and Edema Development

For this series, seven dog hearts were prepared and established in the perfusion device, using varied attachment techniques. Hearts were each perfused for 10 hours at one of 3 flow rates: 10, 15, or 30 mL/100g heart tissue/min, thereby creating conditions where a range of nutrient flow rates at the cellular level would be expected. Perfusion solution used in this series was modified Celsior Solution. At the end of the perfusion period, a measurement of flow distribution was made, using the technique described above. Hearts were removed from the device, drained of residual perfusion solution, and reweighed. Samples of left ventricular tissue were harvested, immediately freeze-clamped, and cooled in liquid nitrogen, then labeled and bagged. The tissue was stored in a -80° C freezer until use. For metabolic measurements, samples were subsequently extracted with perchloric acid. Purified extracts were reconstituted in D_2O and pH was adjusted to 7.0-7.4 for Magnetic Resonance Spectroscopy (MRS).[15] MR Spectra were then acquired with a 14.1 Tesla Varian spectrometer operating at 600 MHz over a spectral width of 8000 Hz. Lactate-to-alanine ratios were measured and compared with 1H proton spectra as measures of cellular metabolism during perfusion as previously described.[16] Separate samples (~2-3g) were collected for measurement of water content. The samples were placed in an oven and tissue weights were recorded until no further weight loss was observed. The percentage change between the initial and final weights was calculated for each sample and data from hearts with high and low nutrient flow were compared.

1.9.5 Summary

The experiments suggested that myocardial perfusion in the organ transport device was influenced by the delivered flow rate and conditions under which the ascending aorta is interfaced with the device. In our model, device flow rates corresponding to 10ml/100g/min or less tissue perfusion resulted in higher percentages of non-nutrient flow. Non-nutrient flow was also increased when the aorta was attached at low flow rate conditions. These same perfusion changes were observed to a more extreme degree when the aortic valve was excised. The results suggested that initial pressurization of

the aortic root results in stable coronary flow and tissue perfusion even if the device flow rate is subsequently decreased. The reason for this was not entirely clear. However, one clue may be that initial higher pressurization of the aortic root, by high perfusate flow delivery, may be required to achieve valve leaflet seating. Once satisfactory closure is obtained, lower flows may then be sufficient to maintain valve competence. At low flows and correspondingly lower aortic root pressures, satisfactory valve leaflet apposition may not be achieved, rendering the valve incompetent with resultant low nutrient flow.

Some clues as to the origins of the observed non-nutrient flow were provided by this study. A system leak would result in reduced preservation solution volume and would be easily detected; however, this was not observed. Intracardiac shunting through coronary arteriovenous connections is another potential source of non-nutrient flow. Under normothermic blood-perfused conditions, the physiologic shunt across the myocardium appears minimal. Human data from Ravin et al suggests only 0.26% of the total cardiac output bypasses the myocardial capillary network.[17] This would represent about 5% of coronary blood flow. The intracardiac shunt fraction during hypothermia has not been previously quantified. Our data from hearts where the aortic valve was sewn closed suggests that the intracardiac shunt was at most 9.7% of delivered flow, assuming that perfect competence was obtained by this manipulation. Measurements made when aortic valve incompetence was guaranteed through excision of the valve nearly eliminated myocardial capillary perfusion. It is likely that aortic valve incompetence of lesser degrees also influences tissue perfusion, perhaps proportionally. Lower flows delivered by the device, either during aortic attachment or during the course of machine perfusion, result in lower distending pressure in the ascending aorta, a condition more likely to result in incomplete aortic valve closure. As a result, the data suggested that the majority of non-nutrient flow was due to aortic insufficiency.

Aortic valve incompetence in a hypothermic perfusion device may have minimal clinical consequence for standard ischemic intervals since even in the worst case scenario, i.e. absent coronary (nutrient) flow, standard static storage conditions would result in reasonable organ preservation in a controlled temperature environment. However, as the storage interval is extended, or if the organ

harvested starts with suboptimal cardiac function, this scenario becomes more problematic, and inadequate myocardial perfusate delivery might result in an injured heart. Data from the third phase of experiments in this study supported this hypothesis. In this phase hearts were perfused for prolonged intervals under conditions that resulted in high or low fractions of non-nutrient flow. In every case, high levels of non-nutrient flow correlated with the expected low levels of tissue perfusion, measured by beads entrapped in the myocardium.

Proton magnetic resonance spectroscopy (^{1}H MRS) analysis of tissue samples from these perfused hearts found other interesting associations. Myocardial lactate accumulation, expressed as lactate to alanine ratio, was higher when tissue perfusion was low and much lower when tissue perfusion matched the delivery rate of the device. It has been previously shown that the lactate to alanine ratio is significantly reduced with perfusion preservation, and is associated with improved cardiac function during early reperfusion in a swine model.[15] Thus, designing the perfusion protocol to optimize tissue perfusion would seem to be a desired goal.

However, ongoing perfusion preservation for prolonged intervals has the potential to create myocardial edema, as identified by other investigators.[20,37,65-67] In our study, after 10 hours of simulated transport, hearts with higher degrees of myocardial perfusion and low non-nutrient flow fractions had greater weight gain and higher water content compared to hearts with the reciprocal perfusion pattern. The clinical relevance of these seemingly contradictory results is unclear. Small animal heart perfusion preservation studies and clinical data from kidney transplantation suggest that increased weight gain may be of little importance. However, unlike renal allografts, transplanted hearts must function immediately and myocardial edema, if not reversed prior to weaning from cardiopulmonary bypass, might result in impaired graft function.[32-35] It was possible that edema observed in some of the perfused hearts could be a function of the preservation solution used in the study. Celsior does not contain a large molecule that would exert an oncotic pressure effect within the coronary vasculature, and may therefore be prone to developing myocardial edema. Additional studies utilizing University of Wisconsin-Machine Perfusion Solution (UW-MPS), which provides superior oncotic properties, have shown either minimal weight

change or an overall weight loss for perfused hearts. In a comparison study, published in Transplantation Proceedings, we have shown that the inclusion of the oncotic agent hydroxyethyl starch (HES) in an extracellular solution (UW-MPS) prevents tissue weight gain during the perfusion interval. Furthermore, perfusion preservation with an extracellular solution without a colloid component (Celsior) leads to significant myocardial edema development.[73]

1.10 Specific Aims

Successful cardiac transplantation depends on the ability to adequately protect the donor heart during procurement and transport. To date this has been accomplished by infusing a cold preservation solution into the heart and storing the heart in an ice chest, a process which is referred to as static preservation. This method has limitations and has led to the development of techniques that provide a continuous flow of oxygenated solution to the donor heart during transport known as machine perfusion preservation. This new strategy may allow ongoing oxidative metabolism in the myocardium which can support essential cellular processes. However, there are several factors that have yet to be evaluated for the adoption of this technology. This project examines myocardial metabolism during machine perfusion preservation in the small animal model to identify substrates in the preservation solution that contribute to ongoing oxidative metabolism. Translational experiments in the large animal model evaluate a new perfusion strategy to reduce the ischemic time during transplantation. These experiments will utilize the preservation solution composition determined from the small animal model. Additional experiments will extend this concept to the human model. Myocardial metabolic data will be used as a means to determine organ viability.

The specific aims of this research project are:

1. To identify substrate components to add to the preservation solution to enhance oxidative metabolism during machine perfusion.
2. To develop and test retrograde perfusion to enhance perfusate delivery throughout the myocardium to enhance oxidative metabolism during machine perfusion.
3. To test Magic Angle Spinning magnetic resonance spectroscopy as a method to determine metabolic state of stored donor hearts prior to transplantation.

Information from these studies may enable us to develop improved analytical methods during machine perfusion preservation, leading to superior results in cardiac transplantation.

1.11 Techniques to study these phenomena

The following sections outline the principal methodologies used in these investigations to evaluate myocardial flow distribution and performance and TCA cycle metabolism. The isolated heart model is now over a century old, but techniques to precisely quantify perfusate flow distribution and ventricular efficiency are much newer. Furthermore the analysis of TCA cycle metabolism utilizes a novel techniques. ^{13}C magnetic resonance spectroscopy (MRS)), ^{1}H magnetic resonance spectroscopy, and, magic angle spinning MRS techniques are new methods to elucidate these phenomena. These approaches have not previously been applied to these questions of machine perfusion for heart transplantation.

1.11.1 Determining Flow Distribution

Colored microspheres were used to quantify nutrient flow to the tissues (IMT Laboratories, Irvine, CA) as previously described.[15] To perform this analysis, the heart was gently repositioned from the perfusion chamber to the sampling container; the side branch of the perfusate delivery line was opened, and the recirculating line was clamped to allow delivery of the bead-containing solution into the device through the inflow cannula for 2 minutes at the same flow rate without recirculation. A sample of the bead

solution was collected for bead quantification. After 2 min, the inflow was switched to the alternative (washout) solution to clear any beads from the tubing by flowing at the same rate for a time calculated to deliver 120 mL of solution from the chamber. At this point, the heart was returned to the initial perfusion chamber and the second randomly selected flow rate was dialed in. The bead solution was changed to one with a different color of beads. After 20 min the sequence was repeated. This process continued until all 6 flow rates were completed. After the final washout, the heart was removed from the sampling chamber. The heart was dissected, and a 1-cm slice at the midventricular level was obtained to quantify segmental perfusion. Tissue samples (2-3 g) of endocardial and epicardial tissue from the anterior, lateral, and inferior left ventricle, the left and right ventricular septum, the right ventricular free wall, and the left atrial appendage were harvested and stored. In addition, fluid samples from the overflow and perfusion device chambers were collected, and the final volume of both chambers was measured. Bead counts of fluid samples and digested tissue were quantified with a NuFlow cytometer (IMT Laboratories, Irvine, CA).

Myocardial tissue perfusion rates and the fraction of non-nutrient flow were calculated as previously described8 by measuring the number of beads collected in the sampling container for each flow rate and dividing this by the total number of delivered beads. The nutrient flow fraction was then calculated as 1 – the fraction of beads collected in the sampling container (i.e. the non-nutrient flow fraction) for each flow rate. Nutrient flow was then determined by multiplying the nutrient flow fraction by the delivered volume per 100g cardiac tissue per minute for each flow rate. Nutrient flow calculations were based on the bead data from the sampling container only. Subsequent bead loss into the perfusion chamber was measured but assumed to represent nutrient flow since it occurred after the washout period. Regional myocardial perfusion was determined in tissue by digesting the tissue, recovering entrapped beads and by dividing the entrapped microsphere count per gram myocardial tissue by the expected nutrient flow per gram myocardium.

1.11.2 Quantifying Metabolism

1.11.2.1 ^{13}C Magnetic Resonance Spectroscopy

Magnetic resonance spectroscopy (MRS) is a method to ascertain chemical information by using the magnetic properties of atomic nuclei. In the presence of a static magnetic field and a radiofrequency (RF) excitation field, measurements of the spectrum of a nuclear resonance can provide insights into the chemical reactions of tissue metabolism.

When applying ^{13}C to MRS, a few inherent characteristics of this carbon isotope need to be considered. First, ^{13}C has a low natural abundance, about 1.1%. Since ^{12}C cannot be detected by MRS, an enriched substrate (^{13}C) is needed for metabolic studies. As well, ^{13}C MRS has a poor sensitivity (concentrations of 0.2 mM and above are required for *in vivo* metabolic studies). However, through careful design of studies, the principal physiologic substrates can be distinguished and the physiologic concentrations of these substrates fall within the detectable range. This problem is eliminated when tissue extracts are used (*in vitro*), a strategy employed in the research herein. ^{13}C offers a wide chemical range (200 ppm) which enables easy resolution of structurally similar compounds.

Applying MRS to ^{13}C provides a powerful method to analyze TCA cycle metabolism. In principle, specific ^{13}C labeling of selected carbons from different substrate sources is required. As these substrates are metabolized to acetyl-CoA, specific labeling patterns in the acetyl-CoA carbon nuclei are unique to each substrate (2 carbons with 4 labeling combinations). Acetyl-CoA is then oxidized within the citric acid cycle introducing ^{13}C into all intermediates. The labeling patterns circulating within the citric acid cycle will reach equilibrium and will be reflected in TCA cycle intermediates such as α-ketoglutarate, which exists in equilibrium with glutamate providing easily obtainable ^{13}C spectra. Analysis of the multiplets of the five carbons in the glutamate spectrum allows the quantification of various sources such as lactate, pyruvate, fatty acids, ketone bodies, and glucose (as determined by their pre-planned ^{13}C labeling patterns). . A review of the glutamate C4 (carbon 4) spectrum can serve as an example. If only glutamate C4 is labeled a single resonance (a singlet) is detected. If the adjacent C3 or C5 are labeled as well, the signal is split into two resonances, a doublet, due to carbon-carbon coupling. If both adjacent carbons are labeled, four

peaks will appear in the C4 spectrum (a doublet of doublets or a quartet). C1 and C2 will not affect the C4 spectrum because the long-range coupling constants are too small. Knowing the origin of each resonance multiplet and summing the peak areas resulting from the same origin enables calculation of the relative utilization of each source. This technique is valuable for detecting changes in relative substrate utilization between differently labeled sources. Unlabeled sources are not directly represented in the spectra. However, using fractional calculations based on the relative total areas of the glutamate C3 and C4 spectra, the contribution of unlabeled sources can be defined. The full analysis utilizes the spectral information of all carbons through a complex system of equations which are solved by commercially available software (TCA Calc). No requirements for metabolic or isotopic steady state conditions for the substrate selection analysis which is termed *non-steady state analysis*[74,75,76]. However, if anaplerotic fluxes are of interest, a *steady state analysis*[77,78] is applied where the calculations are done under the assumption that a metabolic and isotopic steady state was reached. ^{13}C MRS offers the ability to quantify multiple metabolites from a single spectrum, and is particularly valuable because of the low natural abundance of ^{13}C (1.1%). As a result, ^{13}C MRS is well suited to study substrate selection in the heart.

1.11.2.2 ^{1}H Magnetic Resonance Spectroscopy

Proton (^{1}H) magnetic resonance spectroscopy is the application of nuclear magnetic resonance in NMR spectroscopy with respect to hydrogen-1 nuclei within the molecules of a substance, in order to determine the structure of its molecules. In samples where natural hydrogen (H) is used, practically all of the hydrogen consists of the isotope ^{1}H.

Simple NMR spectra are recorded in solution, and solvent protons must not interfere. Deuterated (deuterium = ^{2}H, often symbolized as D) solvents are used for NMR, specifically, deuterated water, D_2O. Deuterated solvents are supplied with a small amount (typically 0.1 %) of tetramethylsilane (TMS) as an internal standard for calibrating the chemical shifts of each analyte proton. TMS is a tetrahedral molecule,

with all protons being chemically equivalent, giving one single signal, used to define a chemical shift = 0 ppm.

Deuterated solvents permit the use of deuterium frequency-field lock, also known as deuterium lock or field lock, to offset the effect of the natural drift of the NMR's magnetic field B_0. In order to provide deuterium lock, the NMR constantly monitors the deuterium signal resonance frequency from the solvent and makes changes to the B_0 to keep the resonance frequency constant.[79] Additionally, the deuterium signal may be used to accurately define 0 ppm as the resonant frequency of the lock solvent and the difference between the lock solvent and 0 ppm (TMS) are well known. Proton NMR spectra of most organic compounds are characterized by chemical shifts in the range +14 to -4 ppm and by spin-spin coupling between protons. The integration curve for each proton reflects the abundance of the individual protons.

Chemical shift values, symbolized by δ, are not precise, but typical - they are to be therefore regarded mainly as orientational. Deviations are in ±0.2 ppm range, sometimes more. The exact value of chemical shift depends on molecular structure and the solvent in which the spectrum is being recorded. Hydrogen nuclei are sensitive to the hybridization of the atom to which the hydrogen atom is attached and to electronic effects. Nuclei tend to be deshielded by groups which withdraw electron density. Deshielded nuclei resonate at higher δ values, whereas shielded nuclei resonate at lower δ values.

The chemical shift is not the only indicator used to assign a molecule. Because nuclei themselves possess a small magnetic field, they influence each other, changing the energy and hence frequency of nearby nuclei as they resonate—this is known as spin-spin coupling. The most important type in basic NMR is *scalar coupling*. This interaction between two nuclei occurs through chemical bonds, and can typically be seen up to three bonds away.[80]

Occasionally, small peaks can be seen shouldering the main ^{1}H NMR peaks. These peaks are not the result of proton-proton coupling, but result from the coupling of ^{1}H atoms to an adjoining carbon-13 (^{13}C) atom. These small peaks are known as carbon satellites as they are small and appear around the main ^{1}H peak i.e. satellite (around) to them. Carbon satellites are small because ^{13}C only makes up

1.1% of the atomic carbon content of carbon, the rest of the carbon atoms are predominantly NMR inactive Carbon-12. Carbon satellites always appear as an evenly spaced pair around the main ^{1}H peak. This is because they are the result of 1.1% of the ^{1}H atoms coupling to an adjoined ^{13}C atom to give a wide doublet (^{13}C has a spin of a half). Note, if the main ^{1}H peak has proton-proton coupling, then each satellite will be a miniature version of the main peak and will also show this ^{1}H coupling, e.g. if the main ^{1}H peak is a doublet, then the carbon satellites will appear as miniature doublets, i.e. one doublet on either side of the main ^{1}H peak.

In the experiments described herein, both proton and carbon spectroscopy techniques are used to quantify metabolites. Proton spectroscopy is rapid and can readily detect many pertinent molecules such as lactate. Carbon spectroscopy techniques are applied in studies where the heart is supplied with 13C enriched compounds to quantify relative substrate contributions to metabolic pathways. The two techniques are complementary and together provide a more complete assessment on intermediary metabolism

1.11.2.3 Magic Angle Spinning Magnetic Resonance Spectroscopy

^{1}H high-resolution magic angle spinning (MAS) MRS, which requires minimal tissue, reduces processing time, and generates a wide array of metabolic data. A single, cylindrical, anterolateral left ventricular biopsy (approximately 10 mg) is obtained with a small Tru-Cut biopsy needle and immediately stored in liquid nitrogen at -80°C for MAS MRS
(Figure 1.11).

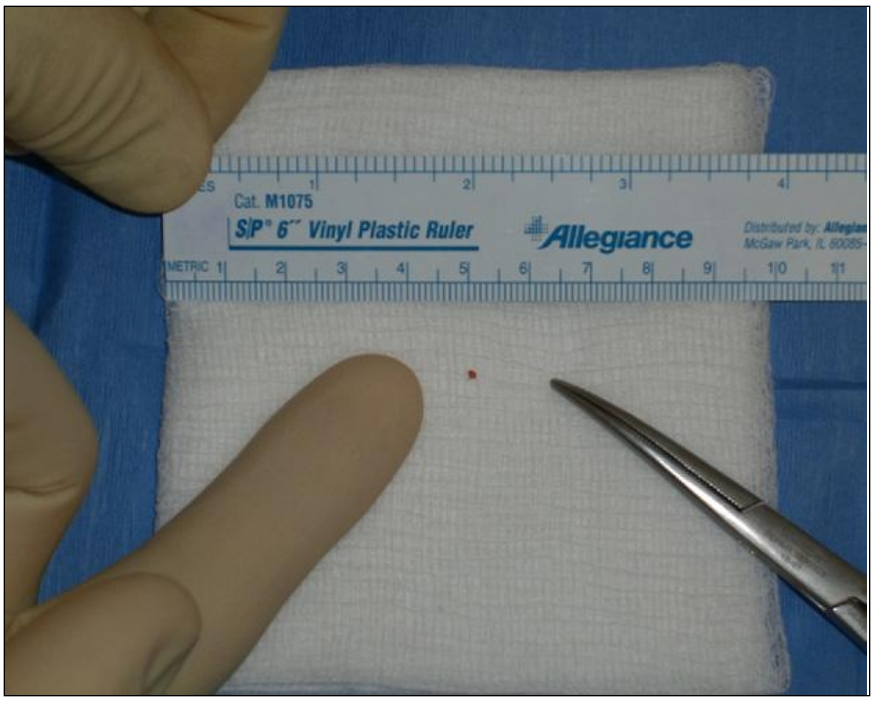


Figure 1.11 Left ventricular ^{1}H MAS MRS biopsy sample.

Tissue is placed in a 50-µl rotor with 5 µl of D2O as a lock solvent. MAS MRS is carried out with a gHx nanoprobe on a spectrometer (Varian 14.1T VNMRS; Varian, Inc., Palo Alto, CA). Spectra are acquired with the "presat" pulse sequence using a 1-second delay, a 3.2-second water pre-saturation pulse and a 1.8-second acquisition time, for a Tr of 6 seconds. Line broadening can be minimized by rotating the sample at 54° 44'' to the external field – the so called magic angle. This strategy has been applied to a variety of intact biologic systems. We demonstrated its ability to determine differences in preservation strategies in our preliminary studies.

Composite MAS spectra are analyzed using commercial software, and data from >70 different resonances can be adequately resolved for analysis. Resonance areas are analyzed by a partial least-squares (PLS) algorithm6 to identify biomarkers that independently predicted the storage method. The total spectral acquisition time for each sample was <10 minutes. The peak-picking PLS algorithm has

been used to identify 5 resonances that are sufficient to categorize the spectra as representing either static storage or perfusion preservation with 100% efficiency. These resonances are at 0.89 ppm (triglyceride methyl protons), 2.02 ppm (protons alpha to a triglyceride double bond), 3.92 ppm (creatine), 4.63 ppm (unassigned) and 5.22 ppm (glycerol protons) (Figure 1.12).

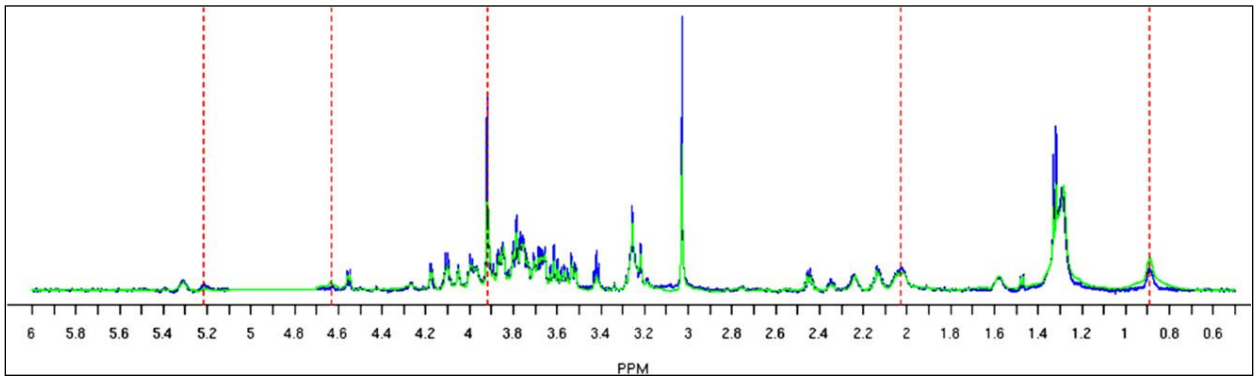


Figure 1.12 Composite ^{1}H MAS MRS spectra: green composite spectrum– static blue composite spectrum–perfusion. The five different resonances identified by the PLS algorithm are marked by the dashed red lines. These correspond to 0.89 ppm (triglyceride methyl protons), 2.02 ppm (protons alpha to a triglyceride double bond), 3.92 ppm (creatine), 4.63 ppm (unassigned) and 5.22 ppm (glycerol protons).

The algorithm selects the minimum number of resonances that can accurately classify the spectrum. For example, lactate resonances were significantly higher in the static group, but lactate was not a sufficiently powerful discriminator to be included in the model. MAS MRS is rapid and requires minimal tissue. Acquired spectra provide a broad array of metabolic information. PLS-discriminant analysis can accurately identify metabolic differences from varying cardiac preservation methods using relatively few resonances. MAS MRS also can quantify intracellular lactate and alanine (measurements that have been associated with myocyte viability in this model), although lactate resolution is difficult due to the presence of –CH2 groups from fatty acids, limiting its application for analysis of intact tissue.

The ability to use minute tissue samples and obtain metabolic data in short time intervals may provide clinicians with important real-time information on the status of a donor heart prior to implantation without a deleterious extension of the ischemic interval.[81]

1.11.3 Myocardial Function

1.11.3.1 Evaluation of myocardial contractility

The heart's function as a pump for the systemic circulation is carried out by the left ventricle (LV). The main factors that define output from the LV are:

1. Preload – The filling condition or the LV dimensions, which relate to the fiber length of the individual myocyte when the heart is maximally filled with blood at the end of the diastolic phase of the cardiac cycle.
2. Afterload – The impedance against which the LV must eject, usually related to the aortic pressure, the elasticity of the aorta and the viscosity of the blood.
3. Contractility – The intrinsic inotropic state of the myocardium that reflects cardiac performance independent of loading conditions.

The performance of the LV has been studied extensively and some indices of mechanical performance (especially "contractility) have become generally accepted. A reliable index must be sensitive to the inotropic state of the heart but independent of preload and afterload. During one beat, the heart goes through two main phases: diastole, the relaxation and filling phase, and systole, the contraction and ejection phase. Similarly, the heart changes from minimal volume at end-systole to maximal volume at end-diastole and from minimal pressure during diastole to maximal pressure when ejection starts. Plotting the dynamics of pressure vs. volume creates a closed loop, called the pressure-volume (PV) loop. Sagawa[82] and colleagues made the first in-depth analysis of the pressure-volume-time relationship. At end-diastole (approximately the right lower corner of the PV loop) a nonlinear curve is obtained identical to the passive pressure-volume curve (or "Frank Starling" curve, see Figure 1.11).

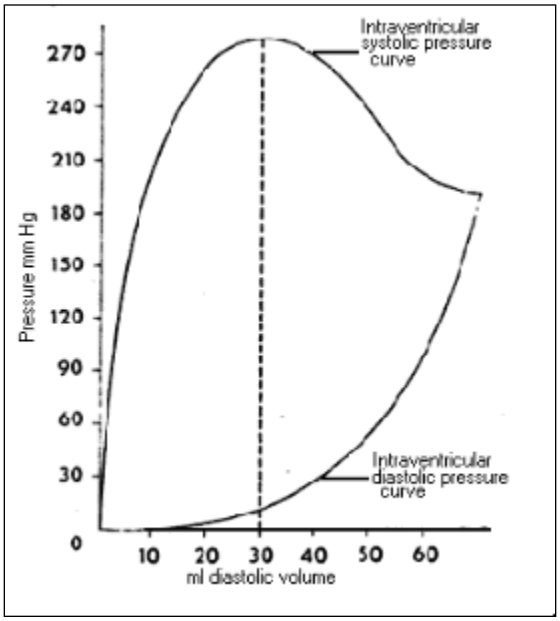


Figure 1.13 Frank Starling curve

As contraction begins, the curve the curve increases its slope E(t), shifts leftward and its volume axis intercept Vd(t) becomes more linear (Figure 1.11). The curve reaches its maximal slope, E_{max}, at end-systole (left upper corner of the PV loop). The maximal slope, determined by the contractile state of the ventricle, is sensitive to inotropic changes, but insensitive to preload and afterload. Administration of positive inotropic agents, such as epinephrine, increases contractility as evidenced by increased E_{max} with no significant change in the volume axis intercept, V_d[82,83]. This index of LV mechanical performance was the first true measure of contractility, independent of preload and afterload. It has been validated in isolated dog hearts[84], intact animals[85,86,87], conscious animals[88], and humans[89,90]. However, E_{max} has been found to have some limitations, in particular, loss of linearity at the low extremes of end-systolic pressure. Nevertheless, it remains a useful parameter for quantifying LV function[87,91]. A distinction should be made however, between E_{es} and E_{max}. E_{max} is the maximal value of the time varying elastance ($E(t) = P(t)/[V(t) – Vo(t)]$) whereas Ees is the set of individual end-systolic points (max of P/[V- Vo]). Both

parameters describe the same phenomenon, but differences may arise, especially *in vivo*, when preload and afterload are interrelated[92]. Most experimental studies use E_{es} rather than E_{max}, although the latter notation is frequently applied. This convention was followed in the studies presented here.

Another approach to evaluate contractility is to examine the relationship between the filling conditions (the end-diastolic volume) and the stroke work (the integral of the PV loop), which describes the mechanical output of the heart to supply the circulation. An exponential relationship is generally observed between stroke work and end-diastolic pressure. However, a linear relationship is obtained when relating end-diastolic volume (or dimension) and stroke work (Figure 1.11). Glower[93] named this relationship the *preload recruitable stroke work (PRSW)*. PRSW is preload-independent and afterload-insensitive, but is responsive to inotropic state. Therefore it can serve as an index of myocardial performance. It tends to remain linear over a wide range of contractile states. This index has been validated in animals[88,94] and humans[91]. We have applied PRSW as the principal measurement of contractility in these studies. The relationship is readily obtainable over a wide range of contractility conditions, appears to remain linear even at extremes of contractility, and may be easier to measure than E_{max} where defining the point of maximal elastance can be challenging. .

CHAPTER 2

EFFECTS OF EXOGENOUS SUBSTRATES ON MYOCARDIAL METABOLISM IN A SMALL ANIMAL MODEL OF MACHINE PERFUSION

2.1 Introduction

Cardiac transplantation has emerged as a life saving procedure for patients with end stage heart failure over the past 40 years[95]. Over that time the general technique of cardiac preservation has changed little. Hearts today are preserved by flushing them with a cold potassium-based crystalloid solution, and then storing them in a container filled with preservation solution that is placed within an ice chest to maintain organ hypothermia during transport. However, the current technique has limitations, and graft storage and transport beyond five hours results in significantly reduced one-year survival[96]. Realizing the limitations of current methods of hypothermic static for cardiac transplantation, some have suggested that providing continuous perfusion of an oxygenated solution to the coronary bed may significantly improve outcomes[97]. The oxygenated solution may allow ongoing aerobic metabolism in the myocardium, which could lead to better preservation of cellular ATP stores and of membrane conditions. As well, the continuous perfusion may wash out metabolites that accumulate during the ischemic process such as lactate[15] or adenosine. Machine perfusion systems can also precisely control the temperature of the circulating solution and the myocardium, and may avoid myocyte damage arising from very low temperatures that are frequently seen with static (ice chest) systems[98]. Thus, providing an ongoing supply of oxygenated substrate to the myocardium may diminish the ischemic burden to the heart, reduce reperfusion injury, and avoid thermal damage.

A sizeable volume of literature has been published evaluating continuous machine perfusion of the heart in a variety of experimental animal models. Virtually all reports describe beneficial effects of machine perfusion preservation compared to hypothermic static storage. Temperature control is vastly superior with a machine perfusion technique and potentially deleterious effects of very low temperatures ($<1^{o}C$) that occur with standard static storage methods may be averted[98,99]. Increased myocardial oxygen

utilization during storage[16,49] is uniformly observed with machine perfusion and superior oxygen utilization after reperfusion of the transplanted heart is also described[67]. Myocardial metabolism seems better maintained with machine perfusion, with investigators observing a reduction in intracellular lactate[15,16], an increase in glucose production[12] and greater fatty acid turnover in the heart[30]. These metabolic events appear to translate into improved intracellular high energy phosphate levels[12,14,49], and a reduction in oxidative stress in the myocardium[25]. Studies have found improved early post-reperfusion ventricular performance with machine perfusion, even when conventional storage intervals are used[15,16]. Functional benefits are magnified when storage intervals are prolonged[12,28,71]and successful transplantation of large animal hearts has been reported even after 48 hours of preservation[72]. Thus, experimental work supports the concept that machine perfusion can provide superior metabolic support to the stored heart and improve early reperfusion function.

However, machine perfusion is hampered by a lack of data on appropriate preservation solutions for this method. In fact, existing solutions fail to include appropriate substrates that can be completely oxidized by the heart to fully realize the potential of this technique. This is perhaps not surprising, as the fundamental premise behind conventional preservation strategies is to reduce metabolism. Machine perfusion techniques that supply oxygenated solutions offer the prospect of harnessing the energy produced by ongoing aerobic metabolism to maintain or repair the stored tissue. As such, the preservation solution should contain a metabolic substrate that can be oxidized to take advantage of this opportunity. Many clinically-available organ preservation solutions contain no substrate that can participate in oxidation through the tricarboxylic acid cycle (TCA cycle), the main cellular engine for energy production. Some solutions contain glucose, but recent data has suggested that glucose participates minimally as an oxidative substrate in models of machine perfusion[16]. The current study was designed to test the utilization of glucose as an oxidative substrate over a clinically relevant range of concentrations in a model that mimics machine perfusion for cardiac transplantation. Pyruvate, a simpler substrate that may have advantages as a metabolic fuel was also tested as an alternative preservation

solution component. In additional experiments, varying concentrations of acetate were tested as a metabolic substrate. This simple two-carbon molecule is readily metabolized to acetyl-CoA, but bypasses the glycolytic enzymes required for glucose oxidation. Glucose and pyruvate, have the potential to participate in anaplerotic pathways - non-oxidative metabolic events that serve to replete citric acid cycle intermediates (in this case via formation of oxaloacetate form pyruvate via *pyruvate carboxylase*). Acetate, however, does not participate in any anaplerotic reaction, leading to the possibility for depletion of citric acid cycle intermediates over time. In a final iteration, varying concentrations of acetate were studied with the addition of 5 mM propionate. Propionate was chosen as it can serve as an anaplerotic substrate (undergoing carboxylation through *propionate carboxylase* to succinate). Groups with the combination of acetate plus propionate thereby test the importance of an anaplerotic additive in enhancing metabolism during machine perfusion. The study was designed to directly measure substrate incorporation into the citric TCA cycle using magnetic resonance spectroscopy.

2.2 Materials and Methods

2.2.1 Animal protocol

Male Sprague-Dawley rats weighing 220 to 540 g were given access to food and water ad libitum and used in an institutionally approved animal research protocol. All animals were cared for in compliance with the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources (National Academy of Sciences) and published by the National Institutes of Health (NIH publication number 86-23, revised 1996).

Rats were anesthetized with an intraperitoneal injection of 4% chloral hydrate (1mL/100g). After median sternotomy and an intrahepatic injection of heparin (1000 U/kg), the inferior and superior vena cavae were ligated. The left atrium was incised to decompress the heart. A diastolic arrest was achieved by administering ice-cold preservation solution through the ascending aorta. The heart was then rapidly

excised, cooled in saline slush (to simulate clinical heart procurement), and then perfused as described below.

2.2.2 Perfusate composition

University of Wisconsin Machine Perfusion Solution (UW-MPS) was utilized as the primary preservation solution vehicle in this study. For these experiments, we obtained a special order from the manufacturer that contained no glucose (see Table 2.1). Solutions were supplemented with 2.5, 5, 10, or 20 mM of glucose or 5, 10, 20, or 40 mM pyruvate. As such, the pyruvate groups contain an amount of carbon that matches the respective glucose groups. All glucose solutions were labeled uniformly with ^{13}C, and all pyruvate solutions were provided as 3-^{13}C pyruvate. For the acetate portion of the study concentrations of 2, 4, 8, and 16mM were selected. These concentrations remained the same for the propionate study, with the concentration of propionate 5mM. All labeled substrates were obtained from Cambridge Isotope Laboratories (Andover, Mass).

Table 2.1. UW-MPS preservation solution composition

Component	Concentration (mM unless otherwise specified)
Na^+	100
K^+	25
Ca^{2+}	0.5
Mg^{2+}	5
Cl^-	1
$H_2P0_4^-$	25
HEPES	10
Adenine	5
Glutathione	3
Mannitol	30
Glucose	0*
Gluconate	85
Ribose	0.5
Hydroxyethyl Starch	5%
Osmolality	300±15 Osm
pH	7.4±1.5

*Standard UW-MPS contains 10 mM glucose. The base solution used in these studies did not contain any unlabeled glucose.

2.2.3 Preservation techniques

Groups of male Sprague-Dawley rat hearts (n = 4 per group) were harvested as described above and perfused by continuous, oxygenated perfusion with a calibrated roller pump (Masterflex, Cole Parmer Instrument Company, Vernon Hills, IL) at 0.5 ml/min for 360 minutes at 8±2ºC. Hearts were randomly assigned to receive either glucose or pyruvate supplemented UW-MPS solution as the perfusate. After 360 minutes of perfusion hearts were removed from the perfusion apparatus, weighed, and small samples of fresh LV tissue were collected, weighed, and placed in an oven for desiccation. The remainder of the LV was immediately freeze-clamped and processed for metabolic analysis as described below. The fresh cardiac tissue was weighed daily until a constant weight was reached during consecutive measurements. The myocardial water content was then calculated as ((wet weight – dry weight)/wet weight).

2.2.4 Assessment of metabolism during the preservation interval

Coronary flow, and inflow and outflow partial pressure of oxygen (pO_2) were measured, and myocardial oxygen consumption (MVO_2) was calculated from coronary flow and inflow-outflow pO_2 differences as described below[49].

$$MVO_2 = (pO_2(in) - pO2(PA)) * CFR * 3.1x10\text{-}5 / 25.4 \quad [2.1]$$

Where pO_2(in) is the partial oxygen pressure in the inflow perfusate, pO_2(PA) is the partial oxygen pressure in the pulmonary artery expressed in mmHg, CFR is the coronary flow rate expressed in mL/min. The solubility of oxygen per mL perfusate is $3.1x10^{-5}$ mL/mmHg at 37°C and a conversion constant from volume to concentration is 25.4 mL/mmole. To express MVO_2 per gram dry weight a final correction is applied based on the heart weight and the measured wet/dry ratio. MVO_2 is thereby expressed in µmol oxygen/ min/ g dry weight.

At the end of the preservation interval, hearts were freeze-clamped with aluminum tongs and cooled in liquid nitrogen. Cardiac tissue was extracted with perchloric acid and lyophilized. One portion of each extract was reconstituted with D_2O for initial proton (^1H) MRS. The remainder of the extract was then purified by passage through a Dowex 50WX2-100 resin (Sigma Chemical) to remove carboxylic acids, and then lyophilized. Purified extracts were reconstituted in D2O, and the pH was adjusted to 7.0 to 7.4 for subsequent magnetic resonance spectroscopy (MRS).

For quantification of substrate metabolism, initial ^1H spectra were acquired with a 14.1 Tesla Varian spectrometer operating a 600 MHz over a spectral width of 8000 Hz. After Fourier transformation, spectra were analyzed with the use of a commercially available line fitting program (ACD Labs, Toronto, Canada) to determine enrichment of lactate and alanine. Glycolysis from catabolism of exogenous, labeled glucose leads to enrichment of glycolytic intermediates, which are quantified from the incorporation of ^{13}C into the number 3-carbon in lactate or alanine. Enrichment from metabolism of exogenous pyruvate will result in similar labeling. The contribution of ^{13}C in both lactate (which reflects glycolytic activity) and alanine (which reflects non-glycolytic pathways) was calculated by comparing the ^{12}C-C3–bound protons to the ^{13}C-C3–bound protons (which produces upfield and downfield resonances attributable to heteronuclear coupling).

To better quantify oxidative metabolism, ^{13}C enrichment of the 4 carbon (C4) of glutamate was done by ^1H MRS on extracts that had been passed through an ion exchange resin. This technique takes advantage of the equilibrium between the TCA cycle intermediate α-ketoglutarate and the much larger intracellular glutamate pool. Any ^{13}C labeled glutamate C4 must be derived from acetyl-CoA generated by pyruvate dehydrogenase either from labeled glucose or pyruvate in the preservation solution since nonoxidative metabolism does not enrich glutamate C4. ^1H MR spectra of extracts were obtained with a 14.1T Varian Inova spectrometer (Varian Instruments, Palo Alto, California) operating at 600 MHz with a 4 second delay between proton pulses and a 0.5 second water saturation delay. Spectra were collected with 24K data points using 64 frequency-induction decays per sample. Data from each extract were

acquired both with and without ^{13}C decoupling centered on the glutamate C4 resonance (34.2PPM). Decoupling removes the heteronuclear interaction between ^{13}C C4 nuclei and the attached ^{1}H nuclei, resulting in a single resonance. Fractional ^{13}C-enrichment of glutamate was determined by calculating the increase in the intensity of the H4 (protons bound to carbon 4 of glutamate) signal associated with ^{13}C - ^{1}H decoupling[100].

The decoupling experiment determines enrichment of glutamate C4 by exogenous substrate that occurs through exchange with the TCA-cycle intermediate alpha-ketogluterate. Not all glutamate may participate in this exchange mechanism due to compartmentalization within the cardiac myocyte, potentially underestimating the contribution of exogenous substrate to myocardial oxidative metabolism. ^{13}C MRS was therefore performed on the same extracts followed by glutamate isotopomer analysis assuming steady-state and non-steady state conditions[77,101]. Isotopomer analysis uses multiplet data from multiple glutamate carbons and substrate ^{13}C enrichment data to determine the total contribution of exogenous substrate to oxidative metabolism and anaplerotic reactions. Substrate contribution data from these experiments was than combined with MVO_2 data to calculate TCA cycle flux, anaplerotic flux, and substrate oxidation rates[102].

2.2.5 Statistical analysis

Results are reported as mean and SEM. Groups were compared by one-way analysis of variance with the use of commercially-available statistical software (SigmaPlot, SPSS, Inc, Chicago, IL). A p-value $< .05$ was considered significant.

2.3 Effect of Glucose and Pyruvate on myocardial metabolism

2.3.1 Perfusion Parameters and MVO_2

All hearts achieved a perfusate temperature of less than 10ºC and maintained this temperature throughout the perfusion interval. There were no differences in MVO_2 amongst any of the groups. Within groups, MVO_2 did not change over time (data not shown). There were no changes in preservation solution pH at any time point. Myocardial water content at end-experiment was similar among groups. See Table 2.

Table 2.2. Perfusion parameters and MVO_2

Group	Temperature (ºC)	Perfusate pH	Water Content	MVO_2 (µmol/g dry weight/min)
2.5 mM Glucose	8.6 ± 0.2	7.26 ± 0.01	0.78 ± 0.01	2.3 ± 0.3
5 mM Glucose	8.2 ± 0.3	7.27 ± 0.01	0.77 ± 0.01	2.1 ± 0.1
10 mM Glucose	8.6 ± 0.1	7.28 ± 0.01	0.76 ± 0.01	1.9 ± 0.2
20 mM Glucose	8.9 ± 0.1	7.24 ± 0.02	0.77 ± 0.01	1.9 ± 0.2
5 mM Pyruvate	8.6 ± 0.2	7.26 ± 0.01	0.78 ± 0.01	2.1 ± 0.2
10 mM Pyruvate	8.5 ± 0.1	7.22 ± 0.01	0.79 ± 0.01	1.7 ± 0.1
20 mM Pyruvate	8.9 ± 0.5	7.26 ± 0.01	0.78 ± 0.01	1.9 ± 0.1
40 mM Pyruvate	9.0 ± 0.1	7.23 ± 0.01	0.79 ± 0.01	1.8 ± 0.3

Data are Mean ± SEM.

There were no significant differences in perfusion conditions, myocardial water content, and oxygen consumption between groups.

2.3.2 Anaerobic Metabolism

Representative ^{1}H spectra from perchloric acid extracts of heart tissue are shown in Figure 2.1. In the left panel a spectra from the 5 mM Glucose group and the right panel from a heart in the 10 mM Pyruvate group. A moderate amount of lactate enrichment but minimal alanine enrichment is seen in the Glucose group spectrum. Greater lactate enrichment and markedly increased alanine enrichment (represented by the larger satellite peaks) is seen in the Pyruvate group spectrum.

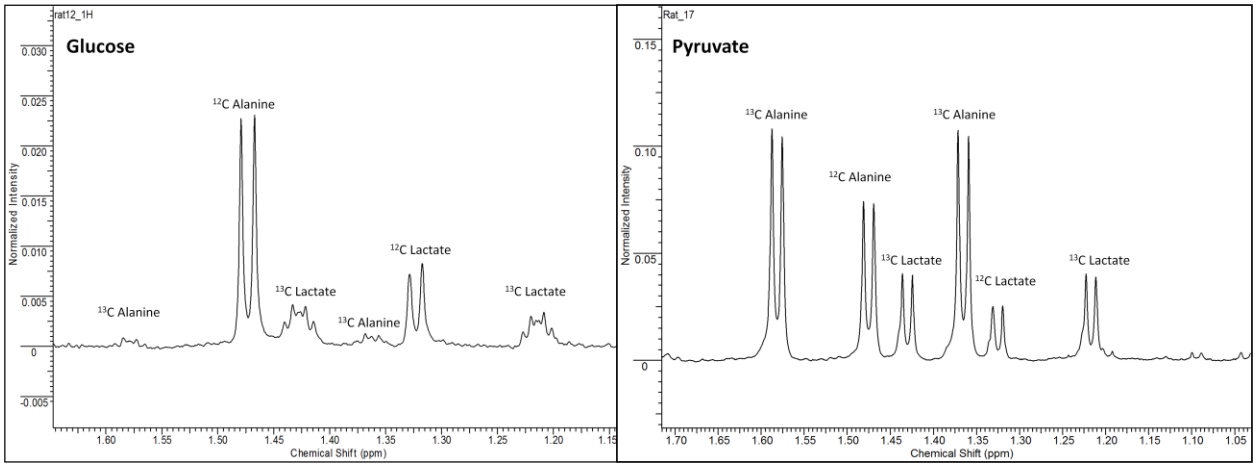


Figure 2.1 Representative ^{1}H spectra from initial perchloric acid extracts of a heart in the 5 mM Glucose group (Left) and 10 mM Pyruvate group (right).

The lactate/alanine ratios measured in all groups were consistent with an oxidative metabolic profile but tended to be lower in pyruvate groups. This suggests a preference for pyruvate to undergo metabolism through the TCA cycle and other non-*lactate dehydrogenase*-dependent pathways. See Table 2.3. In all glucose groups, a moderate enrichment of lactate (37-62%) was observed, consistent with metabolism through glycolytic pathways. ^{13}C enrichment of alanine was much less (11-30%), suggesting minimal participation in non-glycolytic pathways including oxidation within the TCA cycle[103]. In contrast, lactate enrichment in all pyruvate groups was higher (70-81%) although these differences

reached statistical significance only compared to the 2.5mM and 5mM glucose groups. However, alanine enrichment was significantly greater in all pyruvate groups (72-87%). This suggests greater participation of pyruvate in non-glyolytic pathways. While oxidative metabolism through the TCA cycle would appear to be a likely candidate explanation, pyruvate might also undergo transamination to alanine. To directly quantify oxidation, the analysis of enrichment of TCA cycle intermediates was performed as described above.

Table 2.3. Metabolic results

Group	Lactate/Alanine	Alanine Enrichment	Lactate Enrichment
2.5 mM Glucose	1.0 ± 0.3	0.15 ± 0.01	0.47 ± 0.09†
5 mM Glucose	0.6 ± 0.1	0.11 ± 0.03	0.37 ± 0.13†
10 mM Glucose	1.2 ± 0.1	0.18 ± 0.02	0.57 ± 0.06
20 mM Glucose	1.2 ± 0.2	0.30 ± 0.06	0.61 ± 0.05
5 mM Pyruvate	0.4 ± 0.1	0.72 ± 0.02*	0.70 ± 0.05
10 mM Pyruvate	0.2 ± 0.1	0.73 ± 0.01*	0.70 ± 0.03
20 mM Pyruvate	0.7 ± 0.1	0.77 ± 0.03*	0.77 ± 0.04
40 mM Pyruvate	0.2 ± 0.1	0.87 ± 0.02*	0.81 ± 0.02

Data are Mean ± SEM.

* - $p<.001$ versus all glucose groups, † - $p<.05$ versus all pyruvate groups

2.3.3 Oxidative (aerobic) metabolism

Labeled substrates can only enrich TCA cycle intermediates by participating in oxidative metabolism. TCA cycle enrichment, as measured by ^{13}C incorporation into C4 of glutamate, identified significant differences amongst groups. In glucose groups, less than 11% of glutamate C4 was enriched (range 2.4-10.7%), consistent with minimal oxidation of glucose through the principal aerobic pathway.

There was no relationship between glucose concentration and TCA cycle intermediate enrichment across the range of glucose concentrations studied. In the pyruvate groups, enrichment of the total intracellular glutamate pool increased significantly up to the 20mM concentration but was lower in the 40mM group. Glutamate enrichment measured in the 10 and 20mM pyruvate groups was significantly greater than that observed in all glucose containing groups, reflecting greater aerobic metabolism of this substrate ($p<.05$). See Figure 2.2.

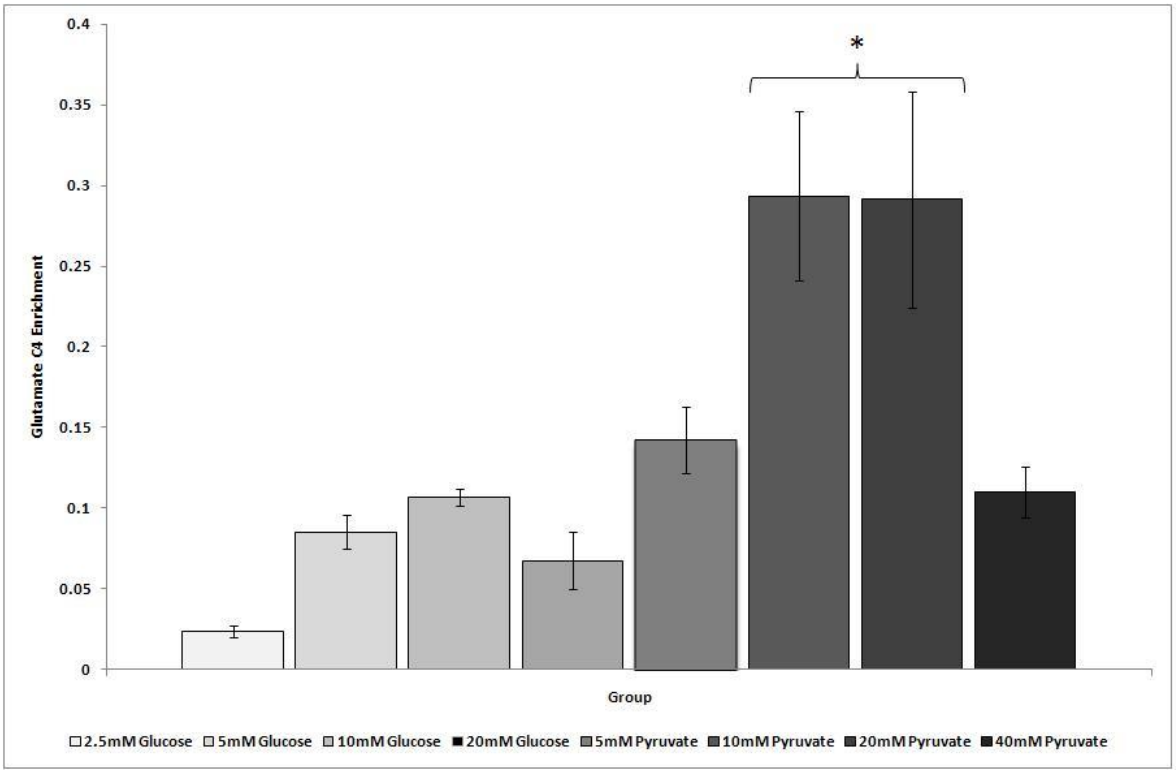


Figure 2.2 Glutamate C4 enrichment in all glucose and pyruvate groups. Data are shown as mean ± SEM. * identifies groups that exhibited significantly greater enrichment compared to all other groups by one-way ANOVA ($p<0.05$).

^{13}C MRS supported these findings but also suggested that the absolute contribution of exogenous substrate to oxidative metabolism was much higher in the pyruvate groups. Although C4 enrichment was only about 30% even in the highest pyruvate groups, isotopomer analysis indicated that most oxidized substrate in the pyruvate groups was derived from exogenous pyruvate in the preservation solution (65.8-81.8%). This contribution remained less than 25% for all glucose groups (13.5 -23.0 %, p<.001 versus all pyruvate groups). See Figure 2.3.

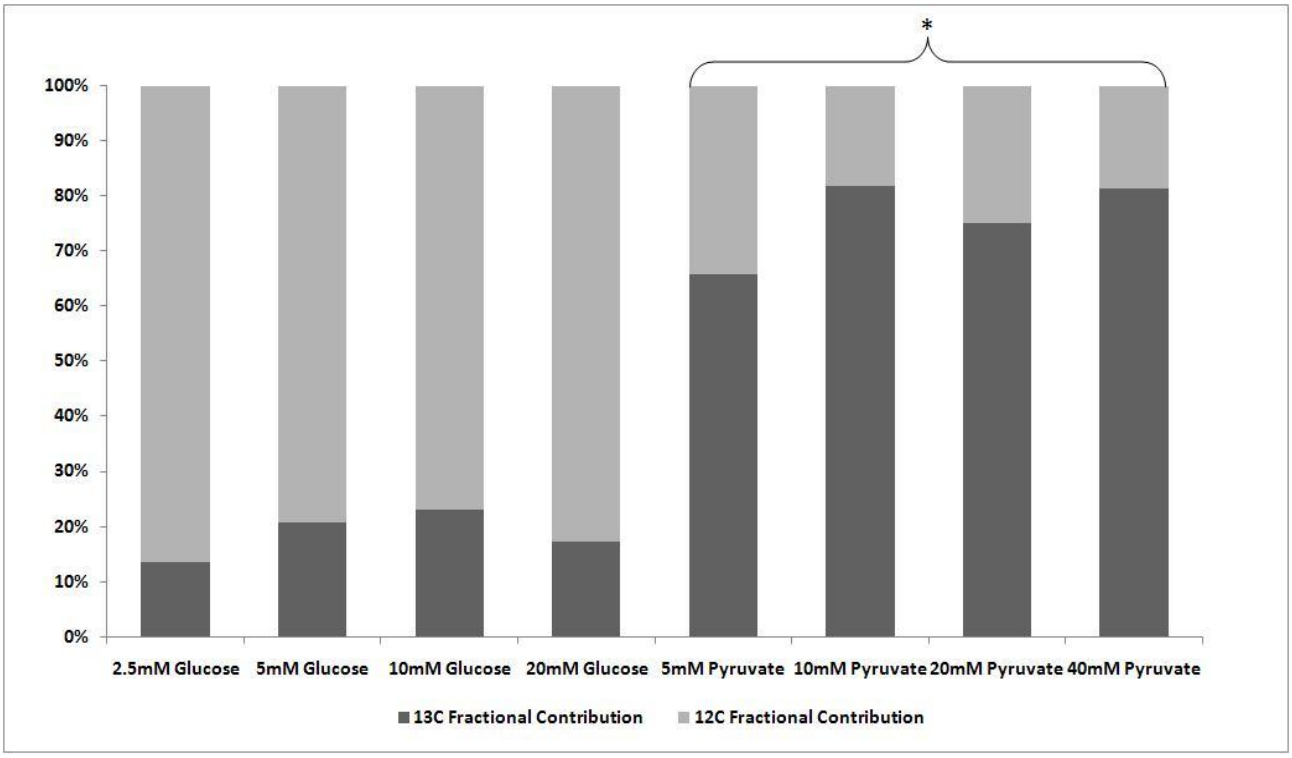


Figure 2.3 Fractional contributions of unlabeled (^{12}C) and labeled (^{13}C) substrates to TCA cycle intermediates. * identifies groups that exhibited significantly greater ^{13}C enrichment compared to all other groups by one-way ANOVA (p<0.001).

Combining the isotopomer data with MVO_2 information produced calculations of metabolic flux of exogenous labeled substrates (either glucose or pyruvate in the solution) and endogenous unlabeled substrates within the myocytes. No differences in flux rates were observed amongst the glucose groups, and no differences were observed amongst the pyruvate groups respectively. However, exogenous substrate flux was significantly higher in all pyruvate groups compared to all glucose groups. Conversely, flux of endogenous substrates through the TCA cycle was decreased significantly in all pyruvate groups. Anaplerosis, expressed as fraction of citrate synthase flux, was also low in all glucose perfused groups (0.02-0.08) but was important in replenishing the TCA cycle intermediate pool in all pyruvate perfused groups (0.33-0.47) ($p<.05$ versus all glucose groups). See Table 2.4.

Table 2.4. Tricarboxylic acid cycle flux and anaplerosis

Group	Exogenous Substrate Flux	Endogenous Flux	Total Flux	Anaplerosis (Fraction citrate synthase flux)
2.5 mM Glucose	0.12 ± 0.01	0.85 ± 0.09	0.97 ± 0.10	0.02 ± 0.01
5 mM Glucose	0.15 ± 0.04	0.59 ± 0.01	0.74 ± 0.04	0.02 ± 0.02
10 mM Glucose	0.16 ± 0.02	0.58 ± 0.06	0.74 ± 0.04	0.08 ± 0.02
20 mM Glucose	0.11 ± 0.03	0.56 ± 0.09	0.67 ± 0.10	0.03± 0.02
5 mM Pyruvate	0.56 ± 0.04*	0.27 ± 0.04*	0.82 ± 0.08	0.33 ± 0.04*
10 mM Pyruvate	0.57 ± 0.03*	0.11 ± 0.02*	0.68 ± 0.03	0.40 ± 0.07*
20 mM Pyruvate	0.59 ± 0.10*	0.16 ± 0.05*	0.76 ± 0.06	0.47 ± 0.12*
40 mM Pyruvate	0.59 ± 0.11*	0.12 ± 0.02*	0.71± 0.12	0.33 ± 0..08*

Results represent contributions of exogenous (^{13}C labeled) and endogenous (unlabeled) substrate to TCA cycle flux. The rate of anaplerosis is described relative to the citrate synthase reaction. Data are Mean ± SEM. Units for all groups are µmol/g dry weight/min unless otherwise noted.

*- $p<.05$ versus all glucose containing groups.

2.4 Effect of Acetate on myocardial metabolism

2.4.1 Perfusion Parameters and MVO_2

All hearts achieved a perfusate temperature of less than 10ºC and maintained this temperature throughout the perfusion interval. There were no differences in MVO_2 amongst any of the groups. Within groups, MVO_2 did not change over time (data not shown). There were no changes in preservation solution pH at any time point. Myocardial water content at end-experiment was similar among groups. See Table 2.5.

Table 2.5. Perfusion parameters and MVO_2

Group	Temperature (ºC)	Perfusate pH	Water Content	MVO_2 (µmol/g dry weight/min)
10 mM Glucose	8.6 ± 0.1	7.28 ± 0.01	0.76 ± 0.01	1.9 ± 0.2
2 mM Acetate	7.5 ± 0.5	7.16 ± 0.01	0.77 ± 0.01	1.0 ± 0.1
4 mM Acetate	7.9 ± 0.3	7.16 ± 0.05	0.77 ± 0.01	1.2 ± 0.2
8mM Acetate	8.4 ± 0.7	7.15 ± 0.03	0.78 ± 0.01	0.9 ± 0.1
16 mM Acetate	8.0 ± 0.2	7.14 ± 0.01	0.77 ± 0.01	1.2 ± 0.1

Data are Mean ± SEM.

There were no significant differences in perfusion conditions, myocardial water content, and oxygen consumption between groups.

2.4.2 Anaerobic Metabolism

The lactate/alanine ratios measured in all groups were consistent with an oxidative metabolic profile but tended to be lower in acetate groups. This suggests a preference for acetate to undergo metabolism through the TCA cycle and other non-*lactate dehydrogenase*-dependent pathways. See Table 2.6. In the glucose group, a moderate enrichment of lactate (37%) was observed, consistent with metabolism through glycolytic pathways. ^{13}C enrichment of alanine was much less (11%), again

suggesting minimal participation in non-glycolytic pathways including oxidation within the TCA cycle[21]. Alanine enrichment increased with increasing concentrations of acetate, with only the 16mM concentration reaching statistical significance.

Table 2.6. Metabolic results

Group	Lactate/Alanine	Enrichment (%)	Fractional Substrate Utilization (%)	Exogenous Flux (µmol/min/g dry wt)	Endogenous Flux (µmol/min/g dry wt)
10 mM Glucose	1.3±.1	11±1	23±4	0.09±0.01	0.31±0.04
4 mM Acetate	.73±.2*	12±3	57±5*	0.33±0.07*	0.17±0.03*
8 mM Acetate	.53±.2*	20±10	55±6*	0.20±0.03*	0.12±0.02*
16 mM Acetate	.40±.1*	29±9*	69±3*	0.42±0.04*	0.13±0.01*

Data are Mean ± SEM.

* - $p<.05$ versus all glucose groups

All groups continued to extract oxygen throughout the cold perfusion interval though MVO_2 was lower in the 8mM acetate group. Myocardial water content and lactate accumulation were not different between groups. Lactate/alanine ratios were lower in all acetate groups compared to the glucose control group, again implying a preference for acetate to undergo metabolism through the TCA cycle and other non-*lactate dehydrogenase*-dependent pathways. ^{13}C enrichment of TCA cycle intermediates by MRS increased with higher acetate concentrations in the perfusate, and this increase in ^{13}C enrichment was significantly greater in the 16mM acetate group compared to the 10mM glucose group. Fractional substrate utilization was significantly greater in all acetate groups compared to the 10mM glucose group. Finally, exogenous and endogenous flux were significantly different in all acetate groups

compared to the 10 mM glucose group, further confirming increased endogenous substrate utilization when glucose was the only available substrate in the preservation solution.

2.5 Effect of Acetate plus Propionate on myocardial metabolism

2.5.1 Perfusion Parameters and MVO_2

All hearts achieved a perfusate temperature of less than 10ºC and maintained this temperature throughout the perfusion interval. There were no differences in MVO_2 amongst any of the groups. Within groups, MVO_2 did not change over time (data not shown). There were no changes in preservation solution pH at any time point. Myocardial water content at end-experiment was similar among groups. See Table 2.7.

Table 2.7 Perfusion parameters and MVO_2

Group	Temperature (ºC)	Perfusate pH	Water Content	MVO_2 (µmol/g dry weight/min)
2 mM Acetate	7.5 ± 0.5	7.16 ± 0.01	0.77 ± 0.01	1.0 ± 0.1
4 mM Acetate	7.9 ± 0.3	7.16 ± 0.05	0.77 ± 0.01	1.2 ± 0.2
8mM Acetate	8.4 ± 0.7	7.15 ± 0.03	0.78 ± 0.01	0.9 ± 0.1
16 mM Acetate	8.0 ± 0.2	7.14 ± 0.01	0.77 ± 0.01	1.2 ± 0.1
2 mM Acetate+Propionate	8.0 ± 0.4	7.12 ± 0.00	0.77 ± 0.01	1.3 ± 0.1
4 mM Acetate+Propionate	8.9 ± 0.2	7.11 ± 0.00	0.77 ± 0.01	1.1 ± 0.1
8mM Acetate+Propionate	7.7 ± 0.3	7.14 ± 0.01	0.78 ± 0.01	0.9 ± 0.1
16 mM Acetate+Propionate	7.4 ± 0.3	7.12 ± 0.01	0.80 ± 0.01	0.8 ± 0.0

Data are Mean ± SEM.

There were no significant differences in perfusion conditions, myocardial water content, and oxygen consumption between groups.

2.5.2 Anaerobic Metabolism

All groups continued to extract oxygen throughout the cold perfusion interval though MVO_2 as noted previously was lower in the 8mM acetate group. This may be an artifact. Myocardial water content and lactate accumulation were not different between groups. Lactate/alanine ratios were not different amongst any groups whether or not propionate was added. ^{13}C enrichment of TCA cycle intermediates by MRS revealed only minor differences amongst all groups. No consistent effect on flux of exogenous substrates or flux of endogenous substrates was observed in this study. This implies that efforts to increase metabolism by augmenting TCA cycle intermediates may not achieve this goal. As oxygen consumption was not different with propionate added, an increase in total TCA cycle flux cannot be implied.

Table 2.8 Metabolic results

Group	Lactate/Alanine	Enrichment (%)	Fractional Substrate Utilization (%)	Exogenous Flux (µmol/min/g dry wt)	Endogenous Flux (µmol/min/g dry wt)
2 mM Acetate	0.54±.2	29±5	65±8	0.30±0.04	0.12±0.02
4 mM Acetate	0.73±.2	12±3	57±5	0.33±0.07	0.17±0.03
8 mM Acetate	0.53±.2	20±10	55±6	0.20±0.03	0.12±0.02
16 mM Acetate	0.40±.1	29±9	69±3	0.42±0.04	0.13±0.01
2 mM Acetate + Propionate	0.34±.1	36±5*	49±3*	0.32±0.01	0.25±0.04*
4 mM Acetate + Propionate	0.37±.1	33±5*	52±3	0.29±0.05	0.18±0.01
8 mM Acetate + Propionate	0.42±.1	30±2	66±4	0.28±0.03	0.10±0.01
16 mM Acetate + Propionate	0.49±.2	44±16	52±3*	0.22±0.02*	0.14±0.01

Data are Mean ± SEM.

* - $p<.05$ versus all corresponding concentrations

2.6 Discussion

All existing clinically-used preservation solutions for heart transplantation are designed for use under conditions in which efforts are focused on reducing metabolic demand of the organ during storage and transport through hypothermia and induced (cardioplegic) arrest. The paradigm of machine perfusion is fundamentally different. With machine perfusion, metabolism can continue, and possibly should be stimulated, to allow metabolic processes that may contribute to cellular homeostasis or repair. Prior studies from our laboratory have evaluated myocardial metabolism under conditions of machine perfusion using a commercially available solution with ^{13}C glucose as the provided substrate[15,16]. In these experiments, donor hearts stored with machine perfusion sustained less functional impairment after storage, accumulated less tissue lactate and exhibited less myocardial necrosis without increasing myocardial edema. However, one observation was unexpected in these large animal studies. While hearts in the perfusion preservation group extracted dissolved oxygen continuously throughout the 4 hour storage interval (consistent with ongoing oxidative metabolism) analysis of left atrial tissue extracts by ^{1}H MRS revealed less than 1% glutamate enrichment in these animals. As the natural abundance of ^{13}C is 1.1%, no oxidation of exogenous labeled glucose was detected. Any lactate that was detected was also unlabeled. It is likely that oxygen consumption reflected oxidation of endogenous myocardial substrates (glycogen, triglycerides, etc) and the available glucose in the preservation solution (all ^{13}C-labeled) was not metabolized by aerobic or anaerobic pathways. Therefore, existing commercially-available preservation solutions (which include no metabolic substrates or glucose only) appear poorly suited to take advantage of machine perfusion techniques.

The reasons behind the ineffectiveness of glucose are not established. The heart can oxidize a wide range of substrates and glucose alone can support isolated working hearts. However, in these experiments, machine perfusion was performed under profound hypothermia and depolarized arrest, conditions that have been shown to alter myocardial substrate selection[104,105]. As well, uptake of glucose

may be inefficient at low temperature, particularly when insulin is not available[106]. These findings led us to design the current study that assesses alternative substrates in the preservation solution.

Pyruvate is an attractive option as shown in our prior work[107,108]. It readily enters the cell through a non-energy-dependent process via monocarboxylate transporters[109], bypasses the feedback inhibition of 1,6-phospho¬fructo¬kinase by citrate that regulates glucose metabolism, and stimulates its own entry into the TCA cycle as acetyl-CoA formed via pyruvate dehydrogenase. Pyruvate has been shown to reduce reactive oxygen species (ROS) formation that may further inhibit carbohydrate metabolism in other transplant models[110]. It may reduce ROS formation through the NADH oxidase reaction by altering the cytosolic redox state[111] or act as a ROS scavenger directly by non-enzymatically reacting with H_2O_2 [112]. As well, supraphysiologic pyruvate levels favor glucose entry into the pentose-phosphate shunt leading to generation of the glutathione reductase cofactor NADPH and regeneration of reduced glutathione[113]. One would not expect TCA cycle intermediates to be depleted over time as pyruvate can serve as an anaplerotic substrate via pyruvate carboxylase[114].

The present study confirmed that glucose, even when provided at high concentrations, is minimally effective in participating in oxidative pathways under conditions modeling machine perfusion. Pyruvate appears to represent a better alternative as an exogenous substrate as ^{1}H and ^{13}C spectroscopy demonstrated significantly increased incorporation of labeled carbon into TCA cycle intermediates, a process that can only occur through oxidative metabolism. This is a theoretical advantage, as metabolism could be enhanced allowing greater energy generation to support cellular homeostasis.

It is noteworthy that global MVO_2 was not different amongst any groups studied. Since glucose contributed minimally, this observation implies that hearts perfused with glucose as the provided substrate use endogenous fuels such as glycogen or lipids to support oxidative metabolism. In models of ischemia, metabolism of glycogen[115] or lipids[116,117] can be associated with inferior recovery. With pyruvate

serving as a dominant source of oxidative substrate, one would anticipate less myocyte catabolism of endogenous energy stores.

The degree of glutamate enrichment by ^{13}C decoupled ^{1}H MRS increased with increasing concentrations of pyruvate up to 20 mM, then declined at a concentration of 40 mM. However, the contribution of the pyruvate to myocardial oxidative metabolism by ^{13}C MRS was unaffected over the concentrations studied (65.8-81.8%). This difference between the two MRS techniques may be the result of reduced exchange between TCA cycle intermediates and the intracellular glutamate pool. The reason for this is not known but might relate to changes in the electrolyte composition of the solution as pyruvate is added without removing any other preservation solution component. Pyruvate is neutralized with sodium hydroxide to maintain a normal solution pH, but this increases the solution sodium concentration. Preservation solution electrolyte composition can influence oxidative metabolism in models of machine perfusion[49]. As well, we have observed a plateau of enrichment with similar pyruvate concentrations in models of lung preservation[107].

Anaplerotic pathways are important in replenishing the TCA cycle intermediate pool but usually represent less than 10% of TCA cycle flux in the heart. In the current experiments, anaplerosis in glucose groups was quite low but it was markedly elevated in all pyruvate groups suggesting that pyruvate is more effective in sustaining the TCA cycle and avoiding intermediate losses to disposal pathways. Anaplerosis calculations assume steady-state conditions but the acetyl-CoA enrichment data indicated that steady-state had not quite been achieved. This tends to overestimate anaplerosis[102]. We also assumed that anaplerotic flux was via pyruvate carboxylase and therefore had little effect on TCA cycle flux calculations. Despite these limitations, important differences in anaplerosis appear to be present between glucose and pyruvate perfused groups.

As noted above, Glucose is minimally effective in participating in oxidative pathways under conditions modeling machine perfusion. Acetate, a simple 2-carbon molecule appears better suited as an exogenous substrate as MRS demonstrated significantly increased incorporation of labeled carbon into

TCA cycle intermediates. Associated with this was a reduction in oxidation of endogenous energy stores. It is noteworthy that acetate (2-carbon) even at a concentration of 4 mM led to greater TCA cycle enrichment than glucose (6-carbon) at 10 mM. The flow of carbon from exogenous sources was at least 5-fold greater with acetate. The mechanism behind this observation is unknown but possibly stems from a systemic preference towards smaller carbohydrate energy sources at low temperatures. It may be that bypassing all of the glycolytic enzymes that are required for glucose catabolism renders acetate more efficient under these conditions.

Based on data from proton spectroscopy, enrichment appears to vary with different concentrations of acetate in the solution. At low concentrations of acetate, enrichment of glutamate is low. Supplementing these low concentrations of acetate with propionate leads to a significant increase in enrichment. This enrichment must be due to greater oxidation of acetate. Therefore it is possible that adding an anaplerotic substrate may increase the pool of TCA cycle intermediates and allow greater oxidation of an exogenous energy source. At higher concentrations of the exogenous fuel the need for enhancement of intermediates appears to diminish. Further analysis of carbon spectra, which assesses the oxidation rates at the time of freeze clamping, did not confirm an increase in acetate oxidation with the addition of propionate.

Thus the addition of propionate as an anaplerotic substrate did not lead to an increase in oxygen consumption or an increase in oxidation of exogenous labeled acetate. Our hypothesis was that ongoing oxidation of acetate which has no anaplerotic potential would lead to a depletion of citric acid cycle intermediates over time that could reduce oxidation rates. However, no reduction of oxidation of acetate was identified over the 6 hour study interval (at least as assesses by myocardial oxygen consumption). Therefore, the addition of propionate may have been superfluous under these conditions. Analysis of TCA cycle intermediates could help to verify or refute this theory.

2.7 Summary

These data suggest that myocardial oxidative metabolism is maintained over 6 hours under conditions modeling machine perfusion for transplantation. When glucose is provided in the preservation solution as the only metabolic substrate, minimal oxidative metabolism is observed. Pyruvate appears to be a superior alternative as it is oxidized to a significantly greater extent and replenishes TCA cycle intermediates. As such it should be the dominant source of energy generation, which is the principal goal of any added substrate. Interestingly acetate as an added exogenous substrate also led to enhanced oxidation rates in this study. Thus even simple carbon sources may be advantages under conditions modeling machine perfusion. With the addition of propionate, ^{13}C enrichment of TCA cycle intermediates by MRS revealed only minor differences amongst groups. No consistent effect on flux of exogenous substrates or flux of endogenous substrates was observed by adding this anaplerotic substrate. Efforts to increase metabolism by augmenting TCA cycle intermediates may not be effective. As oxygen consumption was not different with propionate added, an increase in total TCA cycle flux cannot be implied.

Currently available preservation solutions may not be optimal for machine perfusion applications. Further studies are warranted to evaluate other substrate options and to assess the relative effects of substrate modifications on functional recovery after preservation.

CHAPTER 3

DETERMINATION OF OPTIMAL RETROGRADE PERFUSION CONDITIONS

3.1 Introduction

Machine perfusion for preservation of donor hearts has been investigated for nearly 50 years.[56] This strategy offers a number of potential benefits for improving preservation of donor hearts and extending the donor pool including: 1) tolerance of prolonged donor ischemic intervals and long distance procurements. 2) utilization of extended donors 3) resuscitation of injured hearts 4) better donor-recipient matching (such as by human leukocyte antigens).[56,97] It is therefore surprising that this technique has not gained greater enthusiasm despite improved clinical results after kidney transplantation,[97] and improved myocardial preservation in animal models of short and long-term heart preservation.[16,47,61,118]

Virtually all previous studies of machine perfusion preservation of hearts involve antegrade coronary artery perfusate delivery through the aortic root. Our laboratory has previously demonstrated that although antegrade perfusion preservation of hearts is superior to standard hypothermic cold storage this technique may be limited by aortic valve incompetence which may in part explain the reluctance to develop this technology for cardiac transplantation. We demonstrated that this limitation can potentially be avoided with an initial high flow of perfusion solution to allow for aortic valve leaflet closure prior to initiation of standard cold perfusion flow rates.[119] However, adequate aortic valve closure is difficult to verify in the explanted, perfused heart. Also, these experiments were performed in a laboratory setting with a stable perfusate delivery platform. One would anticipate that transport of donor hearts either by air or ground transport could risk further distraction of aortic valve leaflets, worsen aortic insufficiency, thereby decreasing myocardial perfusion and nutrient flow when applying an antegrade perfusion technique. For standard ischemic intervals this would be of minor consequence as hearts would essentially be undergoing static storage. For extended, long term storage or when resuscitating injured donor organs, inadequate myocardial perfusion could have catastrophic consequences.

An alternate strategy for machine perfusion of hearts involves retrograde perfusion through the coronary sinus. Using a standard retrograde cardioplegia catheter, preservation solution can be delivered directly into the coronary venous system.[120] This technique avoids the issue of aortic valve incompetence. Additionally, perfusion with oxygenated blood cardioplegia can be continued during the implantation period, virtually eliminating the detrimental effects of the warm ischemic interval.[121] Retrograde cardioplegia by perfusion through the coronary sinus is used routinely for myocardial protection during cardiac surgery.[122-124] Although retrograde perfusion is a reliable method of delivering cardioplegia for cardiac surgery, its efficacy for preservation of myocardium under deep hypothermia and ex vivo perfusion of hearts is not known. Retrograde perfusion has rarely been used for preserving donor hearts, and optimal perfusion parameters for this application will need to be defined.[125] Additionally, differences in regional myocardial perfusion, particularly of the right ventricle, may be a limitation with retrograde perfusate delivery.[126,127] Because of these concerns, we decided to investigate retrograde coronary sinus perfusion for preservation of hearts for transplantation. The current study was designed to determine the optimal perfusion parameters and evaluate regional myocardial flow during retrograde perfusion of canine hearts.

3.2 Materials and Methods

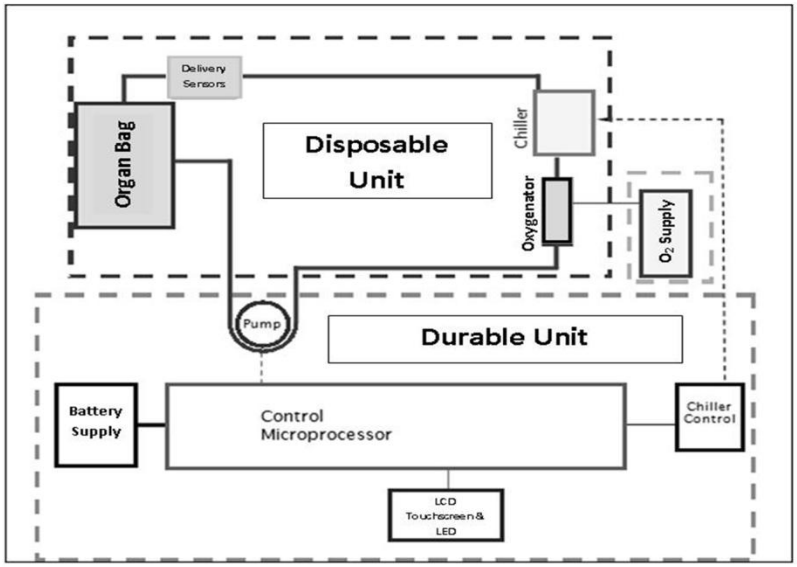
The protocol for this study was approved by the Institutional Animal Care and Use Committee at the University of Texas Southwestern Medical Center. All animals were treated within guidelines set forth in the Guide for the Care and use of Laboratory Animals (National Institutes of Health Publication No. 86-23, revised 1996).

3.2.1 Animal Protocol

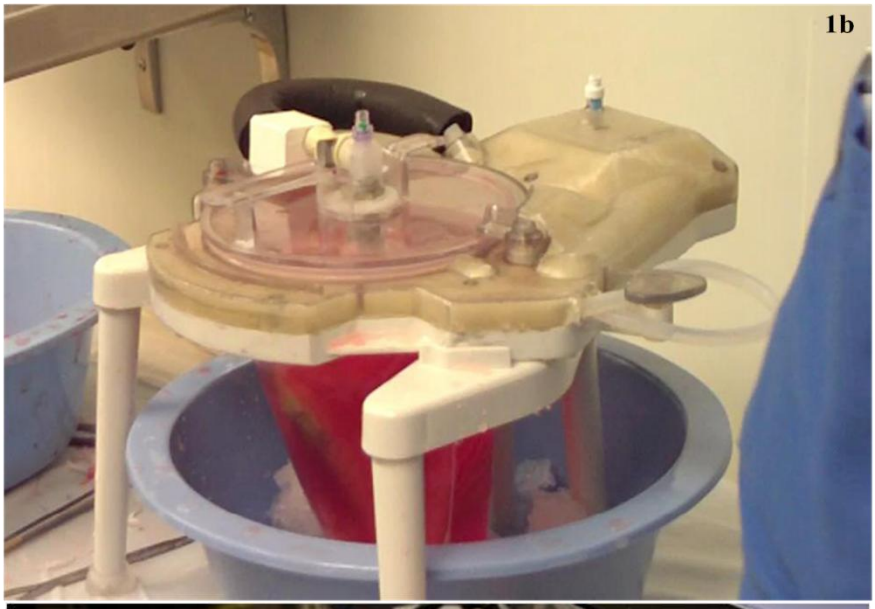
Adult mongrel dogs (n=6, average weight 28.6 kg ± 3.6, range 24.1-34.0 kg) were used in this experiment. In each experiment, hearts were removed from anesthetized dogs and established in a perfusion device that provided continuous perfusion of oxygenated fluid through a calibrated pump system that enabled precise control of flow rate, oxygenation, and fluid temperature (LifeCradle^{TM}, Organ Transport Systems, Inc, Frisco, TX). The hearts were flushed antegrade with University of Wisconsin Machine Perfusion Solution (UW-MPS) to achieve a diastolic arrest, connected to the perfusion device with a coronary sinus catheter (Medtronic Inc, Minneapolis, MN), and perfused with varying flow rates from 10 mL/100g/min to 35 ml/100g heart weight /min at 5 ± 2 °C for 20 minute intervals with UW-MPS. The catheter was secured to the coronary sinus ostium with a 4-0 prolene suture. Also, the balloon was not inflated to avoid malperfusion of the posterior descending and right ventricular venous system. A schematic of the device, and images of the disposable unit and loaded device are shown in Figures 3.1a-c.

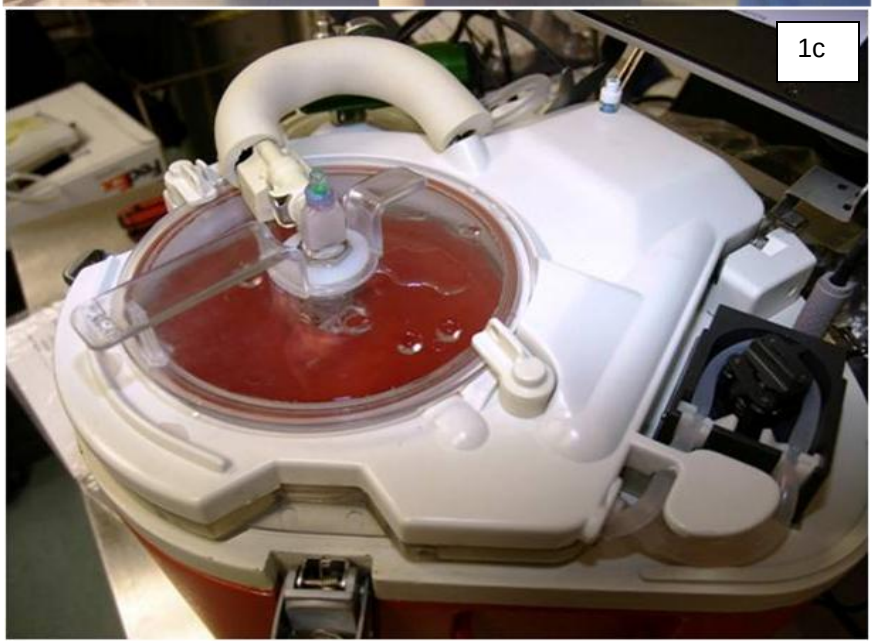
Figures 3.1a-c Perfusion Device

Figure 1a represents a schematic of the device and its components. The disposable unit is shown in Figure 1b. Figure 1c demonstrates the disposable unit loaded into the prototype device with the cover removed during a perfusion experiment.



Figures 3.1a-c Perfusion Device (continued)





Flow rates were selected based on the assumption that capillary delivery of the preservation solution would be less efficient than with antegrade perfusion because of non-nutrient flow through Thebesian

channels and direct venous drainage into cardiac chambers.[128] This range also takes advantage of the entire device flow rate delivery capability.

3.2.2 Data Collection

Colored microspheres were used to quantify nutrient flow to the tissues (IMT Laboratories, Irvine, CA) as previously described.[15] To perform this analysis, the heart was gently repositioned from the perfusion chamber to the sampling container; the side branch of the perfusate delivery line was opened, and the recirculating line was clamped to allow delivery of the bead-containing solution into the device through the inflow cannula for 2 minutes at the same flow rate without recirculation. A sample of the bead solution was collected for bead quantification. After 2 min, the inflow was switched to the alternative (washout) solution to clear any beads from the tubing by flowing at the same rate for a time calculated to deliver 120 mL of solution from the chamber. At this point, the heart was returned to the initial perfusion chamber and the second randomly selected flow rate was dialed in. The bead solution was changed to one with a different color of beads. After 20 min the sequence was repeated. This process continued until all 6 flow rates were completed. After the final washout, the heart was removed from the sampling chamber. The heart was dissected, and a 1-cm slice at the midventricular level was obtained to quantify segmental perfusion. Tissue samples (2-3 g) of endocardial and epicardial tissue from the anterior, lateral, and inferior left ventricle, the left and right ventricular septum, the right ventricular free wall, and the left atrial appendage were harvested and stored. In addition, fluid samples from the overflow and perfusion device chambers were collected, and the final volume of both chambers was measured. Bead counts of fluid samples and digested tissue were quantified with a NuFlow cytometer (IMT Laboratories, Irvine, CA).

Myocardial tissue perfusion rates and the fraction of non-nutrient flow were calculated as previously described8 by measuring the number of beads collected in the sampling container for each flow rate and dividing this by the total number of delivered beads. The nutrient flow fraction was then

calculated as 1 – the fraction of beads collected in the sampling container (i.e. the non-nutrient flow fraction) for each flow rate. Nutrient flow was then determined by multiplying the nutrient flow fraction by the delivered volume per 100g cardiac tissue per minute for each flow rate. Nutrient flow calculations were based on the bead data from the sampling container only. Subsequent bead loss into the perfusion chamber was measured but assumed to represent nutrient flow since it occurred after the washout period. Regional myocardial perfusion was determined in tissue by digesting the tissue, recovering entrapped beads and by dividing the entrapped microsphere count per gram myocardial tissue by the expected nutrient flow per gram myocardium.

Temperature and pressure were measured continuously in perfused hearts. Oxygen consumption (MVO_2) was calculated from inflow pO_2, outflow pO_2 (drawn from the left and right coronary arteries), and the actual delivered flow rate. At end-perfusion, tissue samples of left and right ventricle were collected for proton magnetic resonance spectroscopy (^{1}H MRS). The tissue samples were harvested, immediately freeze-clamped, and cooled in liquid nitrogen. The tissue was stored in an –80°C freezer and extracted subsequently with perchloric acid. Purified extracts were reconstituted in D_2O, and pH was adjusted to 7.0–7.4 for magnetic resonance spectroscopy (MRS). Proton (^{1}H) MR spectra were then acquired with a 14.1 Tesla Varian spectrometer operating at 600 MHz over a spectral width of 8000 Hz. Lactate-to-alanine ratios were compared from ^{1}H spectra as measures of cellular aerobic and anaerobic metabolism during storage. Wet-to-dry ratios and myocardial water content were determined from separate duplicate right and left ventricular samples.

3.2.3 Statistical Analysis

Data were analyzed with SigmaPlot statistical software (SyStat Software, Inc, San Jose, CA) using a two-sided t-test or analysis of variance (ANOVA), as appropriate. When data were collected over multiple time points, a repeated-measures ANOVA was applied. A p-value $< .05$ was considered significant.

3.3 Results

Hypothermia was maintained at all flow rates. The average total perfusion preservation period (donor ischemic time) was 204 ±14 minutes. Heart weight increased significantly over the perfusion interval from 211±30g to 267±43g (Δ 27%, p=.03). Coronary sinus pressures increased with increasing flow rates but this difference did not reach statistical significance (p=.13). Calculated nutrient flow increased over the range of delivered device flow rates. This difference was significant for each 10mL/100g/min flow increase increment (p<.05). MVO_2 was calculated using the left coronary artery outflow sample only. Flow in the right coronary artery was low and did not yield meaningful data. Small increases in MVO_2 were noted up to 20ml/100g/min but this difference was not statistically significant compared to the lower two flow rates (10 and 15mL/100g/min). See Table 3.1.

Table 3.1 Flow rate comparisons

Parameter	Flow Rate (mL/min/100 g heart tissue)					
	10	**15**	**20**	**25**	**30**	**35**
Temperature (°C)	6.0±.8	5.6±.9	6.2±.6	5.6±.6	6.0±.5	5.9±.4
Coronary Sinus Pressure (mm Hg)	10.6±6	12.1±6	14.7±15	18.1±11	23.0±21	23.0±21
Nutrient flow (mL/100g/min)	5.9±2	7.3±3	9.3±3*	10.7±2*	13.5±3*	14.8±5*
Nutrient flow (% predicted)†	47.9±21	54.7±21	43.1±14	41.0±7	43.2±4	41.2±14
MVO_2 (mL/100g/min)	0.12±.05	0.13±.06	0.16±.02	0.14±.04	0.19±.04	0.17±.08

Data are mean ± one standard deviation.

* - p<.05 versus all flow rates 10mL/100g/min less than index flow rate

† - Data are derived from actual delivered flows from microsphere studies.

Total right ventricular (RV), and left atrial (LA) nutrient flows were significantly reduced at all flow rates except 10mL/100g/min compared to left ventricular (LV) nutrient flow ($p<.05$). See Figure 3.2.

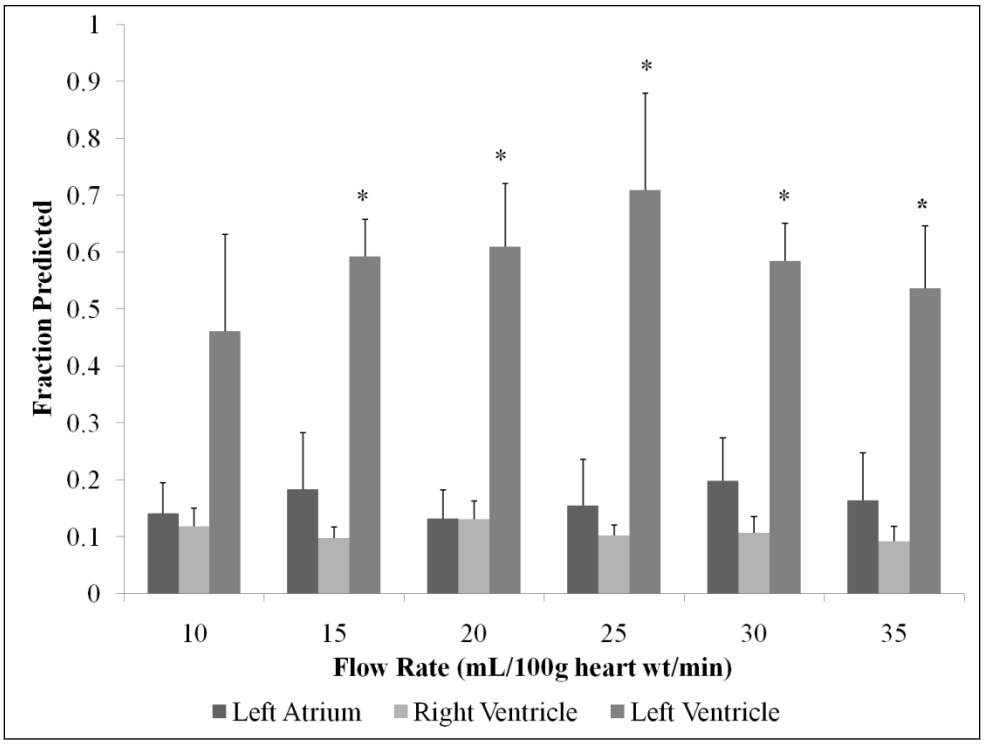


Figure 3.2 Regional myocardial nutrient delivery. * - $p<.05$ versus right ventricular and left atrial nutrient flow

There were no differences in epicardial and endocardial perfusate delivery within all myocardial segments (data not shown). Nutrient flow among all RV segments (RV free wall, RV septum) and LV segments (Anterior, lateral, inferior, septal) was similar (data not shown). Myocardial water content and wet to dry ratios at the end of each experiment were lower in right ventricular samples. See Table 3.2.

Table 3.2 Regional myocardial water content and edema

Region	Water Content (%)	Wet/Dry Ratio
Right Ventricle	77.7±1*	4.5±.3*
Left Ventricle	81.3±2	5.5±.5

Data are mean ± one standard deviation.

*-p<.05 versus Left Ventricle

Lactate/Alanine ratios were low in all LV samples, consistent with ongoing oxidative metabolism. However, lactate/alanine ratios were increased in right ventricular tissue again suggesting reduced RV perfusion though this difference did not quite reach statistical significance (p=.09). The highest lactate/alanine ratios were noted in left atrial samples consistent with reduced oxygen delivery and potentially anaerobic metabolism. This increase was significant compared to LV (p<.01) but not RV tissue (p=.14)). See Figure 3.3. Sample ^{1}H MR regional spectra from each region of myocardium are shown in Figure 3.4.

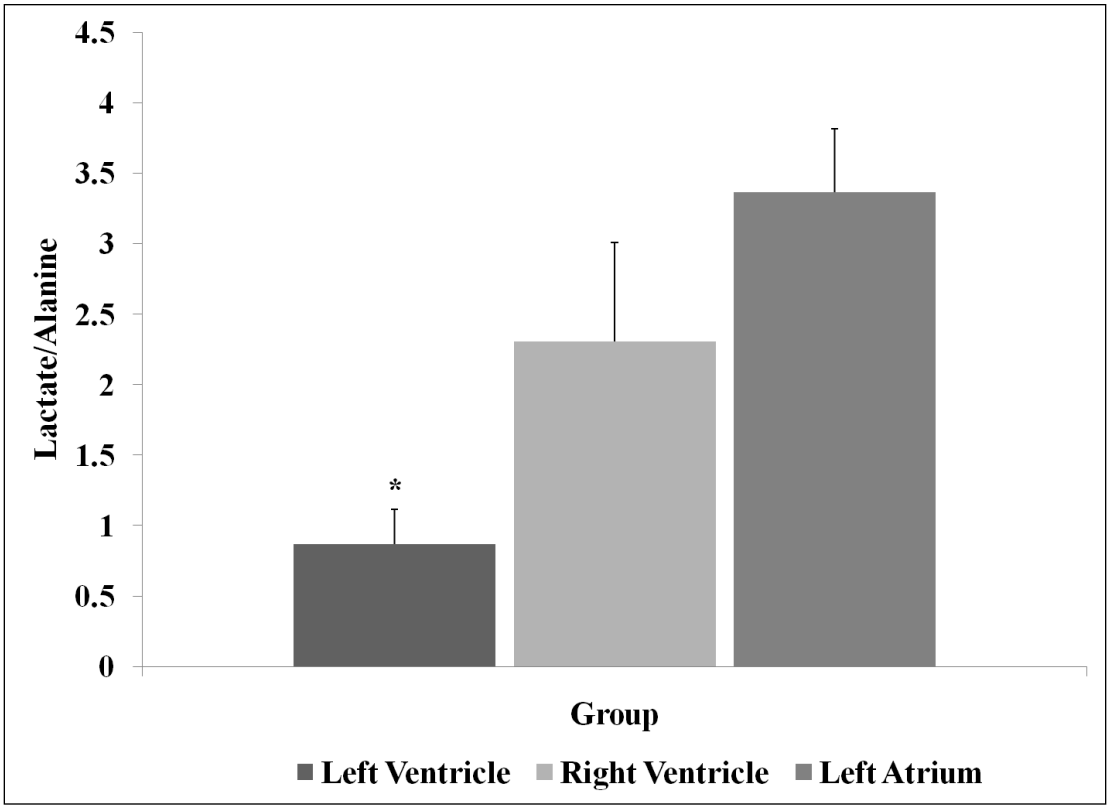


Figure 3.3 Regional myocardial lactate/alanine ratios.

* - p<.05 versus Left Atrium Group

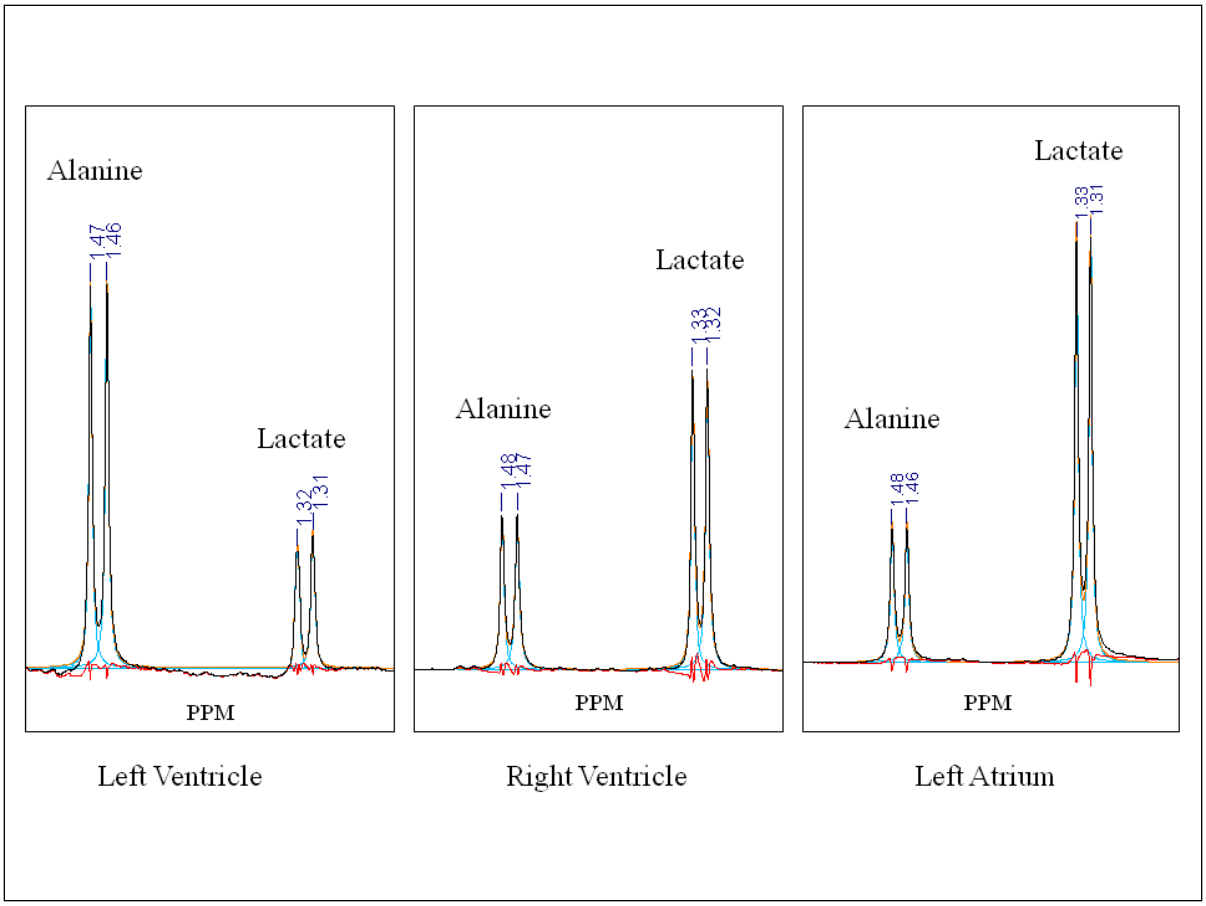


Figure 3.4 Representative ^{1}H magnetic resonance spectra

Representative left ventricular, right ventricular, and left atrial ^{1}H MR spectra demonstrating progressively increasing lactate to alanine ratios in right ventricular and left atrial tissue.

3.4 Discussion

Pratt first described that retrograde perfusion through the coronary venous system could be used to support the heart of a cat.[129] Subsequently, Lillihei at al reported the first clinical application of this technique in a patient with aortic stenosis.[130] Since then, retrograde cardioplegia has evolved as an effective technique for myocardial protection during cardiac surgery. A number of clinical and experimental studies support the use of retrograde perfusion for maintaining the myocardium during both depolarized cardioplegic arrest and normokalemic perfusion.[124,131-133]

The enthusiasm for retrograde perfusion is not uniform. Although retrograde cardioplegia is an established technique for myocardial protection during cardiac surgery, concerns regarding the distribution of retrograde perfusion have been raised. Tian et al reported that global nutrient delivery and myocardial energetics are less well supported by retrograde perfusion compared to antegrade perfusion at the same flow rates and proposed higher retrograde perfusion pressures and flow rates to overcome this limitation.[128,135] Other authors suggest that right ventricular protection, in particular, might be compromised because some portion of the blood supplying the right ventricle drains directly into the right atrium and ventricle, bypassing the coronary sinus.[120] Smith and Partington both reported reduced right ventricular perfusion using retrograde microsphere perfusion of canine hearts, particularly in the presence of patent coronary arteries as would be expected in organs procured for transplantation.[126,136] Concerns regarding right ventricular perfusion by coronary sinus perfusion have not been borne out in most human studies and may be due to differences between canine and human cardiac venous anatomy.[120]

In the current study, retrograde perfusion preservation resulted in excellent LV protection with similar MVO_2 and indices of metabolism compared to our previous antegrade machine perfusion studies except at the lowest evaluated flow rate.[16,119,137] Right ventricular MVO_2 could not be assessed due to inadequate right coronary artery effluent. Perfusion and metabolic parameters as measured by the lactate/alanine ratio and microsphere data also suggest that RV perfusion is impaired to some extent, a phenomenon that appeared to be independent of the flow rate. Left atrial flow also was reduced as

assessed by nutrient flow and metabolic data. This reduction in left atrial flow would not seem surprising since the left atrium is transected during procurement and the lowest resistance drainage from the left atrial venous system would be directly into the perfusate, bypassing capillary beds. This observation does however raise a concern on whether left atrial biopsies could be used to assess myocardial metabolism as part of the transplant process. When retrograde perfusion is applied, metabolic findings were very disparate between left atrial and left ventricular samples. As a result, information gleaned from the left atrium may not reliably predict metabolic conditions in the left ventricle when the heart is protected through retrograde machine perfusion. This difference from our prior observations using antegrade machine perfusion and raises a cautionary note in use of left atrial data to support clinical decisions on adequacy of preservation for clinical cardiac transplantation.

Increased myocardial water content and edema are potential concerns when using machine perfusion for preservation of donor hearts. Left ventricular myocardial water content was higher in the current study compared to values previously reported with antegrade perfusion using the same preservation solution. This might be due in part to the effect of the higher flow rates used during this study compared to antegrade perfusion experiments. Right ventricular myocardial water content was lower which may reflect reduced perfusion of right ventricular capillary beds as documented by the microsphere data. Interestingly, similar increases in myocardial water content in an antegrade machine perfusion model from our laboratory did not impact reperfusion heart function and myocardial water content after transplantation was not different compared to static controls suggesting that the impact of heart weight gain during storage is uncertain.[49]

One of the reported benefits of machine perfusion preservation is the ability to support the metabolic demands of the stored organs during the preservation interval. We have previously reported use of the lactate/alanine ratio by ^{1}H MRS as a simple measure to differentiate aerobic metabolism and anaerobic metabolism.[15,49,137] This ratio takes advantage of the compartmentalization of anaerobic glycolysis and oxidative glucose metabolism.[103,138] Although alanine does not directly participate in

oxidative energy generating pathways (citric acid cycle), observed increases in intracellular alanine compared to lactate seem to be an indicator of increased oxidative metabolism, as well, when assessed directly with labeled substrates.[49] In the current study, LV oxidative metabolism appears preserved and the low lactate/alanine ratios are quite similar to previously reported data for antegrade perfused hearts.[137] Left atrial spectra demonstrated significantly increased lactate/alanine ratios similar to spectra from static storage hearts and are more consistent with anaerobic metabolism and reduced nutrient flow. Lactate/alanine ratios were intermediate for RV tissue suggesting at least reduced perfusion of the right ventricle and right ventricular septum. These data are consistent with findings from the microsphere experiments and indicate oxygenated perfusate delivery to the right ventricle may be insufficient to support oxidative metabolism with this technique. The impact of reduced nutrient delivery during storage on reperfusion RV function is uncertain. RV dysfunction after transplantation is multifactorial. It includes donor factors (RV dysfunction after brain death, donor management strategies), recipient factors (RV strain from increased pulmonary vascular resistance), and technical aspects during reimplantation (warm ischemic time) which may be as important as the metabolic state of the RV during perfusion preservation.[139,140]

This study has several limitations. First, important differences in coronary venous anatomy especially affecting right ventricular drainage have been reported between canine and human hearts.[119,130] MVO_2 calculations represent estimates only of cardiac oxygen consumption since the vast majority of the perfusate appears to have drained through the venous system. Flow rates in this study were assigned in random order and therefore might have affected metabolic data in samples taken from hearts that finished with high versus low flow rates. However, lactate to alanine ratios and MVO_2 data were similar independent of the final experimental flow rate. Only the reduced LV perfusion at 10mL/100g/min by microsphere analysis suggests that perfusion at the lowest examined flow rate may be inadequate to support the metabolic demands of the LV. RV perfusion was reduced independent of the delivered flow rate and perfusion pressure.

3.5 Summary

In summary, retrograde machine perfusion of canine hearts provides effective left ventricular perfusion, maintains myocardial oxygen delivery and supports myocardial oxidative metabolism at flow rates of 15mL/100g/min or higher. Right ventricular perfusion is reduced at all evaluated flow rates and may not be sufficient to support oxidative metabolism of the right ventricle. These data may have important implications for application of machine perfusion to preservation of human hearts for transplantation.

CHAPTER 4

EFFECTIVENESS OF RETROGRADE PERFUSION PRESERVATION AFTER SHORT-TERM STORAGE

4.1 Introduction

This study was designed to test a continuous perfusion strategy for cardiac preservation in a large animal model over a clinically relevant storage interval. It was conducted using techniques that allow the precise quantification of functional recovery using load independent indices of cardiac performance. As noted in Chapter 1, our laboratory has studied the effectiveness of machine perfusion using an antegrade perfusate delivery strategy and has identified advantages of this technique over static preservation, These prior studies will serve as comparator groups for the current analysis done examining machine perfusion with retrograde perfusate delivery.

4.2 Materials and Methods

4.2.1 Experimental Protocol

The protocol for this study was approved by the Institutional Animal Care and Use Committee at the University of Texas Southwestern Medical Center. All animals were treated in accordance with guidelines set forth in the *Guide for the Care and use of Laboratory Animals* (National Institutes of Health Publication No. 86-23, revised 1996).

Thirty-six adult mongrel dogs, of similar characteristics, were used in this experiment. Twelve donor-recipient pairs were randomized to either conventional hypothermic static preservation (n=6) or hypothermic preservation with a perfusion device (n=12) that provided a continuous perfusion of oxygenated fluid through a calibrated pump system that enabled the control of flow rate, oxygenation, and fluid temperature. The flow rate used was based on the results of the tissue perfusion experiments described previously: 15 mL/min/100 g for antegrade studies and 20 m/min/100 g for retrograde studies.

Excised donor hearts were stored for 4 hours, reimplanted into recipient animals, and reperfused for 6 hours. Commercially available Celsior organ preservation solution (SangStat Medical Corp., Fremont, CA) was used for storage of all hearts. Celsior was supplemented with 1 g/L (5.5 mmol/L) of U-^{13}C-labeled glucose to provide a substrate for evaluation of cellular metabolism during the storage period.

4.2.2 Anesthetic Protocol

Each animal was premedicated with 0.07 mg/kg atropine IM and 4.4 mg/kg telazol IM. The animal was intubated and ventilated with 100% oxygen at VT of 10 mL/kg, rate of 10/min, and PEEP of 5 cm H_2O. Anesthesia was maintained with 1% to 4% isofluorane. Central venous pressure, arterial pressure, and a surface electrocardiogram were continuously monitored. Ventilator settings were adjusted based on arterial blood gas measurements to keep the pCO_2 at 35 to 45 mmHg, pH 7.35 to 7.45, and oxygen saturation = 95%.

4.2.3 Donor Protocol

After sternotomy and exposure of the heart, animals were administered 300 units/kg of heparin intravenously and an ascending aortic cardioplegia catheter was inserted. Baseline myocardial function was measured (see below). The aorta was clamped and the heart was arrested with 1 L of cold modified Celsior solution (Table 4.1). The inferior vena cava and right superior pulmonary vein were incised to decompress the right and left ventricles, and the donor cardiectomy was completed.

Table 4.1 Celsior preservation solution composition

pH	7.3
Osmolarity	320–360 mOsm/L
Potassium	15 mmol/L
Sodium	100 mmol/L
Magnesium	13 mmol/L
Lactobionate	80 mmol/L
Mannitol	60 mmol/L
Histidine	30 mmol/L
Glutamate	20 mmol/L
Glutathione (reduced)	3 mmol/L

Animals randomized to the static preservation group were stored in a container filled with 1 L of modified Celsior and placed in an ice chest. Animals randomized to perfusion preservation were attached to the perfusion device via a connector in the ascending aorta providing continuous antegrade flow of oxygenated, modified Celsior solution at a flow rate of 10 mL/100 g myocardium/min at 5 ± 2°C. In the perfusion preservation group, a small caliber polyethylene catheter was placed in the coronary sinus for serial measurements of oxygen tension during preservation; pH, oxygen tensions, and lactate levels in the preservation solution were measured with a commercial analyzer (Radiometer Copenhagen EML 105; Bronshoj, Denmark).

4.2.4 Recipient Protocol

After induction of anesthesia, the recipient animal was placed on cardiopulmonary bypass and the heart was excised to coincide with the end of the donor heart storage interval. The heart was then implanted into the recipient animal using a standard bicaval orthotopic transplant technique. Animals received 1 g of methylprednisolone prior to unclamping of the aorta. After the onset of reperfusion, hearts were defibrillated with 5-20 J, if necessary. An intravenous infusion of dobutamine was started at 5 g/kg/min. The animals were weaned from cardiopulmonary bypass after 1 hour of reperfusion. The left ventricular catheter was reinserted, and the previously placed sonomicrometry crystals were connected to the preamplifier. Pressure-volume data were collected in triplicate over a range of filling conditions produced by draining blood into the cardiotomy reservoir (emptying curves) at 2, 3, 4, 5, and 6 hours postreperfusion. Left ventricular samples were collected for water content analysis.

4.3 Data processing

4.3.1 Data acquisition

Continuous measurements of LV pressure and LV dimensions were acquired and sampled by a data acquisition and signal analysis system (DASA by GOULD Electronics) installed on a personal computer. This system is capable of sampling and storing to hard disk up to 16 channels of analog data with 12 bits A/D resolution (digitized to 0-4095) at a maximal sample rate of 40 KHz. In this research, four channels of data were sampled at 200 samples/second for 20 second time intervals which allowed high quality construction of the pressure and dimension signals (sampling rate was higher than the Nyquist rate, $1/T_{sample} >> 2 * f_0$). A real time scrolling waveform display of up to 8 non-overlapping waveforms was available during acquisition and for monitoring purposes.

The data were read and processed in a computer program (SonoSoft, Sonometrics Corp., London, Ontario, Canada). Each 20 second set of data was analyzed to give beat by beat characteristics including: max and min pressure, max and min dimension, first derivative of pressure with respect to time

(dP/dt), end-diastolic pressure and dimension, end-systolic pressure and dimension, and area of the pressure-volume loop. Further analysis of myocardial performance indices and oxygen consumption are described in the following sections. Least squares linear regression was applied when analyzing linear relationships between parameters of the cardiac cycle when a series of beats were collected over a range of filling conditions.

Data from the same animal at different time points were statistically analyzed by a paired t-test. This technique enables one to test the significance of the effect of an intervention when each subject is observed before and after the intervention. Comparison between groups utilized repeated measures analysis of variance (ANOVA).

4.3.2 Myocardial mechanical performance

LV pressure and dimension measurements were needed for the evaluation of contractility and myocardial performance. Data were acquired once before ischemia and once every hour for the six hour reperfusion interval. Each measurement involved collection of data during a gradual change in workload (controlled by the roller pump with the dog supported by cardiopulmonary bypass) over a 20 second period. This process yielded a series of pressure-volume loops with decreasing area, called an emptying curve. Both pre and post-ischemic measurements were repeated 4-6 times and then averaged. The pressure-volume loop area, end-diastolic dimension and end-systolic points were calculated for each loop.

4.3.2.1 PRSW – Preload recruitable stroke work

The pressure-volume loop area or the stroke work was regressed against end-diastolic volume. Each pressure-volume loop yielded a single point relating its stroke work to its end-diastolic volume. Therefore, the set of loops acquired during an emptying curve yielded a set of points which are shown to be linearly related. Linear regression of these points provides a slope and an x-intercept. The slope is an

index of myocardial performance called PRSW as described in Chapter 1. The x-intercept of the regression line is termed V_d and represents the residual volume in the ventricle when no work is being performed. The end-diastolic point was selected in reference to the value of positive dP/dt. The exact definition varies between investigators. In this study, the end-diastolic point was defined as half the maximal positive dP/dt point.

4.3.2.2 Measurement of Ventricular Performance

LV function was quantified by a load-independent method that has been previously described in a large animal transplant model.[141] Four sonomicrometry crystals (Sonometrics Corp., London, Ontario, Canada) were attached to the subendocardium in the minor axis and major axis of the left ventricle. These remained in situ during storage and reperfusion. A micromanometer-tipped catheter (Millar Instruments, Houston, TX) was inserted into the ventricular cavity through the apex. The catheter was removed prior to harvest and reinserted after reimplantation. Pressure and dimension data were collected at a rate of 250 Hz and digitized. Data were acquired and stored on a computer and later analyzed using commercially available software (SonoLab and CardioSOFT; Sonometrics Corp.). Pressure-volume loops were derived over the cardiac cycle prior to explantation (baseline) and after 2, 3, 4, 5, and 6 hours of reperfusion. At each time point, a series of pressure-volume loops was created over a range of filling conditions. Stroke volume was calculated from the integral (area) of each pressure-volume loop and plotted against the end-diastolic volume of each loop. The slope of this regression is termed preload-recruitable stroke work (PRSW), and is considered a load-independent index of contractility.[142]

4.3.3 Measurement of Myocardial Water Content

Samples of left ventricular tissue were collected, blotted free of blood, weighed, and placed in an oven for desiccation. The tissue was weighed daily until a constant weight was reached during

consecutive measurements. The myocardial water content was then calculated as ([wet weight-dry weight]/wet weight).

4.4 Statistical Analysis

Results are reported as mean and standard error of the mean (SEM). Groups were compared by a two-sided *t*-test using commercially available statistical software (SigmaStat, Chicago, IL). When outcome variables were measured at multiple time points over the reperfusion interval, a repeated-measures analysis variance was applied. Differences between groups were determined with the Student-Newman-Keuls post hoc test and a *P*-value less than 0.05 was considered significant.

4.5 Results

4.5.1 Cardiac Function

Left ventricular function was similar at baseline in all groups of animals. By 6 hours post-reperfusion, LV function was similar in the static and antegrade groups, but was significantly better in the group preserved by the retrograde machine perfusion technique. In general, function was constant over the 6 hour reperfusion interval in the retrograde group, but had slowly declined after about 3 hours in the other groups.

Table 4.2 Transplantation Data

Group	Ischemic Time (min)	Water Content (%)	PRSW (6 hour)
Retrograde	321±6	80.1±.8	72.2±10*
Antegrade	320±4	78.8±.3	38.6±6
Static	318±5	78.3±.2	39.6±11

4.6 Discussion

If perfusion preservation techniques are to enter the clinical realm, initial use of these devices is likely to remain within conventional storage intervals. As a result, we selected a 4 hour preservation interval for this study. The total donor ischemic time in this experiment includes the 4 hours of protected storage and an additional interval of donor ischemia during the implantation operation. This latter portion may provide an important ischemic stress to the donor graft. Studies were conducted using an extracellular preservation solution (Celsior) in both groups. This particular solution was selected based on prior isolated rat heart studies, which identified an increase in metabolism of exogenous glucose and an increase in oxygen consumption when extracellular preservation solutions were selected.[49] intermediates using techniques that have been previously described.[100]

As donor animals were stored for relatively short ischemic interval and all had normal hemodynamics prior to explant, it is perhaps not surprising that functional recovery was good in all groups in this study. Other investigators have found improved functional results with perfusion preservation techniques, but in general, they have selected longer storage intervals where the degree of ischemic

stress is increased. Any system that provides ongoing perfusion with a crystalloid solution has the potential to create myocardial edema, and several previous experimental studies have suggested increased edema in hearts stored with perfusion techniques.[43,143] The current study found no difference in myocardial water content after 4 hours of preservation and 6 hours of reperfusion amongst any of the preservation strategies. This is noteworthy as some of our prior data had raised concerns that a retrograde perfusion technique might increase myocardial water content, with the associated risk of adversely affecting cardiac function. This study suggested that if edema does develop during retrograde machine perfusion it appears to resolve by 6 hours of reperfusion. It is tempting to speculate that resolution of edema may play a role in the eventual superiority of functional recovery in the retrograde group, but serial measurement of water content or heart weight were not feasible within this experimental design. Further studies with longer intervals of storage and reperfusion are warranted to confirm these findings.

It is also noteworthy that the prior studies defining optimal flow rates during machine perfusion applied via a retrograde technique suggested that RV tissue perfusion may be impaired and RV metabolism may be adversely affected. The current study did not suggest any impairment of right ventricular function. However, sensitive measurements of cardiac performance were applied only to the left ventricle and an assessment of RV function likely deserves further study. RV performance is likely to be particularly important in the early period following clinical transplantation, as the right ventricle of the donor is not conditioned, and yet will be placed many times into a recipient pulmonary circulation that has a higher pulmonary vascular resistance due to long standing congestive heart failure.

This study has several limitations. First, this model does not use brain dead donors, which have been shown to display greater myocardial dysfunction and to have an altered metabolic profile.[144] As well, the perfusion preservation group was tested at a single flow rate (15 mL/100 g/min in the antegrade group and 20 mL/min/100g in the retrograde group) and other flow rates, perfusion temperatures, or preservation solution compositions may lead to different results. Finally, a species effect may need to be

considered and direct comparisons to other models using pigs or other species need to be interpreted with caution. Nevertheless, this study suggests that the strategy of continuous preservation of donor hearts stored for transplantation may have benefits to the donor heart, particularly when the perfusate is delivered in a controlled fashion via a retrograde route. Further studies will be required to test this technique under differing perfusion conditions and longer intervals. Perfusion preservation techniques may offer the opportunity to improve results after cardiac transplantation.

CHAPTER 5

EFFECTS OF MACHINE PERFUSION IN A DISCARDED HUMAN HEART MODEL

5.1 Introduction

There is a growing need for new strategies in donor heart transplantation due to the increase of patients on the transplant waiting list, and a paucity of available donors. In order to provide more options for surgeons a new technology has emerged that can shift the paradigm of donor heart placement, known as machine perfusion preservation. This technology is currently only used clinically in kidney transplantation, but results show improved organ function and recovery. Machine perfusion has the capability to increase the donor pool by extending the ischemic interval and procurement of marginal donor hearts. We have previously demonstrated that antegrade machine perfusion is metabolically superior to static preservation, but can cause aortic valve competence and non-nutrient flow. Another machine perfusion method being investigated by our laboratory is retrograde perfusion through the coronary sinus. This technique is used in cardiac surgery, but its application for machine perfusion has not been thoroughly evaluated.

This phase of our research further tested the hypothesis that retrograde perfusion reliably supports myocardial metabolism over an extended donor ischemic interval. It is significant in that it is the first effort to describe the metabolic effects of antegrade and retrograde machine perfusion in man. As well, it applied a new technique (Magic Angle Spinning MRS) in addition to our standard metabolic assessments to test the hypothesis that MAS methods will yield similar results to our standard proven MRS methods, but will require only minute amounts of cardiac tissue for the analysis.

5.2 Materials and Methods

Human hearts obtained from brain dead donors rejected for transplantation were preserved for 12 hours in standard University of Wisconsin Machine Perfusion Solution by one of three techniques:

1. Static hypothermic storage (n=6)
2. Antegrade perfusion (n=8)
3. Retrograde perfusion (n=7)

Hearts from groups 2 and 3 were perfused at 5°C with a pre-clinical heart machine perfusion device. Temperature, flow rate, and perfusion pressure were measured continuously in perfused hearts. After 12 hours, myocardial oxygen consumption (MVO_2) and lactate accumulation in the preservation solution were measured in all groups. Ventricular tissue (~ 2 grams) was collected, at the end of the perfusion interval in each experiment, for proton magnetic resonance spectroscopy (MRS) to evaluate the metabolic state of the myocardium. Another small myocardial biopsy (~ 10 mg) was collected in parallel and subjected to MAS analysis and compared to the results of conventional MRS in tissue extracts. Myocardial water content was measured at end-experiment.

5.3 Results

Donor characteristics, such as age, sex, and ejection fraction, were not significantly different among groups, see Table 5.1.

Table 5.1 Donor characteristics

	Static	**Antegrade**	**Retrograde**
Number	10	9	8
Age	39.3±8	50.4±5	46.4±5
Sex (M/F)	4/6	3/6	3/5
EF (% when known)	53±9	48±12	63±3

During standard transport of organs, organ temperatures can fall to near 0 degrees Celsius with the potential for tissue damage. In the machine perfused hearts temperature was maintained at a significantly higher range of 5-7 degrees Celsius, which may be advantageous and may be preferable to allow ongoing metabolism. Constant temperature is maintained with all methods, Figure 5.1. No temperature corrections were used between groups. Perfusion pressure was increased in the retrograde perfusion group, Figure 5.2, although this difference did not reach statistical significance, it could be a source for myocardial edema over the storage interval.

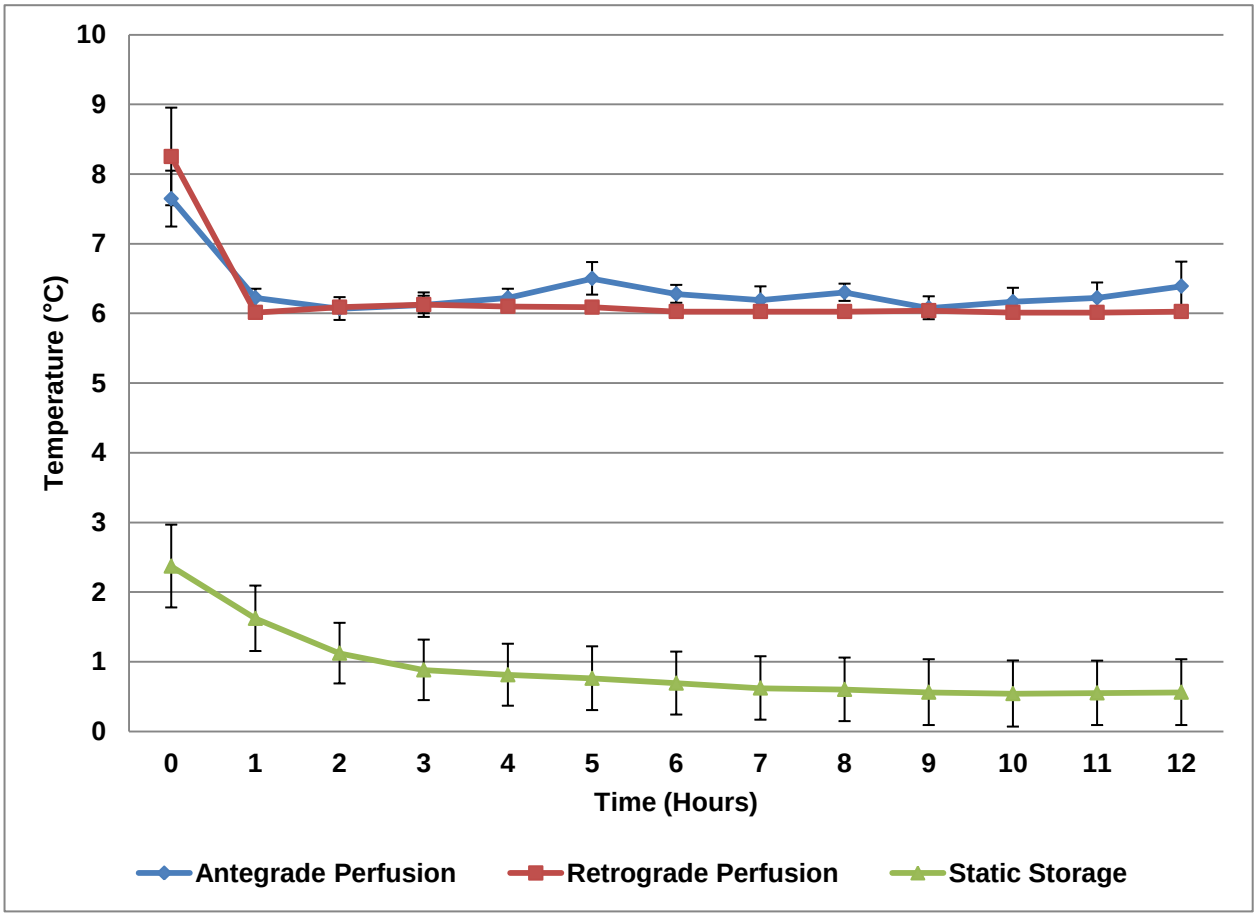


Figure 5.1 Heart temperature during storage.

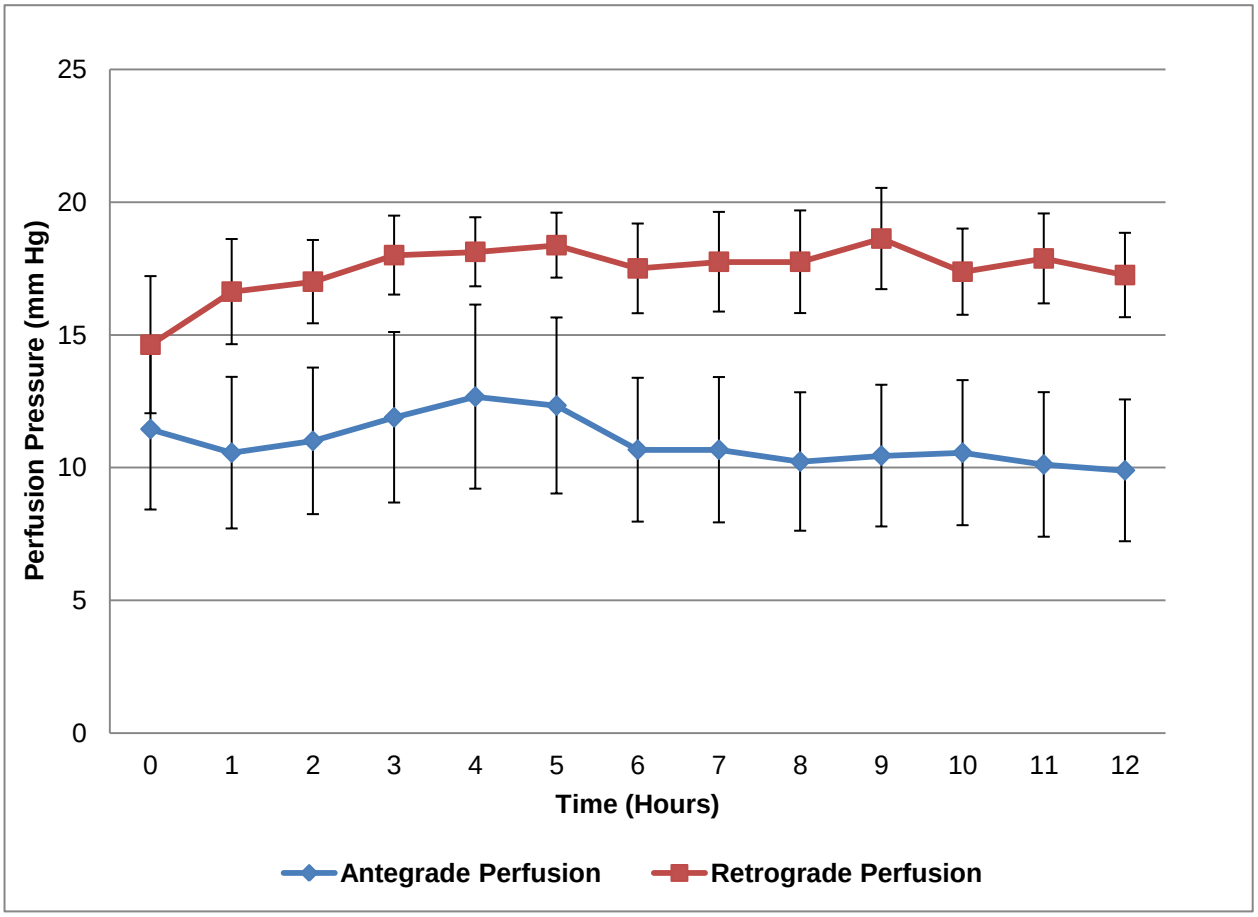


Figure 5.2 Perfusion pressure during storage interval.

Lactate/alanine ratios were lowest in retrograde perfused hearts, 0.5±0.3, and significantly higher in static hearts, 4.7±0.8 ($p<0.05$). Antegrade perfused hearts had a lactate/alanine ratio of 1.2±0.4. A trend towards greater myocardial water content was observed in retrograde perfused hearts, although the difference did not reach significance, see Table 5.2. However, myocardial weight gain was significantly different in the antegrade perfusion group, where a net loss of weight over the storage interval was observed., Figure 5.3.

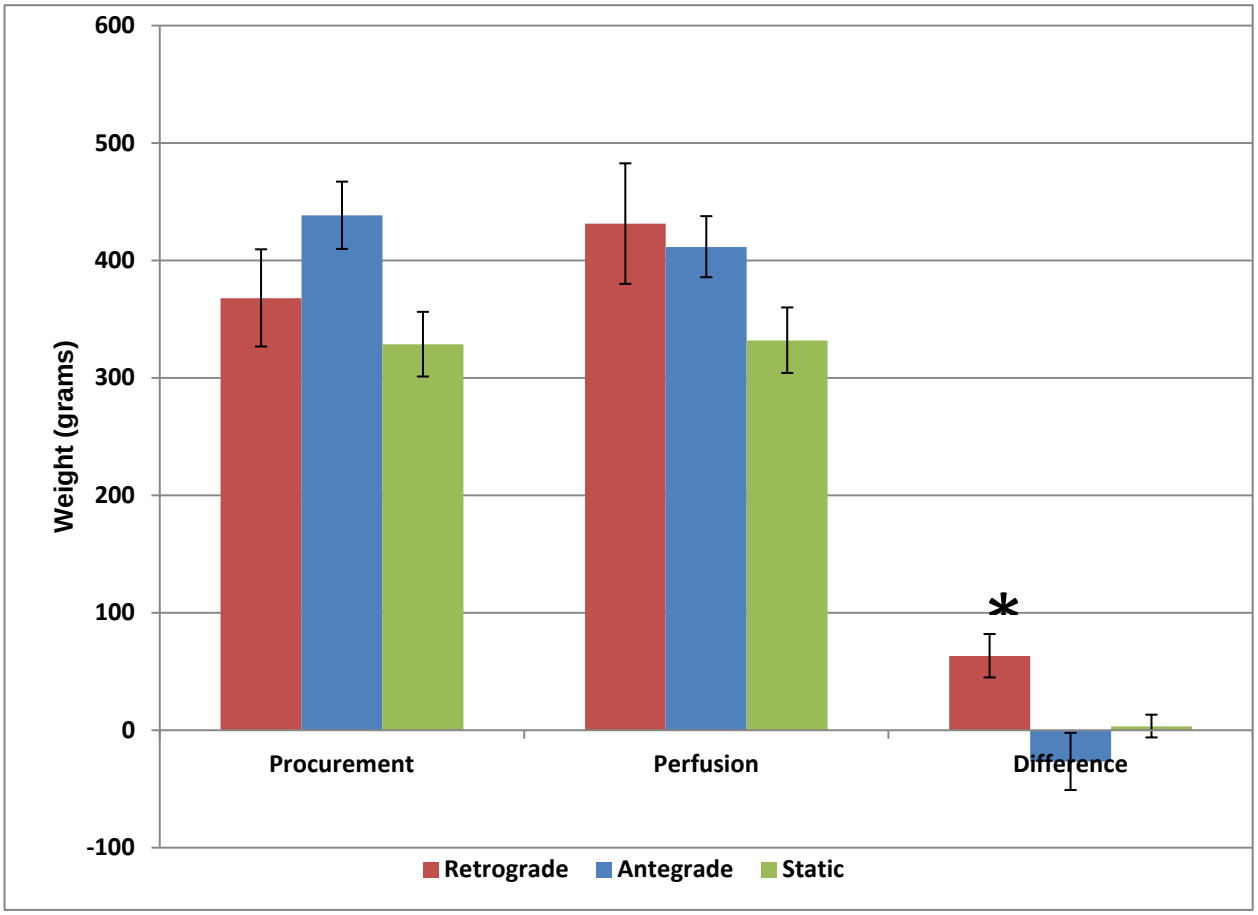


Figure 5.3 Myocardial weight gain after storage interval.

Lactate accumulation in the perfusate (antegrade perfusion 2.0±0.7, retrograde perfusion 1.7±0.1 mM) and MVO_2 (antegrade perfusion 10.6±2, retrograde perfusion 9.1±1 mL O_2/100g/min) were similar amongst machine perfusion groups (p=NS).

The samples analyzed with conventional MRS of tissue extracts of hearts stored with machine perfusion were found to have significantly lower lactate/alanine ratios compared to those undergoing static storage. This relationship was also confirmed in the MAS MRS analysis of small tissue biopsies taken at the same time (data for MAS MRS was available for static storage and antegrade perfusion groups), Table 5.4.

Table 5.2 Metabolic results and myocardial water content

Group	Lactate/Alanine	Water Content (%)
Static	4.7±0.8*	77±2
Antegrade	1.2±0.4	77±1
Retrograde	0.5±0.3	80±1

Data are Mean ± SEM. * - p<0.05 vs all other groups by ANOVA

An example 1 H MRS spectrum of perfused hearts (left panel) versus static hearts (right panel) shows the distinct difference between groups, Figure 5.4.

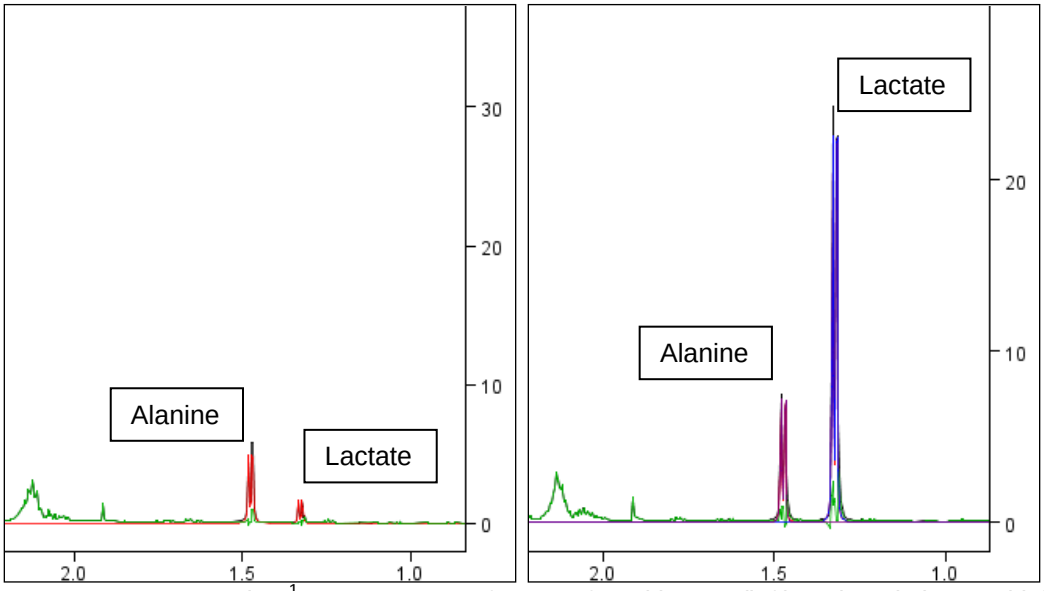


Figure 5.4 Representative ^{1}H MRS spectra from perfused hearts (left) and static hearts (right)

Table 5.3 ^{1}H Magic Angle Spinning MRS

Lactate:Alanine	Antegrade Perfusion	Static
Left Ventricle	1.33 ± 0.53	4.69 ± 0.76*
Left Ventricle MAS	2.41±0.35	3.89± 0.33*

5.4 Discussion

Machine perfusion by either antegrade perfusion or retrograde perfusion technique can support myocardial metabolism of human hearts over extended intervals. Perfusion of the heart, whether by antegrade or retrograde technique, results in a non-ischemic metabolic profile up to at least 12 hours. These data support the notion that prolonged storage intervals may be feasible via this technology in clinical applications. It is noteworthy that relatively normal lactate/alanine ratios were identified in these hearts despite that fact that all had been rejected for clinical transplantation for some reason. Results may be even better in hearts that are deemed acceptable by current criteria. All of these hearts were recovered from brain dead patients, suggesting that brain death does not in itself alter metabolic pathways important to myocardial preservation. While not statistically significantly different, ejection fraction did vary amongst the three groups studied. It is possible that these small differences may reflect some variation in heart quality, but further data are needed to fully evaluate these preservation options.

These data also suggest that retrograde perfusion may be superior to antegrade perfusion for maintaining metrics of myocardial oxidative metabolism, especially the lactate/alanine ratio, although this difference was not statistically significant. However, we did observe a difference in myocardial edema development, as indicated by myocardial water content. This aspect of the study warrants further investigation, as changes in myocardial water content at end-storage do not necessarily imply worse myocardial function after reperfusion.

It is possible that the markedly lower temperatures in the static preservation group contributed to cellular damage that was reflected in some of the metabolic outcomes. However, one would note that lower temperature observed in the static group, if anything, should reduce myocardial metabolism to a very low level. Despite this intracellular lactate levels (as reflected by the lactate:alanine ratio) were significantly higher in this group. Any corrections applied for temperature variation would only magnify this already substantial difference.

Furthermore, this study demonstrates that metabolic information reflecting myocardial intermediary metabolism can be obtained using very small tissue samples analyzed over a short period of time. The comparison with metabolic parameters measured with conventions techniques in tissue extracts was encouraging, but more data will be needed to establish the full potential of this technique and to determine how well these metabolic changes correlate with post-transplant cardiac function.

Machine perfusion appears to be a more viable option, compared to static storage, for long term preservation of human donor hearts.

CHAPTER 6

SUMMARY, CONCLUSIONS, AND FUTURE STUDIES

6.1 Introduction

Previous chapters have presented the background, motivation, design, and execution behind the studies, as well as observations and interpretations of results. In this chapter a general summary of these results will be provided along with an attempt to synthesize the results from all of the experiments into a series of more general conclusions. Finally, a series of suggestions for future directions for this research will be provided.

6.2 Conclusions

The information obtained from these studies will now be discussed in the context of the specific aims presented in Chapter 1.

6.2.1 Specific Aim 1: To identify substrate components to add to the preservation solution to enhance oxidative metabolism during machine perfusion.

Machine perfusion offers the opportunity to capitalize on the greater energetic benefits of aerobic metabolism during the storage interval. Optimizing the preservation solution, to increase metabolism during storage may be advantageous for subsequent functional recovery after re-implantation. As shown in our previous work, extracellular solutions (with higher sodium concentrations) appear to allow greater oxidative metabolism compared to intracellular solutions

Glucose appears to be an ineffective exogenous substrate when added to an extracellular solutions in a model of machine perfusion. Pyruvate as an added substrate increases oxidation of the exogenous fuel and decreases catabolism of endogenous cellular energy stores. Acetate, even when provided in low concentrations, is preferred over glucose as an exogenous substrate and also reduces consumption of endogenous sources in the cell during preservation. Propionate when added as an anaplerotic substrate

does not appear to alter exogenous or endogenous substrate flux under conditions modeling machine perfusion.

6.2.2 Specific Aim 2: To develop and test retrograde perfusion to enhance perfusate delivery throughout the myocardium to enhance oxidative metabolism during machine perfusion.

Retrograde delivery of perfusate in the coronary sinus is feasible, and can avoid the risk of aortic valve incompetence during antegrade perfusion . A retrograde flow rate of 20 mL/100 g/min appears optimal – this rate maximizes tissue perfusion, oxygen consumption, and reduces the rate of myocardial edema.

The RV may be more susceptible to under-perfusion under some conditions with this technique, for reasons yet unknown. Machine perfusion conditions that are associated with lower tissue perfusion may lead to metabolic derangements including an increase in intracellular lactate levels. On the other hand, machine perfusion conditions that are associated with high tissue perfusion carry a risk of myocardial edema formation.

Both antegrade and retrograde machine perfusion can provide excellent LV perfusion appear superior to static storage methods, particularly over longer storage intervals. Under optimal conditions, functional recovery may be superior with retrograde machine perfusion up to six hours of reperfusion.

6.2.3 Specific Aim 3: To test Magic Angle Spinning magnetic resonance spectroscopy as a method to determine metabolic state of stored donor hearts prior to transplantation.

Machine perfusion in human hearts appears to result in very similar metabolic sequelae to those observed in large animal models

Magic Angle Spinning MRS techniques appear promising as a method to quickly evaluate myocardial metabolic state at the end of the storage interval, using minimal amounts of myocardial tissue.

6.3 Potential Future Studies

These studies have advanced our understanding of the effects of enhancing metabolism during the preservation interval, a concept that is critical to the success of any machine perfusion strategy. Armed with this information, we now plan to move forward with testing other substrate additives in order to determine the optimal substrate for enhancing metabolism. We also plan to examine the effectiveness of machine perfusion techniques when applied in a setting that models “donation after cardiac death (DCD)” whereby the donor has undergone an interval of cessation of cardiac activity prior to organ harvest. This results in a donor heart that has sustained a brief interval of ischemic damage. As the perfusion device can deliver oxygenated solution to the heart and washout by-products of metabolism, it may offer an opportunity to render some of these hearts useable for transplantation. If successful, this could substantially increase the donor pool, making more organs available to more patients.

Finally, participation of our research group in the LifeCradle clinical trial, currently scheduled for next year, will also provide valuable knowledge of the potential for machine perfusion preservation in clinical heart transplantation. It is our hope that this process will lead to improvements in the results of clinical heart transplantation, offering improved outcomes to the large population of patients suffering from heart failure.

APPENDIX A

PREVIOUS MACHINE PERFUSION INVESTIGATIONS

Authors	Journal Citation		Species	Duration of preservation	Preservation Solution	Perfusion Temperature (°C)	Benefits of Perfusion
Aizaki M et al.	Transplant Proc	Nov;32(7):2409-10	Canine	12 hours	Celsior and UW	4	Myocardial phosphocreatine, inorganic phosphate, Beta-ATP, and pH levels were higher in Celsior vs. UW
Calhoon JH et al.	Ann Thorac Surg	Jul;62(1):91-3	Canine	12 hours	UW - Viaspan	4	Cardiac output and rate of change of LV pressure were comparable to control. Minimal inotrope doses required for recipient resuscitation
Copeland JG et al.	Ann Surg	Dec;178(6):687-92	Canine	24-28 hours	Krebs	0-3	Perfusate Potassium and CPK levels rose while perfusate glucose fell. No consistent trend in lactate conc. was seen
Ferrera R, Hadour G.	Transplant Proc	Dec;30(8):4340-3	Pig	24 hours	St. Thomas	4	Ultrastructural tissue samples were comparable to normal tissues with slight damage observed in mitochondria
Ferrera R et al.	J Heart Lung Transplant	Aug;19(8):792-800	Pig	24 hours	St. Thomas, "New Perfusion Solution" (NPS)	4	Ultrastructural damage with microperfused NPS samples scored similarly to control whereas the St. Thomas microperfused samples revealed slight damage to cellular components
Fitton TP et al.	Clin Transplant	18 Suppl 12:22-7	Canine	24 hours CHP, 4 hours SP	Not stated	5	CHP stored hearts demonstrated adequate hemodynamic function to support systemic circulation
Fitton TP et al.	Ann Thorac Surg	Nov;80(5):1812-20	Canine	24 hours CHP, 4 hours SP	Modified Belzer	5	Reduced oxidative stress and preserved DNA repair processes (OGG1, MYH, MSH2)
Koike N et al.	J Heart Lung Transplant	Jul;22(7):810-7	Canine	1 hr perfusion with 3 hrs SP	Celsior	4	Cariac output recovery was 80% 1hr after weaning from CPB. End-systolic maximal elastance fell only 2 mmHg/ml compared to baseline of 11.9 mmHg/mL
Hassanein WH et al.	J Thorac Cardiovasc Surg	Nov;116(5):821-30	Pig	12 hours	Normothermic Blood	37	Blood gas measurements over perfusion interval remained within normal limits. After reperfusion hearts returned to normal sinus rhythm without pacing or direct current
Jones BU et al.	ASAIO J	May-Jun;47(3):197-201	Rabbit	8 hours	PEG-Hemoglobin	20	PEG-Hb hearts maintained greater baseline function in developed LV pressure, maximum rates of LV contraction and relaxation, and coronary flow
Wicomb WN et al.	J Surg Res	Mar;40(3):276-84.	Pig/Baboon	48 hours	Unnamed Cardioplegic Solution	4-10	Baboon heart autotransplant saw no loss of function and functional recovery based on tissue histology
Ogiwara H et al.	J Cardiovasc Surg (Torino).	Jun;39(3):313-20.	Canine	12 hrs SP; 1 hr CHP	UW and Modified Kawakami	4	Phosphocreatine, β-ATP, and myocardial pH levels all rose after coronary perfusion recovery. CPK and lactic acid values both decreased dramatically after perfusion.
Hasegawa Y et al.	J Cardiovasc Surg (Torino).	Jun;41(3):363-70.	Canine	12 hrs SP; 1 hr CHP	UW and Diluted Blood (Both Oxygenated)	4 and 15	Phosphocreatine, β-ATP, and myocardial pH levels all rose after coronary perfusion recovery. CPK and lactic acid values both decreased dramatically after perfusion.
Ferrera R et al.	Ann Thorac Surg	May;57(5):1233-9.	Pig	24 hours	St. Thomas	4	Glucose production was increased, noting that St. Thomas solution does not include glucose or sugars.
Hudon MP et al.	J Heart Lung Transplant	Sep-Oct;10(5 Pt 1):704-9	Pig	5 hours	Unnamed Cardioplegic Solution	8-10	Fatty acid turnover was maintained 2 hours post-op.
Kitamura M et al.	ASAIO J	Jul-Sep;38(3):M163-6	Canine	3-5 hours	UW	Not defined	Ability to be weaned from bypass without catecholamine support
Ohtaki A et al.	J Heart Lung Transplant	Mar;15(3):269-74.	Canine	12 hrs SP; 1 hr CHP	Oxygenated UW	4	P-NMR of PCr, α-,β-,γ-ATP revealed significant increases after 1-hour coronary perfusion as compared to static preservation. CPK and Lactic acid values decreased significantly after perfusion
Suehiro K et al.	Ann Thorac Surg	Jan;71(1):278-83	Canine	Up to 60 minutes	St. Thomas	4	All subjects who were successful in ejecting 80 mmHg of afterload for t> 15 min after coronary perfusion were able to be weaned from CPB and Dopamine. These hearts showed significantly higher systolic pressure associated with lower LA pressure
Wicomb W et al.	J Thorac Cardiovasc Surg	Jan;83(1):133-40	Human	Variable from 6 to 15 hrs	Unnamed Oxygenated Cardioplegic Solution	4-10	Heterotopic transplant method used; 3 of the 4 patients were able to accept the heart within the first 20 hours post-transplant
Toledo-Pereyra LH et al.	Ann Thorac Surg	Jan;27(1):24-31	Canine	24 hours	Sacks' solution II	7	Significant damage and lactic acid buildup were observed at the highest pressure, 80 mmHg, whereas minimal to moderate damage was observed in the lower groups, 25 and 50 mmHg respectively. Hearts transplanted from the 25 mmHg group had the greatest post-transplant recovery

Authors	Journal Citation		Edema in Perfusion Group	Other findings
Aizaki M et al.	Transplant Proc	Nov;32(7):2409-10	No significant water difference for either group	Blood washout from coronaries performed earlier with Celsior vs. UW
Calhoon JH et al.	Ann Thorac Surg	Jul;62(1):91-3	No significant water difference for perfused group, subjective edema in nonperfused	Nonperfused hearts were unable to support recipient bypass and led to failure
Copeland JG et al.	Ann Surg	Dec;178(6):687-92	An average of 38% increase in heart weight was observed	Light and electron microscopic analysis were performed on biopsies revealing a few intracellular vacuoles and a large number of capillary pinocytotic vacuoles
Ferrera R, Hadour G.	Transplant Proc	Dec;30(8):4340-3	No significant water difference for either group	ATP, Total amount of Adenine Nucleotides, and Energetic Index decreased between the preserved groups, however, the perfused group remained significantly higher. LV Developed Pressure was higher for perfused group than cold storage over 24hr interval
Ferrera R et al.	J Heart Lung Transplant	Aug;19(8):792-800	Microperfusion storage with St. Thomas solution yielded an average of 40% increase in heart weight whereas microperfusion and simple storage with the unnamed solution led to a net water loss	Post-ischemic coronary flow of microperfused NPS hearts was more than two times greater than microperfused St. Thomas hearts, and equal to that of control
Fitton TP et al.	Clin Transplant	18 Suppl 12:22-7	An average of 18% increase in heart weight was observed	Oxidative stress evaluated by expression of 8-oxoG was significantly increased in the SP group vs. the CHP group
Fitton TP et al.	Ann Thorac Surg	Nov;80(5):1812-20	An average of 27% increase in heart weight was observed	Decreased expression of DNA repair enzymes in SP gorup may be indicative of low temperature environment
Koike N et al.	J Heart Lung Transplant	Jul;22(7):810-7	Water content was 76.3 ± 1.3% after 2hrs of weaning from CPB, significantly different than the SP group 81.1 ± 1.7%	H&E staining revealed myocardial necrosis, dissolution of myocardial cells, and changes in the structural framework. These findings were more severe in the SP subjects than the CPs. Periodic acid Schiff staining showed that glycogen was better preserved in the CP group
Hassanein WH et al.	J Thorac Cardiovasc Surg	Nov;116(5):821-30	No siginficant water difference between blood perfused group and control.	Severe acidosis and edema were achieved in UW stored subjects. Coronary endothelial function evaluated by vasodilatory response of preconstricted vasular rings revealed diminished response in UW stored hearts compared to the blood-perfused group
Jones BU et al.	ASAIO J	May-Jun;47(3):197-201	Qualitatively less edema was observed in PEG-Hb based on greater oncotic properties	Performance testing was doubled from 4 hours to 8 hours with test group and no significant changes were observed
Wicomb WN et al.	J Surg Res	Mar;40(3):276-84.	All groups demonstrated at least a 47% weight gain, the greatest in the pig hearts at 70%	LV pressure buildup in perfused pigs was less than freshly excised hearts. Maintaining temperature above 1°C is critical for functional preservation.
Ogiwara H et al.	J Cardiovasc Surg (Torino).	Jun;39(3):313-20.	No significant water difference for any groups	The inclusion of calcium may play an important role in the use of an extracellular perfusion solution
Hasegawa Y et al.	J Cardiovasc Surg (Torino).	Jun;41(3):363-70.	Mean Water Content in diluted blood group (79 ± 0.4%) was significantly higher than UW group (74 ± 1.1%) after perfusion interval	Myocardial Interstitial Tissue Space (ITS) rate of the subepicardium rose significantly after 12hrs and then dropped back down after coronary perfusion, with the diluted blood group being slightly higher. For the subendocardium, levels continued to rise even after perfusion with the UW group increasing dramatically
Ferrera R et al.	Ann Thorac Surg	May;57(5):1233-9.	Hearts preserved in the low pressure group and low flow group contained a severe amount of edema compared to control and simple storage	Microperfusion indicated slight damage to mitochondria, compared to none observed with low pressure perfusion. Overall the total adenine nucleotide levels were not statistically different.
Hudon MP et al.	J Heart Lung Transplant	Sep-Oct;10(5 Pt 1):704-9	Not discussed	Radioionated free fatty acid imaging of I-IPPA may be a useful tool and comparative to tissue lactate levels
Kitamura M et al.	ASAIO J	Jul-Sep;38(3):M163-6	Not discussed	Waterproof packed device that provided sterile in-storage hypothermic perfusion
Ohtaki A et al.	J Heart Lung Transplant	Mar;15(3):269-74.	No significant water difference was obtained after the 1-hour coronary perfusion	Ventricular fibrillation occurred rapidly after removal of recipient cross-clamp, but restarted using DC shock. LVP and LV dp/dt values were significantly better in the perfused group versus statically stored hearts
Suehiro K et al.	Ann Thorac Surg	Jan;71(1):278-83	Not discussed	No thrombi were found in the major coronary arteries and veins after perfusion and transplantation, yet thrombi were found in both ventricles and inferior vena cava.
Wicomb W et al.	J Thorac Cardiovasc Surg	Jan;83(1):133-40	Not discussed	Slight reduction (< 25%) of coronary flow during perfusion for all hearts
Toledo-Pereyra LH et al.	Ann Thorac Surg	Jan;27(1):24-31	Low pressures generated edemic buildup comparable to statically preserved hearts, approximately 10%. Hearts preserved at higher pressures of 50 mmHg and 80 mmHg generated edemic buildup of 20% and 40% respectively	Systolic perfusion pressure increased with higher pressures, and decreased at lower pressures. Perfusion flow of the different pressures converged to approximately 1 mL/min/g during the preservation interval.

Authors	Journal Citation		Species	Duration of preservation	Preservation Solution	Perfusion Temperature (°C)	Benefits of Perfusion
Wicomb WN, Collins GM.	Transplantation	Jul;48(1):6-9.	Rabbit	24 hours	UW	0	Hearts microperfused with fresh UW and UW with PEG20 displayed the best cardiac output and ablility to generate systolic pressures of 100 cm H2O
Wicomb WN et al.	Transplantation	Jan;43(1):23-9	Baboon	48 hours	2 Unnamed Oxygenated Cardioplegic Solutions	4-10	Coronary flow in Group A did not change during the storage interval. Coronary sinus lactate dehydrogenase increased significantly in Group B. Coronary sinus lactate for both groups decreased over time, however the two values differed from each other significantly.
Wicomb WN et al.	Transplantation	Nov;34(5):246-50.	Pig	20-24 hours	Unnamed Oxygenated Cardioplegic Solution	4-10	Mean myocardial oxygen uptake and coronary flow increased significantly over preservation period
Watson DC Jr.	Transplant Proc	Mar;9(1):297-9	Canine	24 hours	Unnamed Oxygenated Cardioplegic Solution	3	All perfused hearts survived transplantation and observatory 24 hour post-op. Mild left ventricular damage was seen.
Peltz M et al.	Surgery	Oct;138(4):795-805	Rat	200 minutes	Celsior and UW	4	MVO2 during storage was much higher in the perfusion preservation groups. Significant utilization of exogenous substrate by oxidative metabolism was identified in the Celsior perfusion group
Tsutsumi H et al.	J Surg Res	Apr;96(2):260-7	Canine	24 hours	Celsior	4	Significantly higher (SH) ß-ATP levels, pH in perfused group.
Nameki T et al.	J Surg Res	Sep;135(1):107-12. Epub 2006 Feb 28	Canine	12 hours	Celsior	4	No significant difference in hemodynamic.
Masters T et al.	J Heart Lung Transplant	May;21(5):590-9	Canine	18 hours	UW	4-4.5	Regained 50-60% original function. Substrate uptake normal >>glycolytic and lipolytic processes functional.
Smulowitz P et al.	ASAIO J	Jul-Aug;46(4):389-96					
Stowe D et al.	Am J Physiol Heart Circ Physiol	Apr 13; [Epub ahead of print]	Guinea Pig			37.2 ± 0.1	
Minten J et al.	J Heart Lung Transplant	Jan-Feb;10(1 Pt 1):71-8	Rat, Dog	24 hours	Bretschneider-HTK	2 to 4	Higher levels of ATP and Creatine Phosphate
Ferrera R et al.	J Heart Lung Transplant	May-Jun;12(3):463-9.	Pig	6,12,24 hours	St. Thomas' solution	4	Higher levels of HEPs. Higher left ventricular developed pressure (LVDP) than cold storage alone.
Tsutsumi H et al.	Transplant Proc	Nov;32(7):2415-6.	Canine	24 hours	Celsior		Myocardial phosphocreatine, inorganic phosphate, Beta-ATP, and pH levels were higher in perfused group vs static preservation (simple immersion)
Tsutsumi H et al.	Int J Angiol	Jan;10(1):15-19	Canine	12 hours	Celsior		Myocardial phosphocreatine, inorganic phosphate, Beta-ATP, and pH levels were higher in perfused group vs static preservation (simple immersion)
Rosenstrauch D et al.	Tex Heart Inst J	30(2):121-7	Pig	not described, probably <30 min	Houston cardioplegia		Hearts could achieve approx 60% of pre-excision stroke work in this device
Osaki S et al.	Ann Thorac Surg	Jun;81(6):2167-71	Pig	30 min warm arrest, then 5, 20 or 60 min of blood CP perfusion	4:1 leukocyte-depleted blood with Modified St Thomas		Hearts reperfused for 20 minutes before transplanation had better functional recovery than those reperfused for 5 or 60 minutes
Rao V et al.	J Thorac Cardiovasc Surg	Sep;122(3):501-7	Pig	5 hours	Arrested with crystalloid CP, perfused with harvested shed donor blood		Both groups perfused during storage. Group perfused at 25oC had improved recovery of developed pressure, both groups had worsened diastolic compliance after transplantation.
Kadipasaoglu KA et al.	Tex Heart Inst J	1993;20(1):33-9	Human	< 90 min	Unspecified CP, modified donor blood for reperfusion		11/12 hearts resumed beating in this modified Langendorff model. They could be supported for 96+/-49 min. Low pressures were generated. O2 consumption was observed. Reperfusion flow rates were 32-150 cc/min/100 g heart tissue.
Kuhn-Regnier F et al.	Eur J Cardiothorac Surg	Jan;17(1):71-6	Pig	14 hours for 3 groups, 3 hours for control group	HTK, mHTK (HTK with hyaluronidase), or mHTK with coronary oxygen perfusion		The perfusion applied inthis study is only gaseous oxygen (no crystalloid). Hearts protected with mHTK and coronary oxygen persufflation (COP) had better functional recovery (CO and +dP/dt), ATP and total nucleotides better preserved with mHTK and COP, lactate levels higher in mHTK and COP (?)
Kay L et al.	Cardiovasc Res	34(3):547-56	Rat	6, 15, or 24 hours	St. Thomas		Flow rate used in the perfused group was 0.3 ml/min (~30 ml/min/100 g). Perfusion with St Thomas slowed the decline in ATP/Pi ratio and led to better LVDP (although recoverfy was substantially impaired after 6 hours). After 15 hours of storage, perfused hearts did not show evidence of damage to the outer mitochonndrial membrane as was seen with static preservation. The degree of activation by creatine of the respiration in skinned cardiac fibers was minimally impaired at 6 hours but significantly depressed at 15 hours in static groups. This change was avoided by perfusion preservation at 15 hours.

Authors	Journal Citation		Edema in Perfusion Group	Other findings
Wicomb WN, Collins GM.	Transplantation	Jul;48(1):6-9.	Not discussed	Stored solutions, 3-6 months, performed poorly, hypothesized to be related to oxidation of glutathione
Wicomb WN et al.	Transplantation	Jan;43(1):23-9	Significant increase was observed in both groups with perfusate Group A having the higher value. Perfusate Group A contained lower contaminant concentrations of Fe++, Pb, and arsentae than Group B	Group B hearts displayed decreased metabolic activity with none being able to be successfully transplanted. This was associated with cellular destruction seen before reperfusion.
Wicomb WN et al.	Transplantation	Nov;34(5):246-50.	Significant edema was observed, 79% weight increase, with loss of 7.5% during reperfusion.	Hystologically the myofibers and nuclei were well preserved with minimal edema and damage. Left ventricular pressure decreased compared to the fresh control hearts.
Watson DC Jr.	Transplant Proc	Mar;9(1):297-9	An average of 34% increase in heart weight was observed	Long term endogenous stores need to be documented
Peltz M et al.	Surgery	Oct;138(4):795-805	The Celsior perfusion group had the lowest myocardial water content of all groups	Exogenous glucose was oxidized significantly in the Celsior perfusion group only. Perfusion preservation groups recovered more quickly after reperfusion, compared to static.
Tsutsumi H et al.	J Surg Res	Apr;96(2):260-7	Histo showed slight vs. severe interstitial edema in perfused vs. static group	CO and LVP recovery rates not statistically significant but better hemodynaics observed in perfused group. Orthotopic Transplantation
Nameki T et al.	J Surg Res	Sep;135(1):107-12. Epub 2006 Feb 28	Lower water content & less swelling in the SI + CP group vs. the CP group.	Found their method to be suitable but optimal ratio of coronary perfusion to simple immersion has not been found.
Masters T et al.	J Heart Lung Transplant	May;21(5):590-9	Not mentioned	ATP and creatine phosphate leves decreased during preservation but returned to control during reperfusion. Apoptosis but no necrosis. Myocardial function decreased with increasing number of apoptotic cells over time.
Smulowitz P et al.	ASAIO J	Jul-Aug;46(4):389-96		REVIEW: Info for introduction and/or source for additional references
Stowe D et al.	Am J Physiol Heart Circ Physiol	Apr 13; [Epub ahead of print]		
Minten J et al.	J Heart Lung Transplant	Jan-Feb;10(1 Pt 1):71-8	Slight increase in tissue water content in both species (dog 76.3% ± 0.4% to 79.1% ± 1.2%). Suggests that functional recovery is directly proportional to ATP concentration though not tested in this study via reperfusion.	Perfused hearts were not immersed in solution. Hearts had significantly higher levels of HEPs than those in cold storage in saline solution only.
Ferrera R et al.	J Heart Lung Transplant	May-Jun;12(3):463-9.	Significantly higher edema in microperfused group resulting in higher hydrostatic pressure in this group.	
Tsutsumi H et al.	Transplant Proc	Nov;32(7):2415-6.	Not described	Note: low flow rates: 0.5 - 0.7 mL/min in perfusion group
Tsutsumi H et al.	Int J Angiol	Jan;10(1):15-19	Not described	Note: low flow rates: 0.5 - 0.7 mL/min in perfusion group. This study is probably a subset of the one described in #9 above.
Rosenstrauch D et al.	Tex Heart Inst J	30(2):121-7	Not quantified, but seen histologically in the one heart that was "re-resuscitated"	This is not truly a study of perfusion preservation - it is a description of a model
Osaki S et al.	Ann Thorac Surg	Jun;81(6):2167-71	Hearts reperfused for 20 minutes before transplanation had better less edema (10% weight gain) than those reperfused for 5 or 60 minutes (20% gain)	Flow rates not described - prefusion pressure of 40 mm Hg applied at 20oC. Lactate extraction after initial reperfusion returned to zero by 20 min. This is fundamentally a study of resuscitation conditions with perfusion preservation.
Rao V et al.	J Thorac Cardiovasc Surg	Sep;122(3):501-7	Not described	No difference between groups in lactate extraction or pH gardient across myocardium. Tepid group (25oC) had higher oxyegn extraction at all timepoints
Kadipasaoglu KA et al.	Tex Heart Inst J	1993;20(1):33-9	Not quantified, but seen histologically	This is not truly a study of perfusion preservation - it is a description of a model
Kuhn-Regnier F et al.	Eur J Cardiothorac Surg	Jan;17(1):71-6	Water content significantly increased after storage and reperfusion in all groups except mHTK and COP (unchanged from pregrafting values)	Air embolism after oxygen persufflation was prevented by perfusion of the coronary arteries with a low viscosity electrolyte solution prior to blood reperfusion. Not truly machine perfusion as only gaseous oxygen perfsued the coronaries in this study.
Kay L et al.	Cardiovasc Res	34(3):547-56	Not evaluated	This study shows that continuous perfusion of the heart even at low flow rates with a crystalloid solution reduces many of the changes seen under ischemic conditions. In particular, damage to the outer mitochondrail membrane (effect of cytochrome c), damage to the inner mitochondrial membrane (increase in initial respiratory rate with only substrates), decrease in delta VMAX, and loss of the stimulating effect of creatine are all reduced. Alterations to the process of channelling of energy from mitochondia occur before any other damage to mitochondrial respiratory function can be seen.

APPENDIX B

LIFECRADLE EVOLUTION

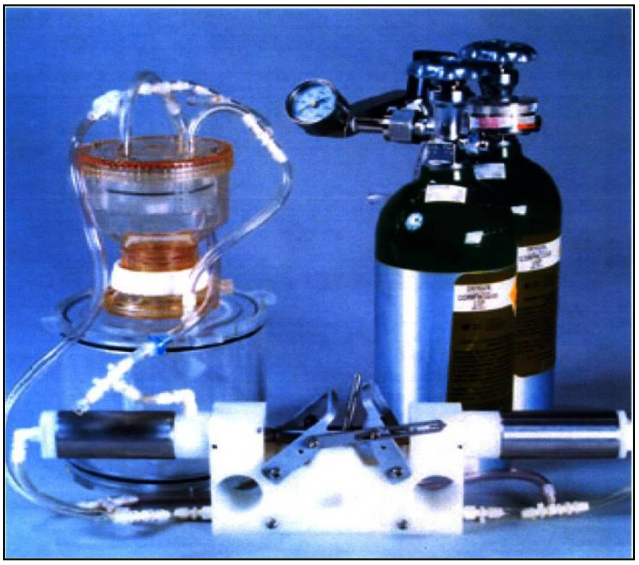


Figure B1: Model 1. Designed & fabricated in 1999 by Les Matthews, Ph.D.

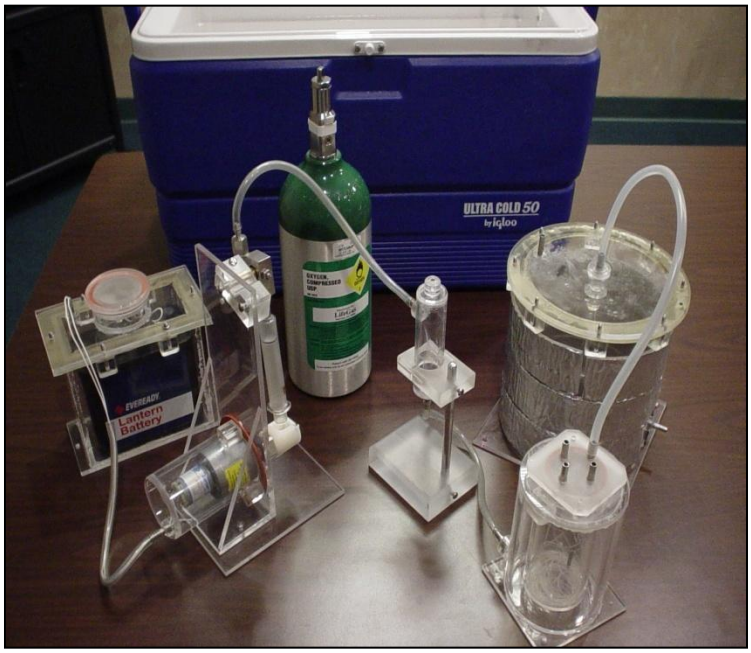


Figure B2: Model 2. Designed in 2000 at Penn State by George Panal-Design Engineer for Artificial Heart

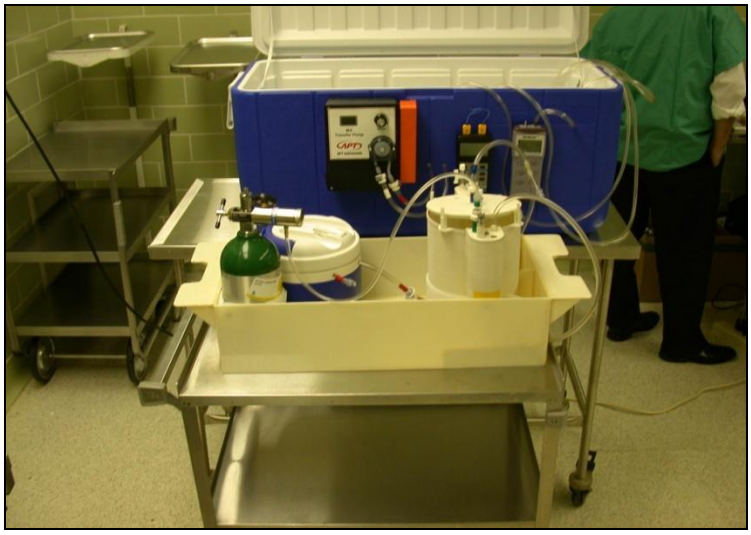


Figure B3: Model 3. Designed in 2001 by Tommy Davis

Figure B4.1: LifeCradle Model 4. Designed by Marshall Wenrich et al. at The RealTime Group, Plano, TX and OTS, Inc., Frisco, TX 2003-2005.

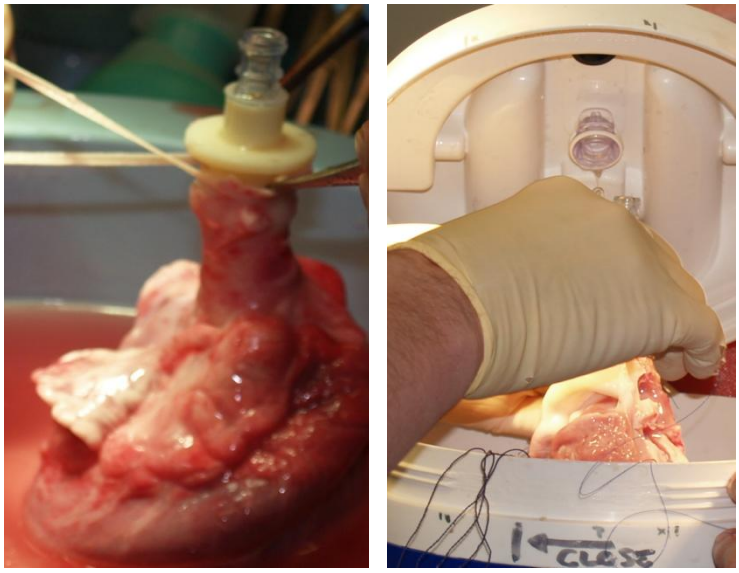


Figure B4.2: (a) Aortic adaptor insertion into aorta. (b) Heart attachment to lid.

Figure B4: Model 5. LifeCradle™ HR. Designed by OTS, Inc.; Probasco; & The RealTime Group 2006-2008

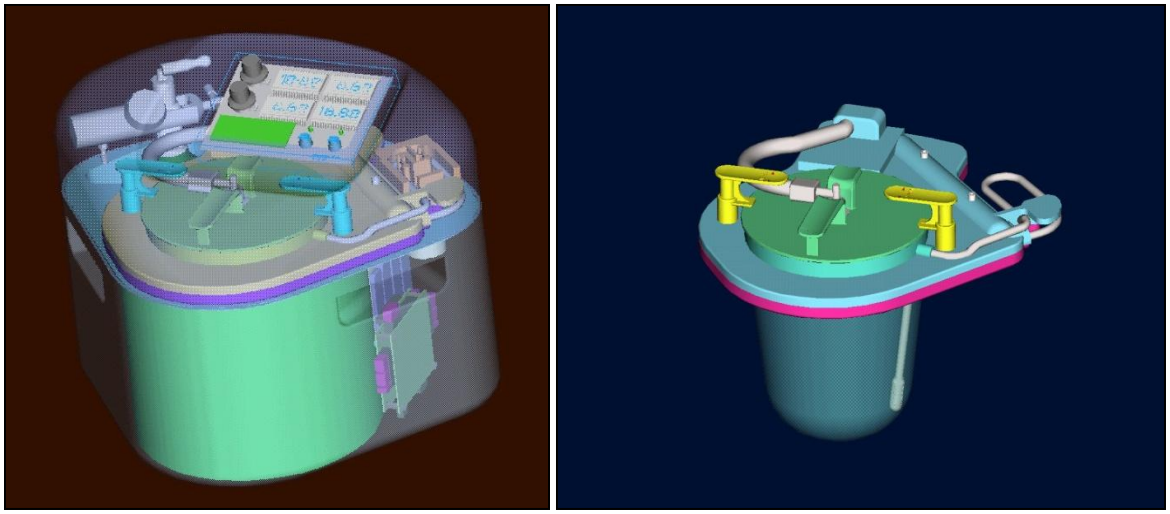


Figure B5: LifeCradle™ HR Components: Durable Unit (left) and Disposable Circuit (right)

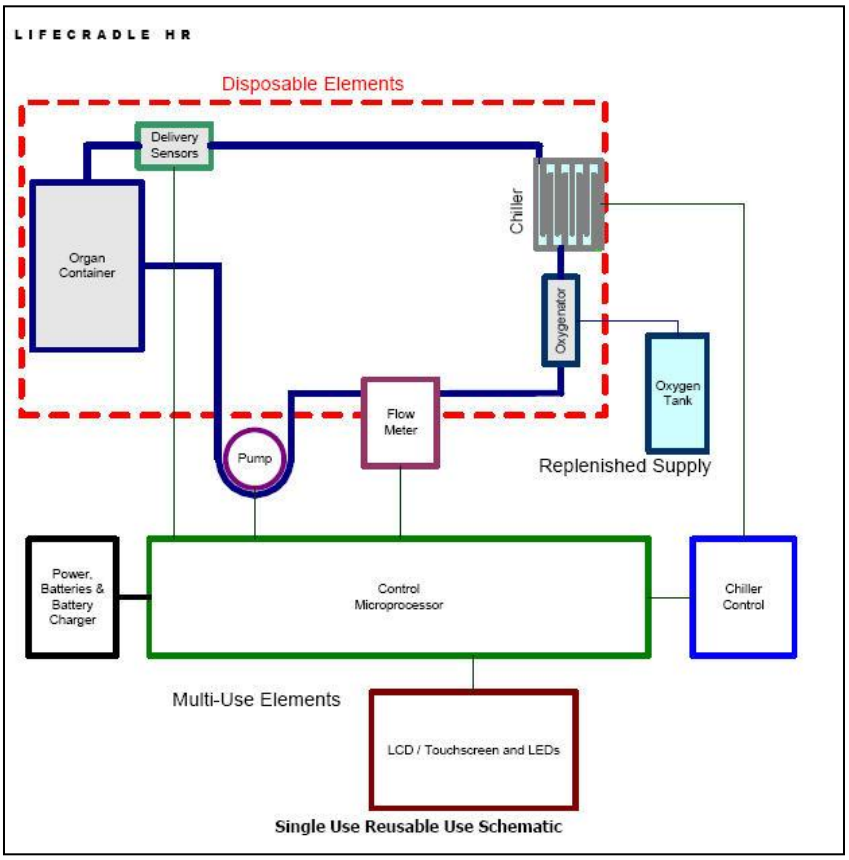


Figure B6: Schematic Drawing of LifeCradle™ HR. (Franklin)

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BIOGRAPHICAL INFORMATION

Michael Cobert has been drawn to the fields of medicine and science since he was a young boy. He earned a Bachelor's degree in Biomedical Engineering from the University of Texas at Austin in May 2006. He spent time there working in Dr. Christine Schmidt's lab with Fransisco (Kiko) Serna and Jon Nichols studying and developing applications for the use of the electroactive polymer Polypyrrole. During his graduate career in the Joint Biomedical Engineering Program with UT Arlington and UT Southwestern Medical Center he was mentored by Dr. Michael Jessen and studied the machine perfusion characteristics of a device for organ transport, on which his Master's Thesis (2008) and this Doctoral Dissertation were based. Michael will continue in Dr. Jessen's lab as a post-doctoral research fellow, and work as an engineer with the medical device company, Organ Transport Systems in Frisco.