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Thyroid Hormone as a Method of Reducing Damage to Donor Hearts after Circulatory Arrest

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

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Abstract

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There is a chronic lack of donor hearts to meet the need for heart transplant both in the US and worldwide. Further, the use of available hearts is limited by the short period between collection and implantation during which the heart can be safely preserved *ex vivo*. Using mid-thermic Langendorff machine perfusion, we have been able to preserve the metabolic function of a healthy heart for up to 8 hours, twice the limit for current static cold storage. We have also been able to preserve the metabolic function of a damaged DCD Heart collected 30 minutes after cardiac arrest for a period of 8 hours. We further investigated whether it was possible to improve the preservation of DCD heart using treatment with 10 µM Triiodothyronine to stimulate the tissue metabolism and we did find a reduction in damage markers in the treated DCD hearts as compared to the untreated group.

Introduction

Organ Transplant is the gold standard of treatment for end stage organ failure, but the need for transplant organs is greater than the supply.[1] In heart failure particularly, although mechanical devices like the Left Ventricular Assist Device (LVAD) and Total Artificial Heart (TAH) have show great promise as both bridges to transplant and destination therapies, a heart transplant is still the best options for most patients. Transplant tends to show better long term survival, as both of these devices cause a certain amount of hemolysis and can experience mechanical failure. They also come with all of the restrictions that accompany a powered, if portable, life support device.[2,3] As of 2012 there were approximately 3000 individuals on the waitlist for a heart transplant, with the average wait time (not accounting for prognosis and situational variables) being coming out somewhere between 1-2 years.[4]

Organ Donor Types

There are two distinct sources of donor organs for human organ transplant: Living Donor Donation and Deceased Donor Donation. Both are used for organs like kidney where a living donor can survive with only a single copy, and in kidney they show similar short term survival rates.[5] However, for obvious reasons, Living Donor Donation is not an option for organs like the heart.

For Deceased Donor Donation there are also two distinct categories within that group. Donation after Brain death (hereafter called DBD) is a Deceased Donor protocol where a patient is classified as brain dead, but thanks to adequate patient management, the heartbeat is persevered until the time of donor organ procurement.[6] This is by far the most common method of organ donation.[7] Non-Beating Heart donation, or more commonly called Donation after Circulatory Death donation (here after called DCD) is the second type of Deceased Donor Donation. This form of donation has been increasing over the last decade for most organ types, with the number of DCD donations between the years 2006-2010 being more than triple the number in the preceding 5 year span.[1] However, DCD

donation is not considered a viable option for human heart transplant. The assumption is that the injury sustained during circulatory death and the subsequent period of warm ischemia is too great to make the organ useful or safe for transplant.[7,8] For these reasons, with occasional rare exceptions in infant heart transplant, DCD hearts are not used for transplant at the present time.[9] However, in a retroactive analysis of non-heart DCD donation over the five year period from 2006 to 2011 Noterdaeme et al. observed that around 11% of DCD donations had a reasonable short period of warm ischemia (less than 30 minutes), and that if those organs could be procured and used, then over that same period of time, that increase in organ collection would lead to a 6% increase in transplant rate, a 15% increase in donor pool, and potentially as much as a 40% decrease in waitlist mortality.[10]

Ethical Aspects of Organ Donation and Consequences for DCD Donation

In organ donation there is a concept called the "Dead Donor Rule." This rule states that organ donation procedures cannot kill the donor, or in other words, that the donor must already be "dead." This raises the question of "what is 'Dead'?" particularly in the case of heart transplant where we are taking a heart that is still viable or "alive" to some extent and transplanting it into another individual. There are a number of criteria that can be used to determine death. The major distinction revolves are the difference between irreversible cessation of body function and permanent cessation of body function. In irreversible cessation of body function, no known natural occurrence or medical intervention can restore function to the system. In permanent cessation function still will not resume spontaneously, but medical intervention could restore function, however there is no intention of such interventions being applied. In the case of DBD donation, it is assumed that there is both permanent and irreversible cessation of brain function. Even though there is still normal circulatory function the donor is "brain dead." However in the case of DCD patients where this is not a cessation of brain function, the distinction between what is permanent and what is irreversible becomes quite important.

If we use irreversible cessation of brain function then DCD donation of any kind is nearly impossible, as it can take more than an hour for all brain function to stop irreversibly. If we use irreversible cessation of the circulatory system as a criterion, then DCD heart donation is by definition impossible, as the heart has stopped and cannot be restarted by any known intervention, and is a "dead" organ that is useless for transplant. Transplant concerns aside, since circulation can be restored by an external total artificial heart, irreversible cessation becomes a fairly useless standard in general. This leads us to consider permanent cessation criteria. The distinction between cessation of brain function and cessation of circulatory function is only largely important for what sort of support and treatments can be used during donation procedure. Since that is beyond the scope of this study, and because the interest here is focused on heart donation, I will focus on permanent cessation of circulatory function. The major concern with permanent cessation is that circulation will not restart spontaneously. Although interventions could restart it, function will not and cannot restart without these interventions. Using the criteria of permanent but not irreversible cessation of function allows for DCD donation in general due to the relatively short time span (irreversible cessation often taking much longer) and DCD heart donation specifically, since a permanently but not irreversibly stopped heart can be resuscitated in a transplant recipient.[11]

To facilitate the Dead Donor Rule, as interpreted through permanent cessation of circulation, DCD donation involves a "stand off" period. This 5-10 minute period of time following asystole exists to confirm that the heart will not spontaneously resume beating. After this period has passed, the organs for transplant may be collected, and placed in storage for transplant to the recipient. This stand off period, combined with the 10-20 minutes when the donor is prepped for the donation surgery, leads to total of 15-30 minutes of warm ischemia at body temperature in DCD transplant.[8]

Organ Preservation and its Impact on DCD Donation

Regardless of their donation protocol, current best practice is to store donor organs on ice until transplant.[12] Although cold storage at near 0 ^{o}C reduces the metabolic activity of the organ by nearly 12 fold, it is still anoxic storage and adds a "cold" ischemic insult.[13] This is not of huge concern in DBD transplant, but in DCD donation, it adds a second insult to the prolonged warm ischemic injury. Although many organs tolerate this to some extent, the heart does not. In fact, the heart is particularly sensitive to warm ischemic time.[11–13] Preclinical work, largely in greyhounds, has shown that cardioplegic cold storage impairs the reanimation of the DCD heart. DCD hearts preserved with cold storage in these experiments, and tested on an *ex vivo* working heart apparatus showed increased lactate production, reduced left ventricular pressure, and reduced fractional shortening compare to uninjured control hearts. However, normothermic perfusion storage resulted in outcomes closer to the uninjured control.[12,14] To that end, there have been test of *ex vivo* oxygenated perfusion storage. The goal of this type of storage is to provide the organ with oxygen and nutrients to support its metabolic needs during the time between collection and implantation. It is under investigation for many types of organ transplant, but has the potential to be especially useful for DCD hearts.[15]

One early study testing *ex vivo* storage and resuscitation of DCD hearts was done in a greyhound model using warm blood perfusion. It found that that DCD hearts perfused in this manner for 4 hours were comparable in function to non-DCD hearts in normal cold storage for 4 hour in terms of many functional parameters, with one exception.[14] Perfused DCD hearts had comparable fractional shortening and rate of pressure change, but much lower cardiac power than non-DCD cold storage, although they were still superior to DCD cold storage. Cardiac power was calculated as a function of [CO*(MAP – LAP)] where CO=Cardiac Output, MAP=Mean Arterial Pressure, and LAP=Left Atrial Pressure. This has units of Pressure*Volume/Time or equivalently Energy/Time which are the units of

power. Since this uses mean arterial pressure, this parameter can be interpreted as the average energy output from the heart into the circulatory system over time. A reduction in this parameter indicates that the heart is not working as efficiently. The authors suggest, although did not test the hypothesis that this reduction in power might be the result of myocardial edema in the perfused hearts.[14] Indeed, myocardial edema is one of the most commonly reported problems in cardiac machine perfusion.[15] Most subsequent studies have been preformed with cold crystalloid perfusion, and, with the exception of increased edema, are often equal to or superior to standard cold storage. In the case of DCD hearts in perfusion seems to be demonstrably superior.[12] Studies have shown the composition of the perfusion solution is an important component in reducing this edema, and that Polyethylene Glycol is a particularly effective oncotic additive.[16,17]

Ischemia and Mitochondrial Damage

Mitochondrial damage is well associated with ischemia, if not with DCD donation specifically.[18,19] More extensive the mitochondrial damage, has been correlated with greater cardiac dysfunction post reperfusion.[20] Preliminary data (not shown) from as yet unpublished observational studies in human tissue DCD heart samples and quantitative studies in rats show an increase in mitochondrial damage in myocardial tissue in cases of prolonged warm ischemia. One small (n=3) sample showed a reduction of nearly 40% in ADP stimulated oxidative phosphorylation in 30 minute cardiac arrest rat hearts as compared to control hearts. It has been well shown that in that Mitochondria Permeability Transition Pore plays a significant role in ischemic and reperfusion injury.[21] Aside from the potential release of mitochondrial DNA, Cytochrome C and other pro-apoptotic damage markers, the opening of this pore destroys the proton gradient that mitochondria use to generate ATP.[22] It is a reasonable hypothesis that, in the case of DCD donation, mitochondrial death and injury contribute to an energetic crisis in the cardiomyocytes, leading to reduced heart function upon reperfusion and contributing to reperfusion

injury. Unlike in other organs, the high energy demand in the heart may make this crisis greater than the tissues ability to recover once the heart is transplanted and functioning to perfuse the body.[23]

The Effect of Triiodothyronine on Cardiac Energetics

Using additives is a common practice in standard cold organ storage solution. Often these additives are antioxidants, apoptotic pathway inhibitors, or enzyme activators.[24] One possible additive is the thyroid hormone Triiodothyronine (T3), which is a major regulator of cellular energetic. Early research into the topic showed that mitochondria do have receptors for T3 hormone, and linked T3 to the initiation of mitochondrial DNA transcription.[25] Later work showed at least three pathways through which T3 up regulated genes leading to mitochondrial biosynthesis: direct activation of mitochondrial DNA transcription through mitochondrial receptors, activation of nuclear receptors to up regulate the production of nuclear DNA transcribed mitochondrial proteins, and through the nuclear up regulation of intermediate factors that promote mitochondrial biosynthesis.[26] One of these intermediate pathways is the upregulation of the transcription co-activator peroxisome proliferator-activated receptor gamma coactivator -1α (PGC-1α) via an upstream thyroid response element.[27] PGC-1α co-activates a number of transcription factors tied to cellular energetic including NRF1/2, ERR-α/β/γ, PPAR- α/δ, and FXR which regulate energetic processes such as mitochondrial biogenesis, fatty acid oxidation, and triglyceride metabolism.[28,29] Figure 1 shows some of the varied activators and targets of PGC-1α. Aside from effects on mitochondrial biogenesis, PGC-1α targets also mediate the switch from glucose as a preferred metabolic substrate to fatty acid oxidation.[30] Although in general, fatty acid oxidation is the preferred method of ATP generation in cardiomyocytes, increased glucose oxidation occurs in the heart in response to acute ischemia and has also been linked to heart failure and cardiomyopathy.[31] By increase levels of the key enzymes in fatty acid β-oxidation, PGC-1α has the potential to increase ATP output in the energy depleted heart.[32]

Working Hypothesis

During hypothermic preservation, metabolically active additives act extremely slowly. However, at normothermic or midthermic conditions, metabolic activity can be stimulated more vigorously.[13,33] Given an adequately oxygenated midthermic system, metabolically active T3 has the potential to be a therapeutic additive in the DCD heart by stimulating mitochondrial biogenesis and greatly increasing available ATP in the energy depleted tissue.

Fig 1. PGC-1α Pathway

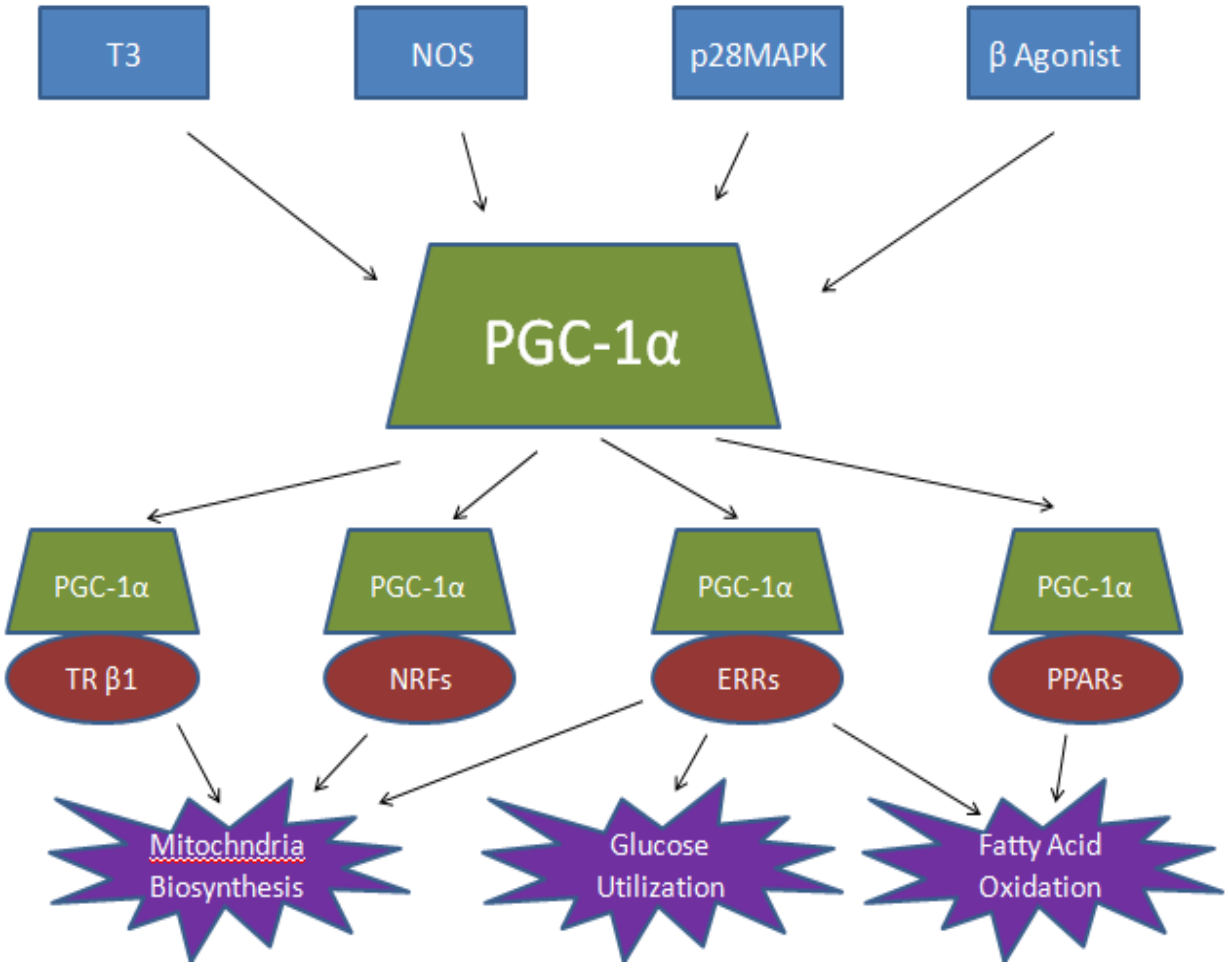

Figure 1: Thyroid hormone (TH), Nitric Oxide Synthase (NOS), p38MAP Kinase, and Beta Adrenergic agonists can activate PGC-1α, which is turn acts as a co-transcription factor with Thyroid Receptor β1 (TR β1), nuclear respiratory factors (NRFs), estrogen-related receptors (EERs), and various Peroxisome proliferator-activated receptor (PPARs).

Materials and Methods

Animals

Adult male Sprague Dawley rats aged 8-12 weeks were used in these experiments. All animal experiments were conducted under the guidelines on humane use and care of laboratory animals for biomedical research published by the US National Institutes of Health. The Virginia Commonwealth University Institutional Animal Care and Use Committee approved the study protocol.

Experimental model

Hearts were collected using either a DCD modeling transplant protocol for the experimental group or a beating heart protocol for control hearts. The control hearts were collected while still beating with the animal under anesthesia and ventilation. In the DCD model, the animal was ventilated and then the diaphragm was paralyzed with 0.1 ml of a 0.9% NaCl solution containing vecuronium (40 µg/rat). Ventilation was removed, and the heart was collected after a 30 minute stand off period.

In both models, the inferior vena cava was cannulated during collection. The heart was then removed, the aorta was cannulated and secured with a metal catheter. The heart was then flushed with 1 ml of cold normal saline, tap dried, weighed, and then connected to the machine perfusion system.

Perfusion pressure and flow were monitored using AD Instruments Power Lab hardware and Lab Chart 7 Pro software. Measurements of flow and perfusion pressure were used to monitor the status of the heart and apparatus, and both inflow and outflow samples were taken at two hour intervals. Samples of the perfusate were collected every two hours and stored at -80 ^{o}C. pO_2, Hemoglobin and lactate levels were measured using a Radiometer ABL Flex 800 Blood Gas Analyzer (BGA). Calculations of myocardial oxygen consumption (MVO_2) and extraction were made based on these measurements. After 8 hours of perfusion, samples were collected for histology, protein and DNA analysis

Fig. 2 Perfusion Apparatus

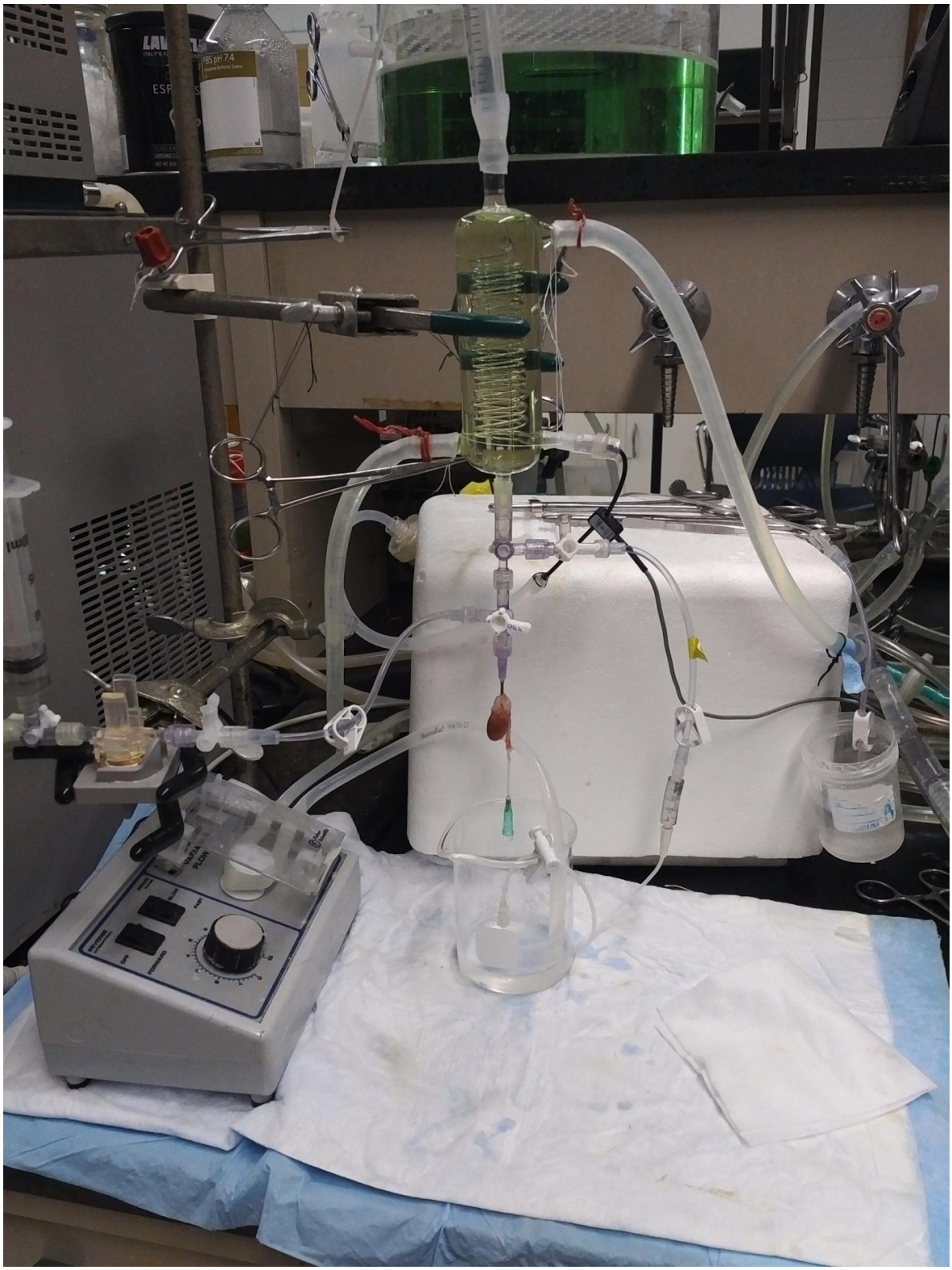

Figure 2 shows the entire system assembled and running. The green color in the glassware is from the circulating coolant. The beaker beneath the heart is a reservoir for the circulating MPS. The particulate filter and oxygen membrane are present, but not visible in this photo.

Machine Perfusion System

Hearts were perfused antegradely on a constant flow, fluid recirculating Langendorff apparatus (see figure 2) at 15 ^{o}C. using a modified Belzer Machine Perfusion Solution (MPS) four 8 hours. Perfusion temperature was maintained using double chambered glassware (figures 3 and 4). The outer chamber of the double chamber glassware contained a continuous flow of liquid coolant circulated at the desired temperature; while the inner chamber contains a serpentine coil that the perfusate travels up before flowing down into the heart, allowing time for the perfusate to temperature equilibrate. The serpentine coil opens into the center of a column of approximately 40 cm in height. This column serves as a bubble trap. At the point where the coil enters the column, air bubbles trapped in the solution are able to travel upwards to the top of the column, while the fluid is forced downward towards the heart. This prevents air that may enter the system from causing embolisms.

Fig. 3 Glassware

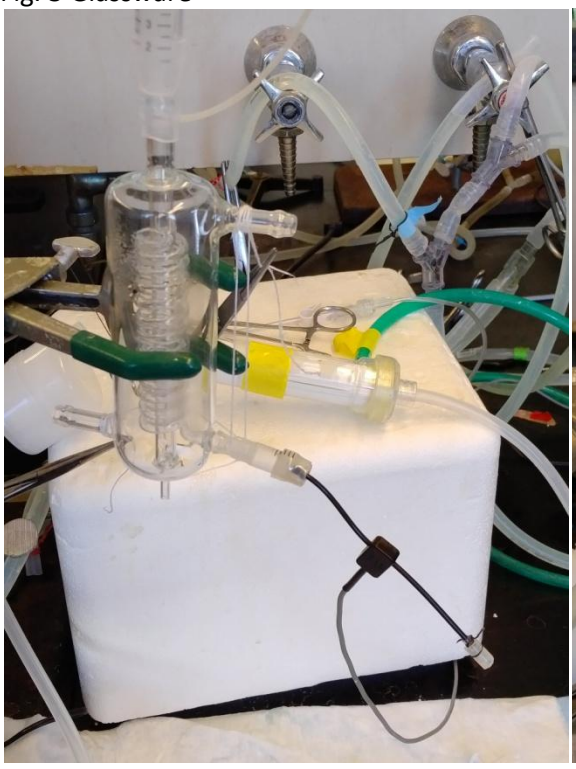

Fig. 4 Flow Probe & Cooling System

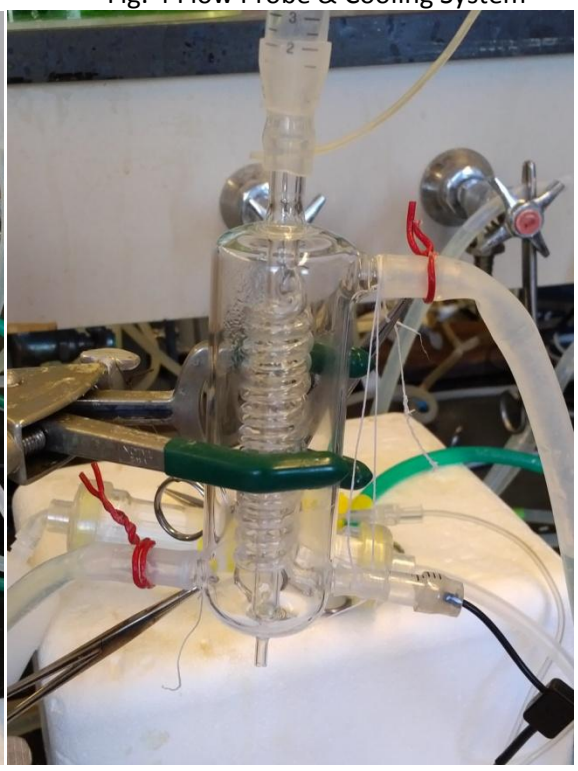

Figure 3 (left) shows the glassware with the flow probe connected to the inflow port. The other two ports are for connecting the cooling system, and fill the outer chamber. Figure 4 (right) shows the cooling system attached.

Directly below the glassware, a set of three-way stopcocks is used to attach the pressure probe, allow access to take input samples, and attach the cannula for the retrograde perfusion (see figure 5). Antegrade perfusion involves cannulating the ascending aorta and tying off below the first aortic branch, and pumping perfusate into the coronary sinus through the aorta. A peristaltic pump was used for this purpose (see figure 6). This creates perfusion pressure in the aorta that closes off the aortic valve, and leads to outflow through the coronary vasculature. The pressure probe is connected to the pressure sealed perfusion circuit at same stopcock the aortic cannula is attached to through a small amount of hard plastic tubing containing a saline solution. Since the solution is incompressible and the tubing mostly rigid, the pressure measured by the probe should be the same as the pressure at the point of connection and in the aorta. Since the vasculature is the only outflow route (at least, while near physiologic pressures) we can conclude that the flow through the heart is equal to the inflow into the aorta. As such, while the flow rate is measured at the point where the perfusate enters the glassware, this measurement should accurately reflect the flow through the vasculature. It should be noted that this is only an accurate measure of flow through the heart when the top of the glassware column is sealed. This is useful since we can much more easily measure the inflow into a sealed system than directly measuring the flow through the heart, but it is the flow through the heart that is critical to calculating MVO_2.

The other stopcock is usually seal, except when we take input samples. When we take these samples, we open a line that allows a small amount of fluid to drain out, and then seal the lower stopcock before we draw the input fluid. This ensures two things: 1) that the fluid drawn for the input measurement is “fresh” i.e. that it actually reflects what is passing through the heart rather than something that may have been stagnating in the access line and 2) that the fluid we draw comes from the column above, and is not being drawn back out of the aorta. This is done quickly to limit the interruption to perfusion to ideally less than 5-10 seconds, and this input is take after the output so as to

Fig. 5) Stopcocks

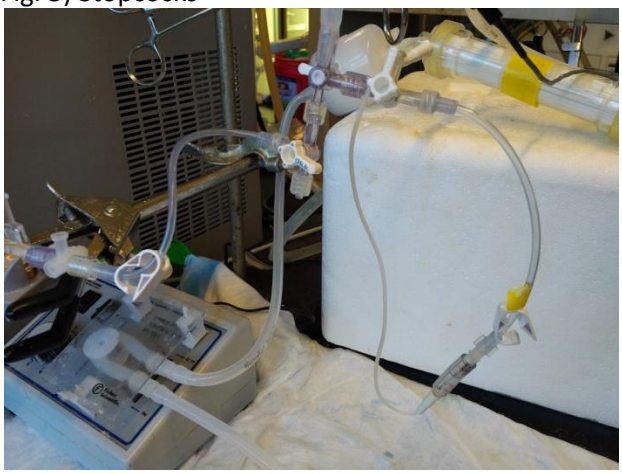

Fig 6) Peristaltic Pump

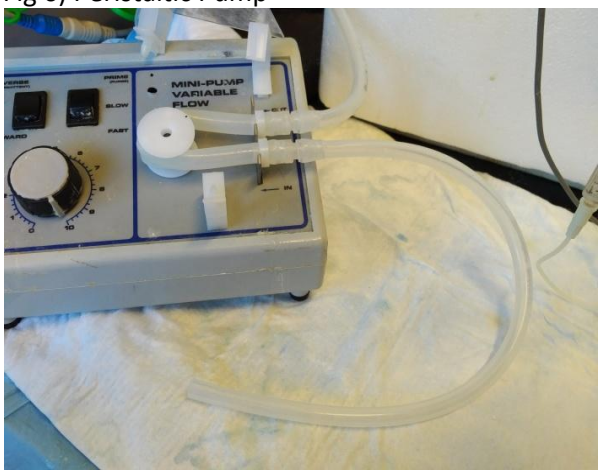

Figure 5 (left) shows the set of 3-way stopcocks. The first, connected directly to the glassware, allows outflow to be shut off to initially fill the system, and allows it to be diverted either downwards towards the heart or out through a secondary tube that bypasses the heart. This can be used to release excess pressure that may have built up in the system. The second stopcock (upper right of the figure) is part of this secondary tube but is used primarily to allow the drawing of input samples from directly before the heart, to allow an accurate measure of oxygen going into the organ. The third and final stopcock is what connect the catheter from which the heart is hung s to the rest of the system. The branch is connected to a pressure transducer through a short stretch of tubing (lower left of the figure). When the system is running, all three directions on this stopcock are left open. Figure 6 (right) shows the connection to the variable speed peristaltic pump. The trailing end of tubing is placed into a beaker of perfusion solution here it draws the solution into the system.

Fig. 7 Filter & Oxygen Membrane

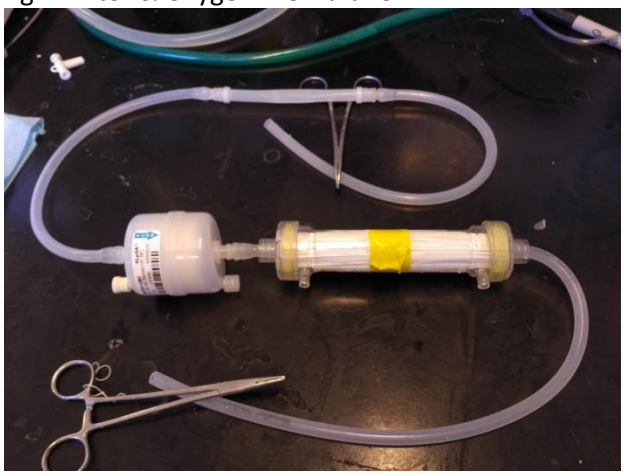

Fig. 8 Connection in Series

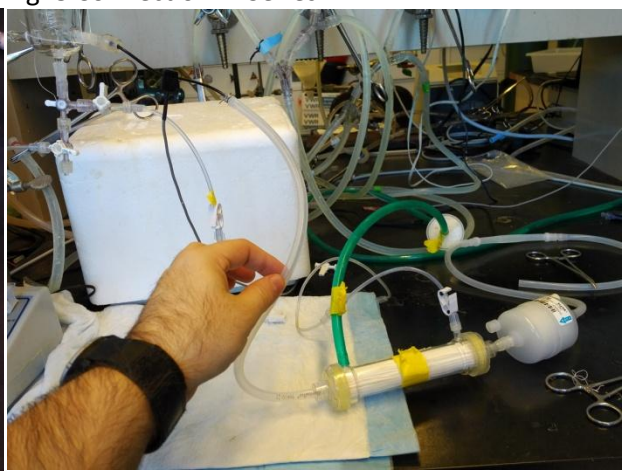

Figure 7 (left) shows the particulate filter and the oxygen membrane (marked with yellow tape) connected in series. Figure 8 (right) shows those components connected to the glassware through the flow probe, with the green oxygen gas line attached to the oxygen membrane.

not allow that brief interruption to influence the output data.

The perfusate is oxygenated using an extra corporeal oxygenation membrane (figure 7 and 8) after being passed through a 20 µm pore filter to remove any excess particulate matter, precipitate, or cellular debris. The membrane is attached to an air tank of 100% oxygen.

Organ Procurement

Our procurement protocol is designed to mimic a clinical DCD transplant setting as closely as is possible in our animal model. Rats were heparinized to prevent blood clotting in the heart and anesthetized using ketamine/xylazine (100/10 mg/kg). At this point the procedure diverges for two of the experimental groups. In control animals, the rat is ventilated and the heart is removed immediately while still beating and oxygenated. In the DCD model, the rat is ventilated and then given vecuronium to paralyze the diaphragm. Once the vecuronium has taken effect, ventilation is removed. This models the removal of life support and the succession of breathing in DCD patients. Then, there is a 30 minute standoff period during which the heart experiences warm ischemia. After the 30 minutes it is removed from the chest. Once the heart is removed, the protocol for the two groups re-converges. During the removal, the IVC cannula is placed, and the heart is flushed with cold cardioplegic solution. After removal, the heart is placed into cold MPS solution while extraneous tissue is removed (for example, pieces of the lung, thymus, descending aorta, and pulmonary artery that are still attached to the heart), and then the aortic cannula is placed. Once the aortic cannula is placed into the ascending aorta, below the first aortic branch, the heart is again flushed with cold MPS through this cannula to ensure that it has been placed correctly. The heart is then weighed and transferred to the Langendorff apparatus.

Fig. 9 Experimental Procedure

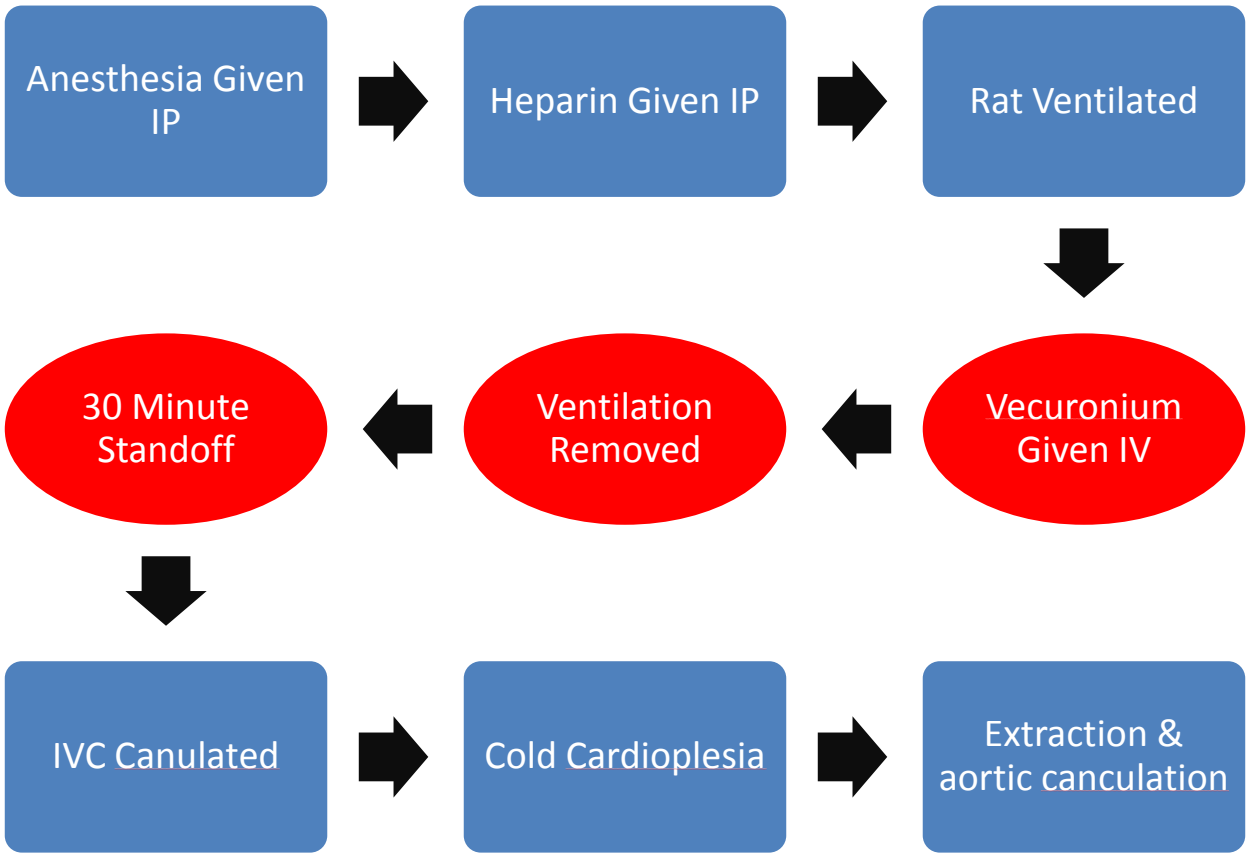

Figure 9 is a chart following the heart procurement procedure. Steps unique to the DCD process are presented in red ovals. These steps are skipped in the DBD procedure where the IVC is cannulated immediately after anesthesia and ventilation.

Perfusion Solution

Table 1 contains the ingredients and ratios for our modified Belzer MPS solution. Final pH of the solution was between 7.35 and 7.45. This pH is achieved by adding 10 N Sodium Hydroxide to the otherwise highly acidic solution, and is maintained by 10 mM of HEPES buffer and 15 mM of phosphate buffer. Final concentrations should be sodium at 101 mM, potassium at 15 mM, chloride at 1 mM, calcium at 500 µM, magnesium at 1 mM, and gluconate at 105 mM. Gluconate acts as a cell impermeant to reduce cellular swelling. Adenosine (5 mM) is added as a vasodilator and substrate for ATP synthesis. Twenty kg/mol polyethylene glycol (PEG-20K) is added at 2.5% weight. PEG is a hybrid impermeant. Unlike pure cell impermeants, the majority of PEG-20K molecules do not pass through the

vascular epithelium. This creates an oncotic pressure localized in the vasculature and helps prevent interstitial edema from high fluid pressure in the vasculature, as well as an oncotic gradient from the cell to the interstitum.[34] Glucose (10 mM) provides substrate for glycolysis, and octanoate (1 mM), a short chain fatty acid, provides substrate for cardiac fatty acid oxidation and metabolism. Dexamethasone (8 mg/L) is added for its anti-inflammatory properties, and the xanthine oxidase inhibitor allopurinol (1 mM), as well as glutathione (3 mM) are added for their respective antioxidant properties. In addition a solution of the following levo-amino acids is added: Arginine (10.0 g/l), Asparagine-H_2O (2.84 g/l), Aspartic Acid (1.0 g/l), Cystine (2.5 g/l), Glutamic Acid (1.0 g/l), Glutamine (0.29 g/l), Glycine (0.5 g/l), Histidine (0.75 g/l) Hydroxy-L-Proline (1.0 g/l), Isoleucine (2.5 g/l), Leucine (2.5 g/l),Lysine-HCL (2.0 g/l), Methionine (0.75 g/l), Phenylalanine (0.75 g/l), Proline (1.0 g/l), Serine (1.5 g/l), Threonine (1.0 g/l), Tryptophan (0.25 g/l), Tyrosine (1.16 g/l), and Valine (1.0 g/l). For thyroid hormone treated groups, T3 is added to the solution at a concentration of 10 μM

Perfusion and Monitoring

Over the course of the 8 hour perfusion, flow through the heart was monitored to ensure hearts were adequately oxygenated and that the flow did not drop below a critical flow (D_{Crit}). The value of D_{Crit}, a critical value of oxygen delivery per mass, was determined experimentally on this apparatus with this solution by prior work in this lab. For DBD hearts the value is approximately 1.4 ml/min/g and for DCD hearts the value is approximately 1.7 ml/min/g. Below this value, the oxygen demands of the tissue are not being met. Above this oxygen consumption is nearly independent of flow. Perfusion pressure was monitored and recorded simultaneously with flow.

Table 1.

Reagent	Concentration	Mol. Wt. (g/Mol)
Allopurinol	1 mM	136.1
Mg-Gluconate	5 mM	207.3
PEG-20K	2.5%	20000
KH_2PO_4	15 mM	136.09
Glucose (Dextrose)	10 mM	180.16
Na- Octanoate	1 mM	166
HEPES	10 mM	238.3
$CaCl_2$ (dihydrous)	0.5 mM	147.0
Na-Gluconate	100 mM	218
Adenosine	5 mM	267
Glutathione	3 mM	307.3
Dexamethasone	8mg/L	392.5

Table 1) a list of in ingredients and concentrations for the modified machine perfusion solution used in these experiments. This is an excerpt from the protocol used to mix the solution in various volumes.

Both the input and the output samples were taken in 1mL plastic syringes. The syringes were stored in ice prior to the sample being taken. To minimize the amount of loss in the dissolved oxygen content after the sample was drawn, the end of the syringe is immediately covered in parafilm and then replaced in the ice while it is rapidly transported to the blood gas analyzer (BGA) machine. Samples are taken consecutively, but not concurrently. Input samples are drawn only after the output has been analyzed and the BGA has been reset to run a new sample. As a result, input values are checked approximately 5 minutes after output values, and output values are measured before the brief interruption of flow that is required when taking an input samples.

Calculation of Oxygen Consumption

Eq. 1) $CO = \frac{VO_2}{C_i - C_o}$

We use the Fick principle to calculate tissue oxygen consumption (VO_2). Although this principle is usually employed *in vivo* to calculate cardiac output (CO), with a measured whole body VO_2, the governing equation can be re-written in such a way as to calculate VO_2 from a known CO. We observe that there is a difference in oxygen concentration in the input solution (C_i) entering into the aorta and the output solution (C_o) exiting the vena cava, but this difference simply gives us a change in concentration, not a volume consumed or a rate of consumption. By multiplying this difference by cardiac output, we can calculate a rate of consumption. We can demonstrate this via dimensional analysis, for the full analysis, see supplemental equation 1. In a clinical environment, we can measure VO_2 using spirometry, and then as in equation 1, we calculate the cardiac output from there. However, our case differs somewhat from the clinical scenario. First, since we are dealing with an isolated organ, rather than the entire body, there is no gas exchange through the lungs, so spirometry to measure VO_2 is impossible. Secondly, we are passing fluid only through the coronary vasculature rather than the entire systemic circuit of an organism. Since the coronary vasculature is what provides the heart, especially the myocardium, with the necessary oxygen and nutrients, this model still supports the use of the Fick equation. Finally, in our case cardiac output is a misnomer, as the cardioplegic heart is not pumping the blood. That said, the importance of the cardiac output term in the Fick equation is not as a measure of cardiac function but as a fluid flow rate. Given that we have direct control of the fluid flow in our mechanically perfused system, the flow (equivalent to cardiac output for this purpose) is an independent variable that we know *a priori.* As we saw during the derivation, we can use this flow (represented as F), along with a measured venous and arterial oxygen concentration (as measured before and after passing through the coronary vasculature) to calculate VO_2.

$$\text{Eq. 2) } VO_2 = F(C_i - C_o)$$

To use this equation we must be able to determine the concentration of oxygen in the solution. The

BGA we use reports gases as partial pressure in mmHg. Conveniently, oxygen concentration is directly proportional to partial pressure of oxygen in the fluid, related by the solubility of oxygen in water. Of course, you must also account for oxygen bound to the carrier protein hemoglobin, and even in our machine perfusion solution (MPS) there is some residual hemoglobin derived from a small amount of blood cells which entered in from the coronary circulation and the LV cavity, particularly in the DCD hearts. However, the amount of measured hemoglobin detected by the BGA was between 0.02 and 0.00 g/dL. Fortunately, this can be determined based on the concentration of total hemoglobin, the oxygen carrying capacity of hemoglobin (1.34 mL/g), and the fractional ratio of oxyhemoglobin to total hemoglobin (oHb/ctHb).

Eq. 3) $$C_{O_2} = \left([Hb]\left(\frac{g}{dL}\right) * 1.34\left(\frac{mL\ O_2}{g\ Hb}\right) * \frac{oHb}{ctHb}\right) + 0.003\ (\frac{mL\ O_2}{dL * mmHg}) * PO_2$$

The carrying capacity of hemoglobin is a constant, and the total concentration of hemoglobin can be measured by our BGA. The ratio of oxyhemoglobin to total hemoglobin can be represented as a sigmoid function of PO_2. The specifics of this function can be determined analytically, but the curve shifts with changes in pH, PCO_2, and temperature. However, within the range of physiological conditions the oxygen disassociation curve asymptotically approaches 1 at PO_2 values higher than about 100 mmHg.[35] In these experiments, PO_2 values generally ranged from 500 mmHg to 200 mmHg and all but the lowest, values were in excess of 100 mmHg. As such, we assume that fraction of oxyhemoglobin is 1 for our calculation when $PO_2 \geq 100$ and as a percentage for the few values under 100 (see supplemental equation 2). The minimum detection threshold of the BGA is 0.01 g/dL of hemoglobin. Any value of hemoglobin less than that was undetected and treated as zero. The measured values of PO_2 from our "arterial" and "venous" solutions (i.e. our input and our output respectively) are then plugged into equation 3 along with the measured hemoglobin values.

Edema Measurements

Transverse sections of the apex and the base of the heart were embedded in paraffin, and then 5 µm slices were fixed to glass slides before being stained with hematoxylin and eosin. Color photographs were taken using a Zeiss AxioCam HR microscopy camera and these photographs were analyzed using the software ImageProPlus v.6. At least five measurements were made on each heart, from two to four different sections of tissue and then averaged together.

Troponin Analysis

Cardiac Troponin I (cTnI) content of the outflow fluid collected at each 2 hour time point was quantified using a commercially available Rat Cardiac Troponin-I High Sensitivity ELISA assay available from Life Diagnostic (catalogue number CTNI-2-HSP).

Mitochondrial DNA Quantification

Mitochondrial DNA was quantified by using quantitative PCR to measure the relative expression of mtDNA as compared to genomic DNA. Primers against DNA repair protein XRCC2 and a sequence from vertebrate mitochondrial DNA were used as genomic and mitochondrial references respectively. Primer efficiency was very close to 2 for both primer sets. PCR was run using Bio-Rad SYBR Green reaction mix. Analysis was done using the ΔΔCt method. Primer Sequences can be found in Appendix 2.

Troubleshooting

During the course of the experiment, the hearts were monitored, and occasional corrections were made to the apparatus if there were indications of a problem. An unusual flow or a sudden change in perfusion pressure could indicate a problem with either the heart or the experimental set up. Extremely high pressure may represent a heart that has embolized, or the system had become blocked. If pressures rose about 110 mmHg for an extended period and no blockage in the system could be

found, the experiment was terminated. A filter was added after a few early attempts showed an uncontrolled rise in pressure after 4 hours of perfusion. The presence of the filter eliminated this problem which indicates that precipitates or cellular components derived from the blood of the donor heart may have been impairing coronary perfusion and increases the resistance of the heart.

An extremely low pressure with a normal flow might indicate a leak from the aorta, in which case the perfusate is not actually passing through the coronary vasculature, and the aorta would be re-ligated. An unusually high flow with low pressure indicated a leak from the system. The leak would be found, and patched if possible, or the leaking component would be replaced if it could not be patched. As mentioned above, both input and output samples were taken at least every 2 hours. These samples were analyzed on the BGA. A sudden or drastic change in oxygen consumption, lactate production, or CO_2 production are all indicators that the heart is being inadequately perfused, and steps such as increasing the fluid flow, or the oxygenation of the fluid need to be taken in an attempt to address that. Samples were taken consecutively rather than concurrently because oxygen would rapidly diffuse out of the plastic syringe. We believe any discrepancy that might result from taking the samples concurrently is much less than the discrepancy in pO_2 content if the input were allowed to sit, even on ice, for the 5 minutes it takes the BGA to run and reset.

In the course of this study only hearts that were adequately perfused in absence of technical problems were used for the data analysis.

Results

Machine Perfusion System Optimization

A major investment in time during this project was optimizing the system used for the experiments. Many early experiments preformed with DCD hearts were terminated after only an hour

or two as pressures rose well above physiologic conditions. This dramatically increased pressure was paired with a reduced flow, and to increase the flow to ensure adequate perfusion would increase the pressure still further, risking damage to the coronary vasculature. So long as this was occurring, we could not run the experiments to their 8 hour conclusion. Since we did not observe this phenomenon dramatically until about 2 to 4 hours into the experiment, the first hypothesis tested was that the heart was metabolizing the adenosine, a substrate and a vasodilatory agent present in the perfusion solution. In particular we discussed the possibility that without the vasodilatory properties of the adenosine, the vasculature was constricting, increasing the resistance and resulting in higher perfusion pressures and lower flows. The attempted remedy to this was to periodically change the recalculating solution to maintain adequate levels of adenosine. This approach only delayed the phenomenon of increased resistance by 1 to 2 hours. While monitoring these experiments, I observed two distinct patterns to the pressure increase. There was a slow and steady increase in pressure over time, but there was also another distinct feature that appeared. At irregular intervals, there would be a transient drop in pressure, followed but a sharp, sudden rise. The pressure tracing from one of these events is shown in figure 10. This pattern implied to me a physical blockage of the vessels. The transient drop would result from whatever debris might be present suddenly slipping deeper into a set of vessels, and the subsequent sharp rise would be the result of those or other vessels now being suddenly blocked off. Whether this debris was some sort of chemical precipitate in the solution or cellular debris deriving from blood cells was unknown, but whatever the cause of the embolisms, adding a filter to the perfusion circuit would eliminate the problem.

Our first attempt was to add a 1 μm pore glass-ceramic syringe filter directly before the heart. This reduced the perfusion pressure dramatically, but also reduced the flow through the heart to almost nothing. After measuring the pressure both before and after the filter, I realized the operating pressure of this syringe filter was much higher than what our system could handle. Although pressures after the

filter were low, in the range of 10-20 mmHg, pressures before the filter were approaching 200 mmHg.

Next, we tried a filter with a larger pore size. Specifically, we used the 20 µm Meissner capsule filter pictured in figure 7, but unlike our final configuration, we initially placed the filter downstream of the oxygen membrane. This solved the problem of the perfusion pressure increase. A gradual increase over the course of the experiment remained, but pressures remained well below physiological pressures, and completely eliminated the characteristic pressure spikes shown in figure 10. However, this configuration presented its own problem.

A Meissner capsule filter holds a volume of approximately 30 ml within its casing. It takes several minutes for this volume to completely turn over, and, much like the syringes used to take samples, the outer plastic housing of the filter is not impermeable to oxygen. The end result of this is that a great deal of oxygen solubilized into the solution was being lost out of solution at the filter level, giving input PO_2 values that were too low to be of use for the experiment. That it was the filter causing this loss was demonstrated by taking a sample and measuring oxygen both before and after the filter and comparing the PO_2 values. Before the filter, PO_2 values were in the range of 700 mmHg, and after the filter, PO_2 values were in the range of 200 mmHg. This problem was resolved by simply switching the order of the filter and oxygen membrane, resulting in the final configuration. This configuration provided adequate flow and oxygen content while solving the problem of the dramatic pressure increases during the experiment.

Fig. 10 Pressure Tracing of Sudden Pressure Increase

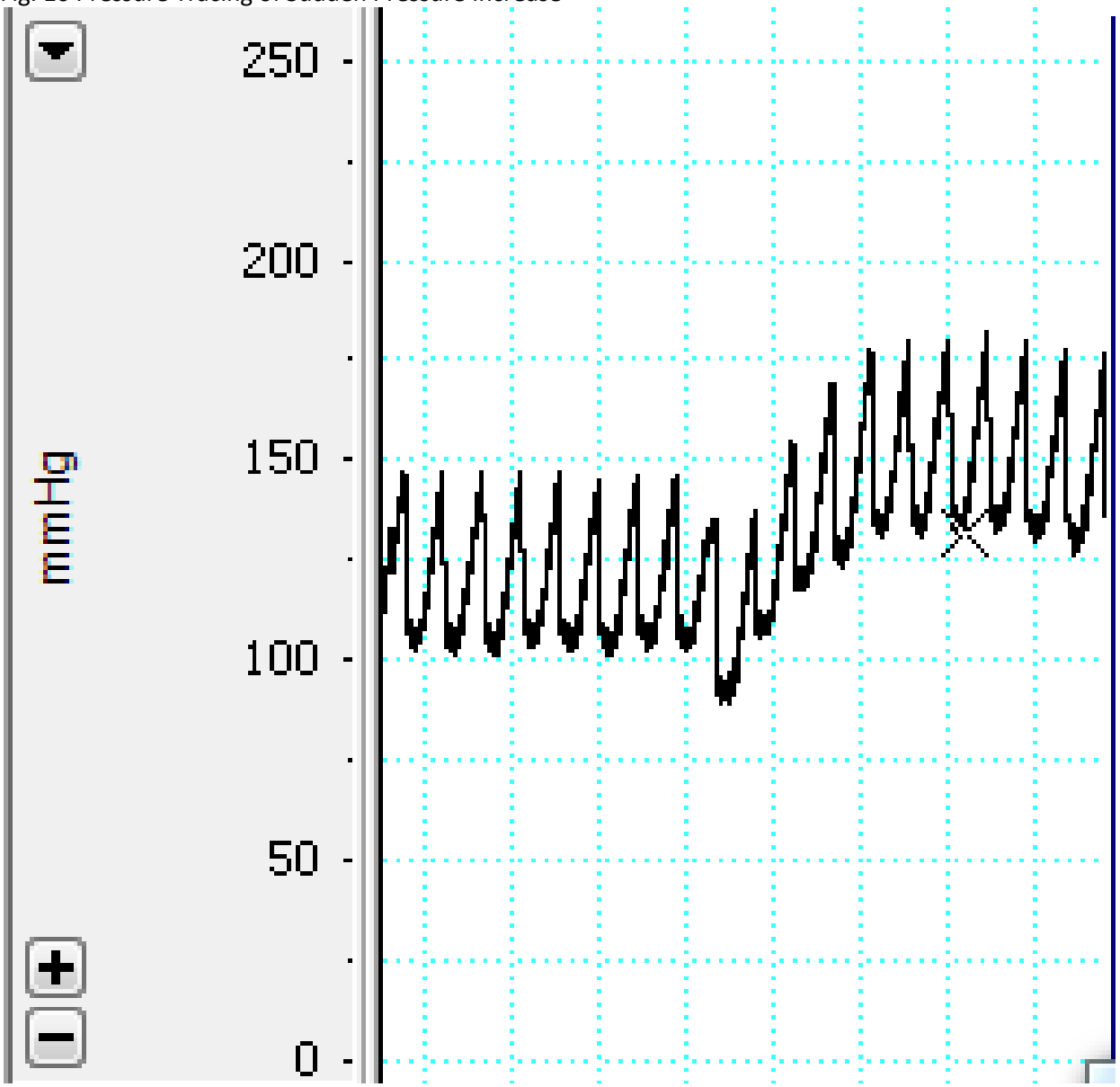

Figure 10 a screen capture showing an example of the unusual rise in pressure prior to adding a filter

Oxygen Consumption

Oxygen consumption showed a trend of higher MVO_2 when compared to the DCD hearts. This confirms our previous observation that DCD hearts have a higher MVO_2 during machine perfusion system organ preservation. The MVO_2 of the DCD hearts decreased with time, while DBD hearts had a trend to that increases MVO_2 over time. The decrease in oxygen consumption was significant between 0 0 hr and 8 hr in DCD+T3 hearts (p=0.032) but not in DCD without T3 (p=0.100). The increase in oxygen

consumption of the DBD hearts was significant in the T3 group (p=0.041) but not in the group without T3 (p=0.143) (Figure 11).

Fig. 11 Myocardial Oxygen Consumption over Time

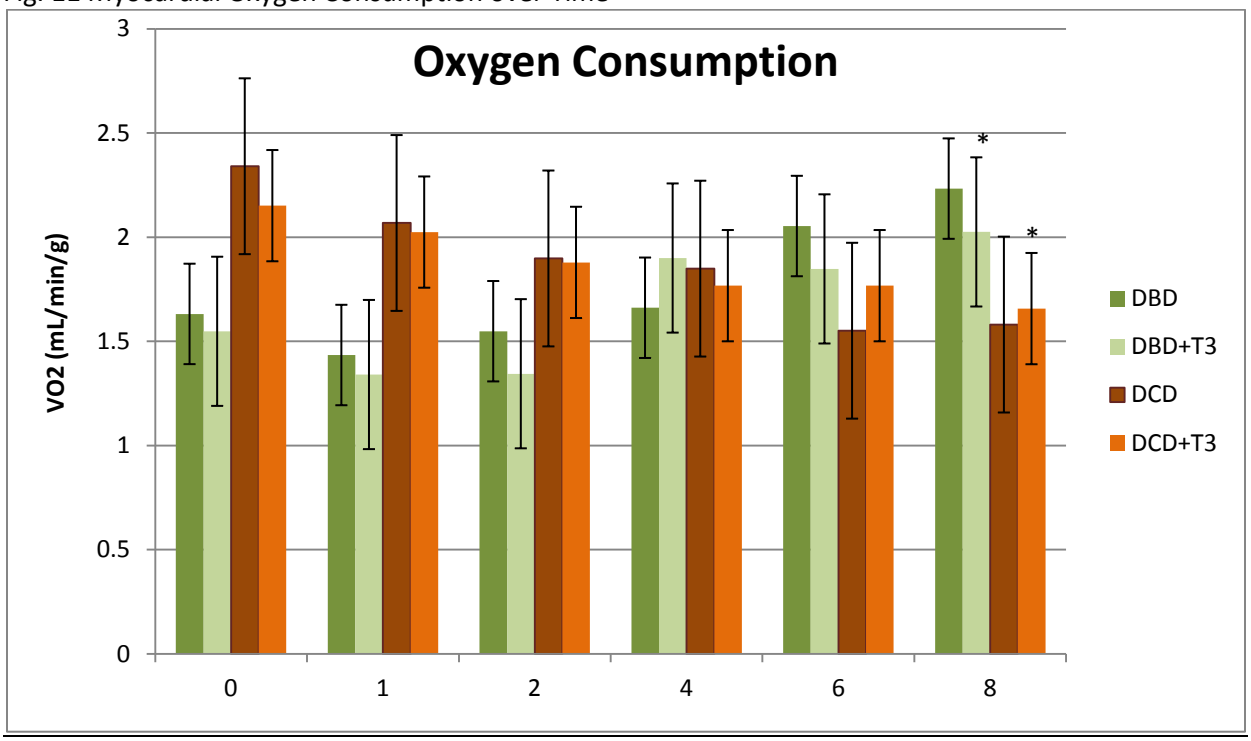

Figure 11 gives oxygen consumption with each hour is grouped together. Each sample group is in the same color in each group. Significant differences ($p<0.05$) vs. time 0 hr are marked with *.

Edema

Cellular edema was analyzed using hematoxylin-eosin (H&E) stained histology slides to measure extracellular space in the fixed tissue. The relative amount of edema was estimated by quantifying the ratio between the unstained extracellular space to the total area of the section. Edema measurements are presented as a percentage of total area. Figure 12 shows the averages plotted on a bar graph. Control hearts were fixed in formalin immediately after collection, rather than being perfused for 8 hours, and serve as a baseline for comparison as a normal tissue. We see a drastic and significant increase in edema in all groups over baseline. DCD hearts show a trend of slightly increased edema over DBD hearts, but the trend does not reach significance (p=0.078).

Fig. 12 Edema by Percent of Space

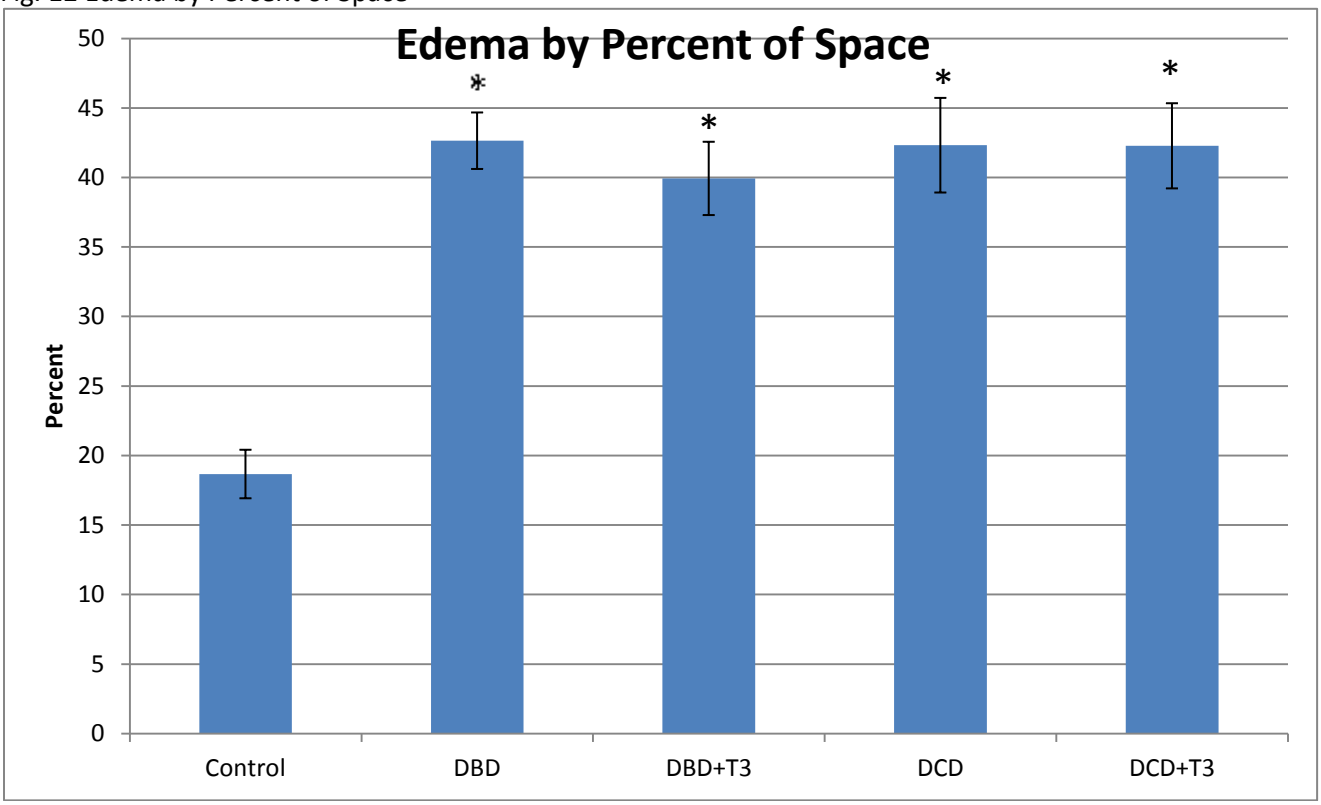

Figure 12 is Percent of extracellular space quantified in histology slides. * indicate significant change from control ($p<0.05$).

Troponin Release

We measured minimal troponin release from the eluate of DBD hearts, and no change in this with T3 treatment. However, we see an increasing release of troponin over time with the DCD heart. This increase is lower in DCD hearts treated with T3, with a statistically significant reduction appearing by 8 hours ($p=0.035$) Figure 13 shows this trend charted over time.

Fig. 13 Troponin Release Over Time

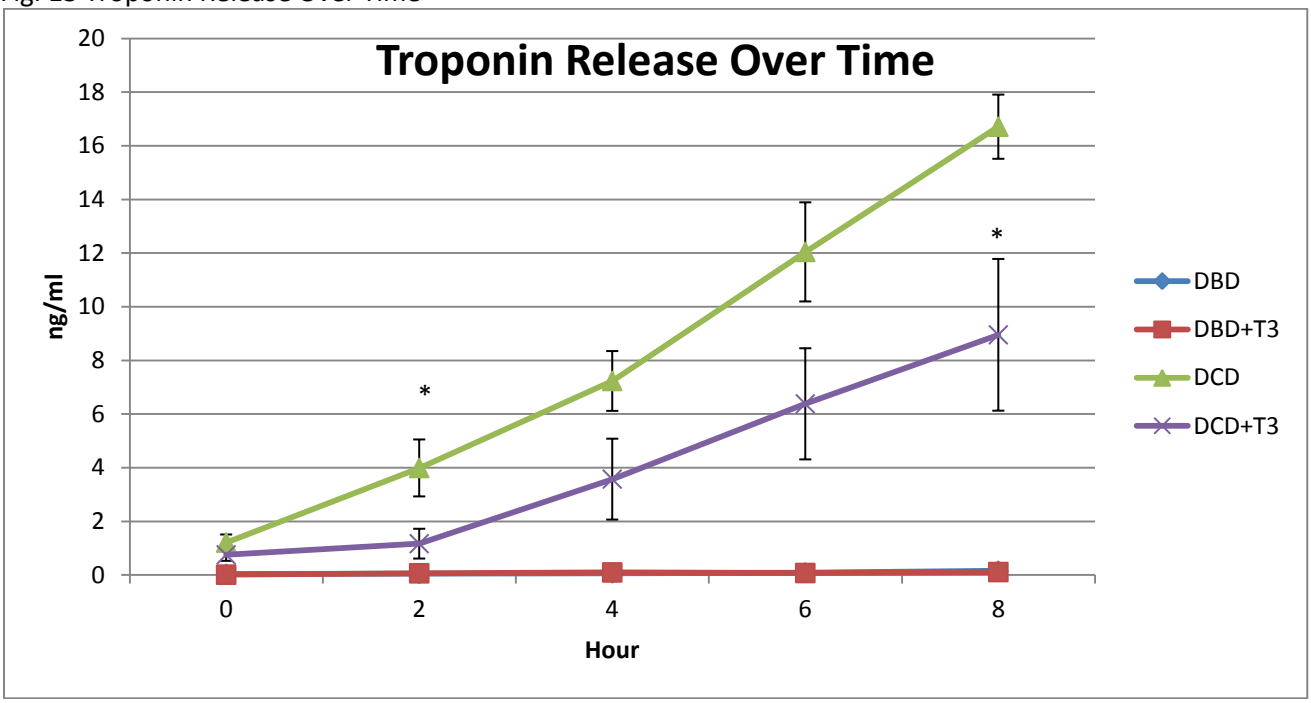

Figure 13 charts the increase of troponin over time. DBD hearts have extremely little troponin release, and all DCD values were significantly higher. * mark hours where DCD+T3 values were significantly lower than DCD without T3 ($p<0.05$).

Lactate

Lactate output was measured over the course of the experiment. At each time point the difference between input lactate and output lactate was calculated. DBD lactate output averaged 0.070 mmol/L over the course of 8 hours. DBD+T3 lactate output averaged 0.094 mmol/L. DCD lactate production averaged 0.043 mmol/L. DCD+T3 lactate production averaged 0.050 mmol/L. There was no statistical significance between any of these groups.

Fig. 14 Average Lactate Production of Each Group.

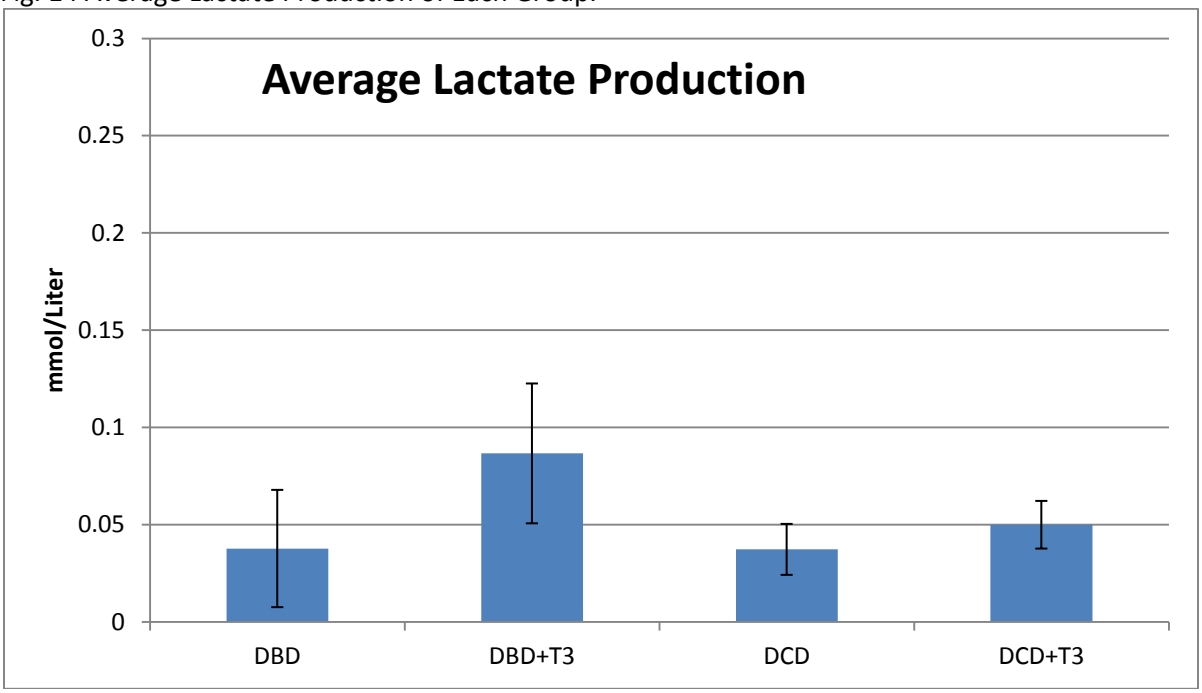

Figure 14 charts the average lactate production of hearts in each group (i.e. the lactate difference between the input and the output). There were no statistically significant differences between groups.

Perfusion Resistance

With direct measurements of both flow and perfusion pressure, the resistance of the heart could be calculated. DBD Hearts had low resistance that increased gradually over the course of the experiment. DCD hearts showed a high initial resistance that decreased over the first few hours and then began to rise. DCD hearts showed a significantly lower ($p=0.002$) average resistance over time than DCD hearts without T3. This trend did not appear in DBD hearts. Table 3 shows total averages and averages by hour of the four groups. Figures 15 and 16 show DBD and DCD resistances respectively charted over time.

Fig 15. DBD Flow Resistance Over Time

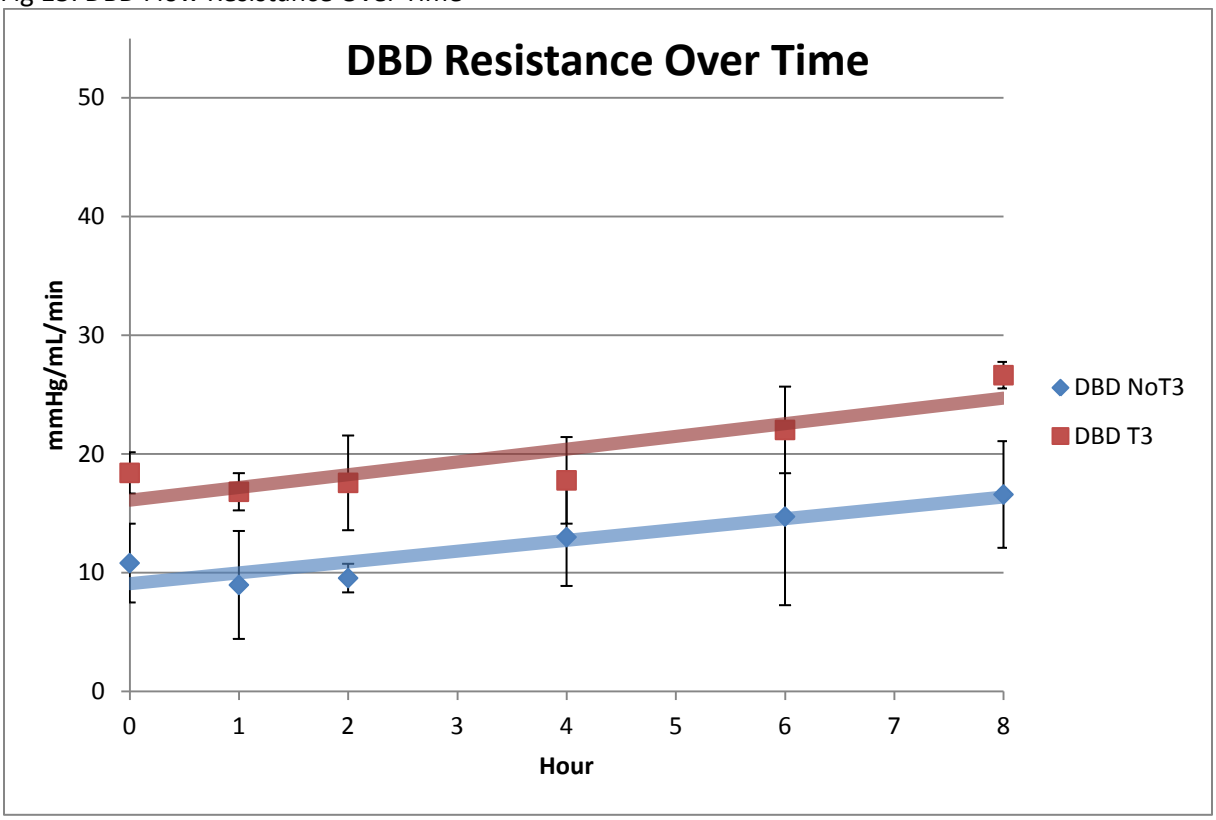

Figure 15 show the resistance over time of the DBD hearts in mmHg/mL/min. Values for individual hearts were plotted and fit with a linear regression

Fig. 16 DCD Flow Resistance Over Time

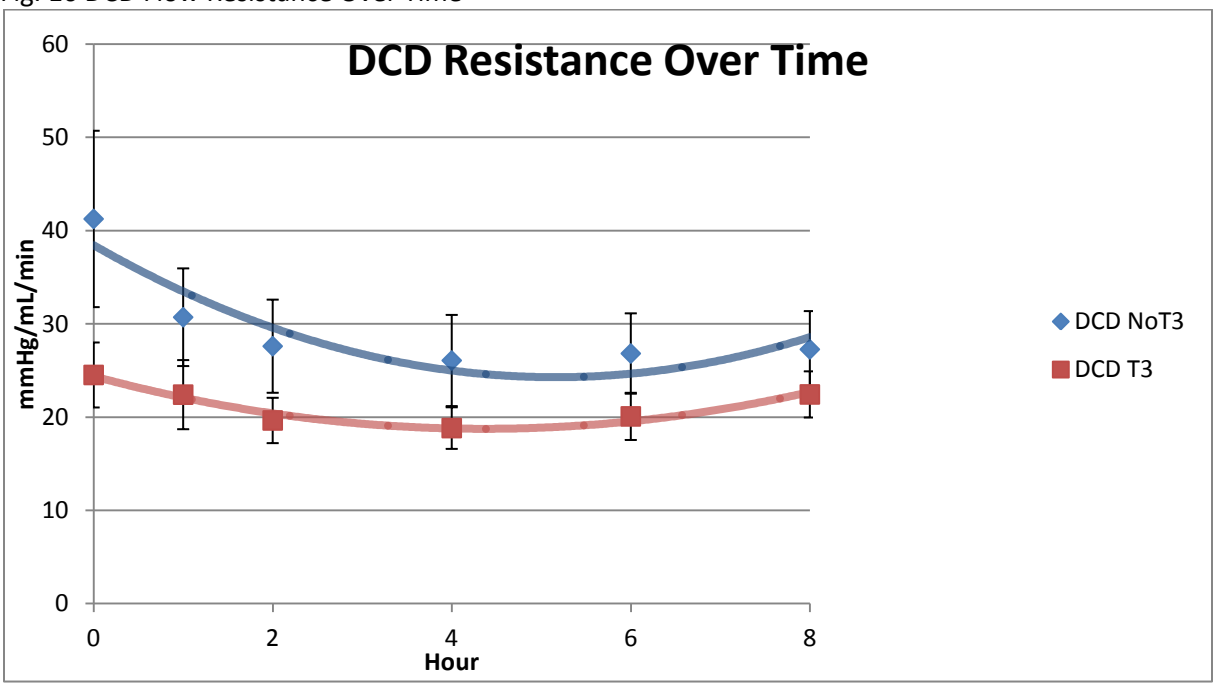

Figure 16 shows the resistance over time between the DCD Hearts in mmHg/mL/min. Values for each heart were plotted and then fit with a quadratic polynomial regression.

Table 3

Average	DBD	DBD+T3	DCD	DCD+T3
Over full duration	17.59	20.71	29.95*	21.14
0 hr	16.89	16.67	41.24	24.5
2 hr	18.13	21.56	27.60	19.64
4 hr	19.85	21.43	26.07	20.18
6 hr	22.19	25.67	26.81	20.07
8 hr	23.12	27.98	27.26	21.24

Table 3 shows the average resistance to flow in mmHg/mL/min. Asterisks represent significant difference from others in the same row ($p<0.05$).

Mitochondrial DNA

Quantification of mitochondrial DNA from tissue at the end of the 8 hour perfusion experiment was done using qPCR to determine the effects of T3 on mitochondrial biogenesis. Relative amount of mtDNA as compared to genomic DNA showed no significant difference in mtDNA between any of the groups.

Fig. 17 Relative Mitochondrial DNA amount

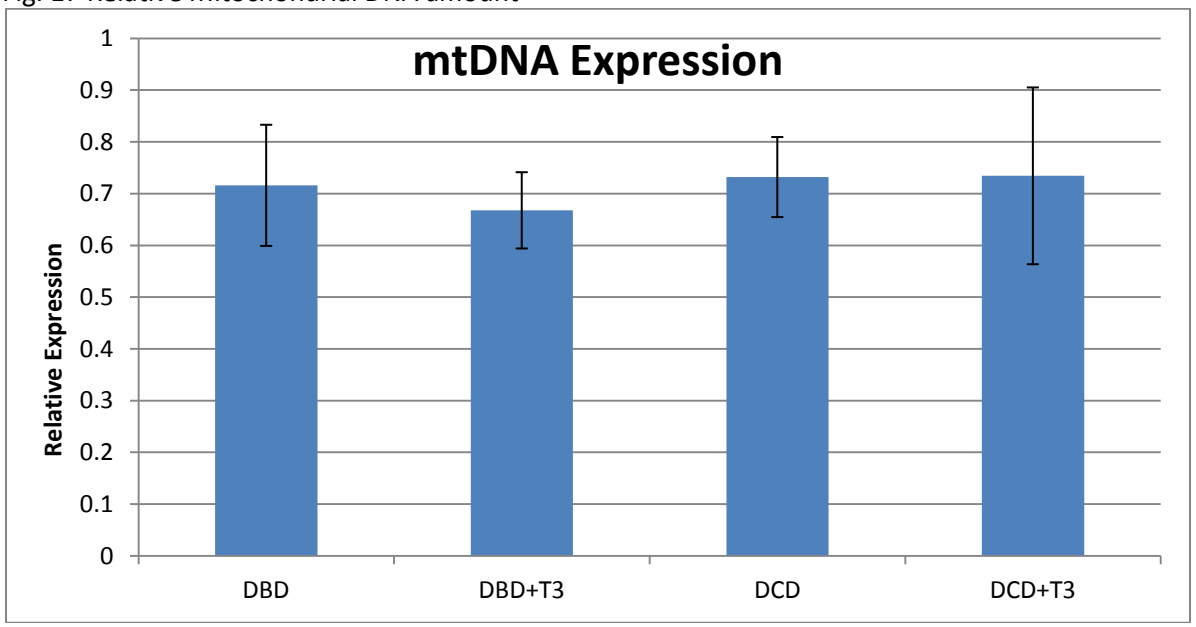

Figure 17 is the quantification of the relative amount of a reference gene sequence in mtDNA normalized by the amount of a reference gene in the genomic DNA

Discussion

Machine perfusion system (MPS) organ preservation is a way to metabolically sustain an organ during the storage phase that precedes the transplantation. MPS preservation is preferable in some instances (e.g. kidney), although in the heart setting has not been clinically tested. In fact there is an undergoing clinical trial to test a particular MPS system that utilized the donor's blood to sustain and monitor the heart heath. In addition, MPS preservation seems to be the only way to efficiently preserve the DCD hearts after they are procured, by reducing the ischemic time to warm ischemia only.

The DBD hearts that we considered here were from deeply anesthetized animals that did not receive any trauma, as one would expect in a true DBD donor. We used this to model a clinical beating heart brain dead donor, for a point of comparison to DCD donation. The hearts were placed on the Langendorff as soon as possible to minimize hypoxia and keep timing consistent within groups. We have observed higher MVO_2 in the DCD heart. This was expected since those hearts have experienced a substantial period (30 minutes) of warm ischemia which probably leads to an oxygen debt that induces a higher O_2 uptake. However, over time, the DBD and DCD heart had an opposite trend, with the DBD hearts increasing their oxygen consumption and the DCD heart decreasing it. However, we did not see any effect of the T3 in this phase of the study. The rise observed in the BD hearts may be explained by the fact that during MPS preservation the heart metabolism is changed. However, due to the fact that lactate levels were consistently negligible during the entire time, we assume that adequate oxygen delivery was maintained during all the MPS preservation time. In opposition, the DCD, which started higher had a lower MVO_2 at the end of the perfusion. This may reflect 1) a stabilization of the metabolism after "paying back" the initial oxygen death, 2) a progressive decrease of cells that utilize the oxygen, 3) or a decrease in viable mitochondria. Also in this case the lactate levels were very low, indicating adequate oxygen delivery and aerobic metabolism.

Edema was similar in all the MPS preserved hearts. The extracellular space was significantly higher in the perfused hearts when compared to control hearts explanted and fixed in formalin (all $p<0.001$). Although the DCD hearts had a trend in increased edema, we did not found any statistical difference. Additional studies looking at more sensitive parameters of tissue edema (like the wet/dry ratio) could be used in future experiments to determine a difference between DBD and DCD. No difference was observed in the preservation in combination with the T3.

Lactate analysis pointed out that the cellular metabolism was adequate during the perfusion preservation. However, we measured the levels of cardiac troponin I (cTnI) in the output solution to detect cellular damage. We observed constant and very low level of cTnI in the DBD during the entire perfusion preservation. This indicates adequate preservation and negligible cellular damage over the entire 8 hour period. The addition of T3 did not affect the cTnI level, and the two groups were overlapping at all the time points. As expected, the DCD process induced an increase of cTnI in the eluate output samples collected from the heart even at time 0 hr, few minutes after starting the perfusion, like caused by ischemic damage in the DCD hearts. The time course analysis revealed that during the MPS preservation, the levels of cTnI in the eluate increase approximately linearly, indicating cellular damage. This may explain the observed decline in the MVO_2 over time in the DCD hearts. However, the T3 delayed the rise of cTnI levels for the first 2 hours of perfusion. Beyond two hour the cTnI levels began to rise and continued to do so through the end of the experiment. Regardless, at the end of the experiment, cTnI levels were still significantly lower in T3 treated hearts than in untreated DCD hearts. This supports the hypothesis that T3 may have a protective activity in the DCD heart. Further, T3 showed a significant reduction in vascular resistance in the DCD hearts. The reduction in resistance, combined with the lack of change in edema between the T3 treated and non-treated DCD hearts likely indicates a reduction in constriction of the vasculature, but the exact mechanism is unclear at this time.

Mitochondrial DNA quantification showed no change between T3 treated and untreated groups. This indicates that over the course of the 8 hour midthermic perfusion there was not sufficient time to induce T3 dependant mitochondrial biosynthesis. This is not entirely unexpected, given the number of proteins that must be transcribed and synthesized in that process and the various intermediate steps in part of the T3 signaling. It is possible that T3 in our midthermic model is stimulating this process but that 8 hours is not long enough for the process to progress to the point of mtDNA replication. Activation of PGC-1α and T3 specific signaling pathways need to be assessed. Longer times could be used to test if this is the case, but longer perfusions times could be counter-productive to the goal of organ preservation.

This study does present some limitations. Firstly, there is a lack of functional data to determine whether the use of T3 improves myocardial function of the DBD or the DCD hearts. Additionally, some of the MVO_2 and edema data presented trends that could provide further insight but that did not reach statistical significance. We believe that increasing the sample number will increase the statistical power of the analysis, and possibly shed more light on these trends. Furthermore, the duration of warm ischemia and of preservation time may be too long to allow functional recovery of DCD MPS preservation. Studies using shorter ischemic times and preservation durations may help determine an optimal window of intervention and viable storage duration needed to ensure and maintain metabolic function after preservation. We did not measure any parameters of T3 activity beyond mitochondrial DNA. Studies of PGC-1α activation or ATP content could help demonstrate biological T3 activity. Finally, the mechanisms of DCD protection by T3 where not investigated. Analysis of cell of death signaling and markers (e.g. necrosis vs. apoptosis vs. autophagy) could be used to investigate this.

Conclusions

Our system of MPS preservation is able to metabolically sustain the DBD and DCD hearts for a period of

8 hours. The DBD hearts show edema but no signs of cellular damage. On the contrary, the DCD hearts had cellular damage that worsened over time. Of note is the fact that T3 reduced the troponin release in the DCD heart, indicating that T3 did in fact improved the organ preservation. As an increase in mitochondrial DNA was not detected, this effect appears to be independent of mitochondrial biogenesis.

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Appendix 1: Supplementary Equations

Eq. S1) $$\left(\frac{mL\ Oxygen\ In}{L\ Blood} - \frac{mL\ Oxygen\ Out}{L\ Blood}\right) * \frac{L\ Blood}{Minute} = \frac{\Delta mL\ Oxygen}{L\ Blood} * \frac{L\ Blood}{Minute} = \frac{\Delta mL\ Oxygen}{Minute}$$

Equation S1 shows in full the dimensional analysis demonstrating that our application of the Fick equation results in a unit of oxygen consumption over time

Eq. S2) $$\begin{cases} \frac{oHb}{ctHb} = 1\ if\ PO_2 \geq 100 \\ \frac{oHb}{ctHb} = \frac{PO_2}{100}\ if\ PO_2 < 100 \end{cases}$$

Equation S2 shows the formal calculation for our approximation of the fraction of Oxyhemoglobin.

Appendix 2: Primer Sequences

Genomic Primer Forward Sequence: CGG AGG AGA ATG TAA GCC CC

Genomic Primer Reverse Sequence: CGG AAG GTA AAG CAG GAG CA

Mitochondrial Primer Forward Sequence: CAC GCT TCT TCG CAT TCC AC

Mitochondrial Primer Reverse Sequence: GGG ATT TTG TCT GCG TCG GA

Vita

William Patrick Adams was born an American Citizen on March 2, 1992 in Lynchburg Virginia. He graduated from the Central Virginia Governor's School for Math Science and Technology, Lynchburg, Virginia and concurrently graduated from Altavista High School, Altavista, Virginia in 2010. In 2014 he was awarded a Bachelor's of Science in Physics from The College of William and Mary, located in Williamsburg, Virginia.