Resuscitation, preservation, and evaluation of hearts donated after circulatory death: an avenue to expand the donor pool for transplantation

by

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Abstract

Cardiac transplantation is the treatment of choice for eligible patients with advanced heart failure; however, it is limited by a critical shortage of suitable organs from traditional brain-dead donors. Organs donated following circulatory death (DCD) have been used to successfully expand the pool of organs available for kidney, liver, and lung transplantation; however, concerns regarding the severity of injury sustained by the heart following withdrawal of life sustaining therapy have deterred the clinical transplantation of DCD hearts. Investigations aiming to optimize the resuscitation, preservation, and evaluation of DCD hearts may facilitate the development of an evidence based protocol for DCD heart transplantation that can be translated to the clinical area and expand the donor pool. Therefore, the objectives of this thesis are to develop a clinically relevant large animal model of DCD and gain a greater understanding regarding the physiologic impact of donor extubation on the DCD heart, demonstrate as a 'proof-of-concept' that utilizing an approach to donor heart resuscitation, preservation, and evaluation that is tailored to the DCD context can facilitate successful transplantation, and finally to investigate ways to optimize the resuscitation, preservation, and evaluation of DCD hearts for transplantation. The results of this thesis may then be used to inform the development of an evidence-based protocol for DCD heart transplantation that can be translated to the clinical area. The clinical adoption of such a protocol has the potential to expand the donor pool and improve outcomes for patients with end-stage heart failure.

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List of Abbreviations

ANOVA: analysis of variance

ATP: adenosine triphosphate

ADP: adenosine diphosphate

A-L: adenosine-lidocaine

C_aO_2: arterial oxygen content

CBF: coronary blood flow

CK-MB: creatine kinase-MB isoenzyme

CO: cardiac output

CPB: cardiopulmonary bypass

C_vO_2, venous oxygen content

CVR: coronary vascular resistance

DBD: donation after brain death

DCD: donation after circulatory death

d*P*/d*t*: rate of pressure change

DPP: direct procurement and perfusion

ECG: electrocardiography

ECMO: extracorporeal membrane oxygenation

EDPVR: end-diastolic pressure volume relationship

EVHP*: ex vivo* heart perfusion

ELISA: enzyme-linked immunosorbent assay

ESPVR: end-systolic pressure volume relationship

Fe: iron, H_2O_2: hydrogen peroxide

HBOC: hemoglobin based oxygen carrier

HPLC: high performance liquid chromatography

IL: interleukin, IQR: interquartile range

IR: initial reperfusion

IRI: ischemia-reperfusion injury,

LAP: left atrial pressure

LC-MS/MS: liquid chromatography-tandem mass spectrometry

LV: left ventricle

MRI: magnetic resonance imaging

MRS: magnetic resonance spectroscopy

MV: mechanical ventilation

MVO_2: myocardial oxygen consumption

NCX: sodium-calcium exchanger

NHE: sodium-hydrogen exchanger

NRP: normothermic regional perfusion

OCS: organ care system

OxPC: oxidized phosphatidylcholine

P_aCO_2: arterial partial pressure of carbon dioxide

P_aO_2: arterial partial pressure of oxygen

PCr: phosphocreatine

Pi: inorganic phosphate

PRSW: preload recruitable stroke work

P_vO_2: venous partial pressure of oxygen

RBC: red blood cell concentrate

RISK: reperfusion injury salvage kinase

ROS: reactive oxygen species

RV: right ventricle

RyR: ryanodine receptor

SAFE: survivor activating factor enhancement

SE: standard error

SVR: systemic vascular resistance

T0: starting point of *ex vivo* heart perfusion

T1: 1-hour following initiation of *ex vivo* heart perfusion

T3: 3-hours following initiation of *ex vivo* heart perfusion

T5: 5-hours following initiation of *ex vivo* heart perfusion

T6: 6-hours following initiation of *ex vivo* heart perfusion

UHPLC: ultra high performance liquid chromatography

VA: veno-arterial

WLST: withdrawal of life sustaining therapy

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Physiologic Changes in the Heart Following Cessation of Mechanical Ventilation in a Porcine Model of Donation After Circulatory Death: Implications for Cardiac Transplantation
American Journal of Transplantation 2016, 16 (3): 783 - 793
DOI: 10.1111/ajt.13543
John Wiley and Sons, License number: 3787131395981

A cardioprotective preservation strategy employing *ex vivo* heart perfusion facilitates successful transplant of donor hearts after cardiocirculatory death
The Journal of Heart and Lung Transplantation 2013, 32 (7): 734 - 743
DOI: 10.1016/j.healun.2013.04.016
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A whole blood-based perfusate provides superior preservation of myocardial function during ex vivo heart perfusion
The Journal of Heart and Lung Transplantation 2015, 34 (1): 113 - 121
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Assessment of donor heart viability during *ex vivo* heart perfusion
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DOI: 10.1111/ajt.13574
John Wiley and Sons, License number: 3865061035477

Impact of reperfusion calcium and pH on the resuscitation of hearts donated after circulatory death
The Annals of Thoracic Surgery 2017, 103(1):122-130
DOI: 10.1016/j.athoracsur.2016.05.084
Elsevier, License number: 4075090438475

Chapter 1:

Thesis Overview

Background

Cardiac transplantation remains the “gold-standard” treatment for eligible patients with advanced heart failure. While the number of potentially eligible recipients and the transplant waiting lists are growing, the number of transplants being performed has remained static over the last 25 years. Consequently, the clinical impact of cardiac transplantation on the treatment of advanced heart failure has become limited by a critical shortage of suitable donor organs and patients awaiting cardiac transplantation suffer a 15% mortality rate.

Cardiac transplantation has been performed almost exclusively with organs procured from donors that have been declared dead based on neurologic criteria (donation after brain death (DBD)). DBD donors have intact cardiorespiratory function, which provides an opportunity to evaluate donor heart suitability for transplantation prior to organ procurement. Acceptable organs are arrested using a cold cardioplegic solution and stored on ice (cold static storage) until they are transplanted. Cold static storage represents the current standard of care for donor heart preservation; however, cold ischemic injury limits the safe preservation interval to a 4 – 6 hour period. Reliance on this standard approach to donor heart procurement and preservation has contributed to the current organ shortage; therefore, exploration of alternative donor sources is warranted to mitigate the growing disparity between the number of eligible transplant recipients and available organs.

Donation after circulatory death (DCD) describes the procurement of organs from donors that have been declared dead based on circulatory criteria. Following donor extubation, hearts from DCD donors are forced to function in an increasingly hypoxemic environment while attempting to maintain systemic oxygen delivery. Therefore, DCD hearts are exposed to a variable period of warm ischemia during the progression to circulatory arrest and declaration of death. In order for hearts from DCD donors to become a reliable source of organs for

transplantation, there is a desperate need for research investigating methods to mitigate the detrimental effects of donor warm ischemia that occurs following donor extubation, avoid incremental ischemic injury during the preservation interval, and assess organ viability prior to transplant.

Hypothesis

Optimizing an approach to donor heart resuscitation, preservation, and evaluation that is tailored to the DCD context (Figure 1.1) can facilitate successful transplantation and represents an avenue to expand the donor pool for transplantation.

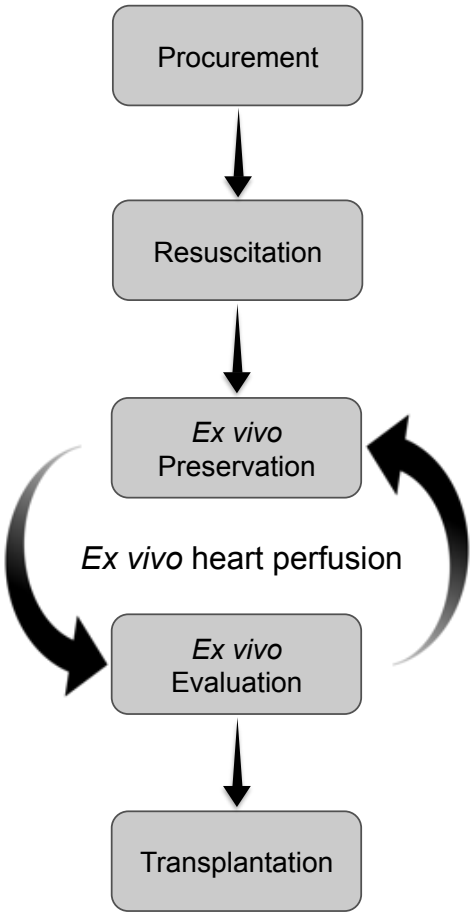

Figure 1.1 Suggested approach to DCD heart transplantation

Objectives

The objectives of this thesis are described below and displayed in Figure 1.2.

1. Provide an overview regarding the current state of DCD heart transplantation (Chapter 2)
2. Develop a clinically relevant, translational, large animal model of DCD (Chapter 3)
3. Investigate the physiologic impact of donor extubation on the DCD heart (Chapter 3)
4. Demonstrate as a 'proof-of-concept' that utilizing an approach to donor heart resuscitation, preservation, and evaluation that is tailored to the DCD context can facilitate successful transplantation (Chapter 4)
5. Optimize the process of DCD heart transplantation by improving the preservation of donor hearts using *ex vivo* heart perfusion (Chapter 5)
6. Optimize the process of DCD heart transplantation by determining the optimal means of assessing organ viability prior to transplantation (Chapter 6)
7. Optimize the process of DCD heart transplantation by improving DCD heart resuscitation at the time of organ procurement (Chapters 7 and 8)

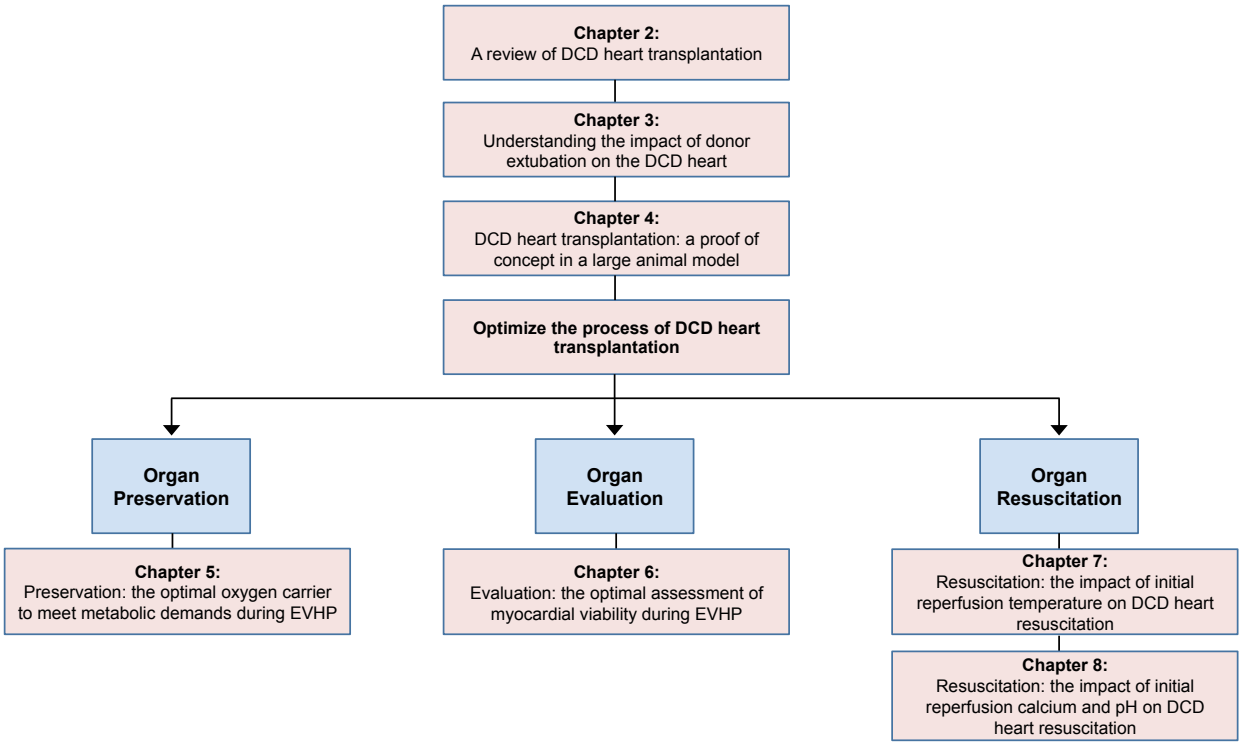

Figure 1.2. Thesis overview

Chapter 2

Transplantation of hearts donated following circulatory death: a narrative review

Submitted

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Contributions of Co-Authors

Christopher W. White: manuscript preparation, manuscript submission, manuscript revisions

Simon J. Messer: manuscript revisions

Stephen R. Large: manuscript revisions

Jennifer Conway: manuscript revisions

Daniel H. Kim: manuscript revisions

Demetrios J. Kutsogiannis: manuscript revisions

Jayan Nagendran: manuscript preparation

Darren H. Freed: manuscript preparation

Chapter 2 Preface

Chapter 2: *Transplantation of hearts donated following circulatory death: a narrative review*, reviews the current state of DCD heart transplantation. This review describes the clinical rationale for expansion of the donor pool beyond those organs currently being accepted for transplantation. This chapter also highlights how hearts from DCD donors differ in many respects from traditional DBD donors, and that simply utilizing the traditional approach to heart transplantation is unlikely to be successful in the DCD context. Therefore, an approach tailored specifically to the DCD setting is proposed. This approach focuses on: 1) optimizing the resuscitation of DCD hearts at the time of organ procurement, 2) optimizing the preservation of DCD hearts prior to transplantation, and 3) optimizing DCD heart evaluation to identify viable organs and minimize the risk of post-transplant graft dysfunction.

Abstract

Purpose

Cardiac transplantation has become limited by a critical shortage of suitable organs from brain-dead donors. Reports describing the successful clinical transplantation of hearts donated following circulatory death (DCD) have recently emerged. This narrative review describes the history and current state of DCD heart transplantation.

Source

A literature search of PubMed was performed to identify articles relevant to the topic of DCD heart transplantation.

Principal Findings

Hearts from DCD donors suffer significant ischemic injury prior to organ procurement; therefore, the traditional approach to the transplantation of hearts from brain-dead donors is not applicable in the DCD context. Advances in our understanding of ischemic postconditioning have facilitated the development of DCD heart resuscitation strategies that can be used to minimize ischemia-reperfusion injury at the time of organ procurement. The availability of a clinically approved *ex vivo* heart perfusion device now allows DCD heart preservation in a normothermic beating state and minimizes exposure to incremental cold ischemia. This technology also facilitates assessments of organ viability to be undertaken prior to transplantation, thereby minimizing the risk of primary graft dysfunction. The application of a tailored approach to DCD heart transplantation that focuses on organ resuscitation at the time of procurement, *ex vivo* preservation, and pre-transplant assessments of organ viability has facilitated the successful clinical application of DCD heart transplantation.

Conclusion

The transplantation of hearts from DCD donors is now a clinical reality. Further research investigating ways to optimize the resuscitation, preservation, evaluation, and transplantation of DCD hearts is vital to ensure a broader application of DCD heart transplantation in the future.

Background

Cardiac transplantation remains the “gold-standard” treatment for eligible patients with advanced heart failure, and more than 112,000 heart transplants have been reported to the International Heart and Lung Transplant registry since its inception in 1983 (1). Such registry data suggests that the donor population is aging, and medical comorbidities (diabetes mellitus, hypertension, chronic obstructive pulmonary disease) are becoming more common among recipients (1, 2). In addition, 50% of recipients have undergone previous cardiac surgery and greater than 40% of patients are now bridged to transplant with some form of mechanical circulatory support (1). Despite these changes in the donor and recipient populations, post-transplant outcomes continue to improve and the median survival in the current era is 11 years (1).

While the number of potentially eligible transplant recipients and the transplant waiting lists are growing, the number of transplants performed in the United States has remained static over the last 25 years (2,253 ± 144 / year) (3). The current cardiac transplant waiting list exceeds 4,000 patients and is associated with a pre-transplant mortality rate of 12-15% (4). Similarly in Canada, the annual increase in the number of patients listed for heart transplant far exceeds the increase in the number of donors, and over the last 10 years the mortality rate for patients awaiting cardiac transplantation was 16% (5, 6). Overall, the clinical impact of cardiac transplantation on the treatment of advanced heart failure is limited by a critical shortage of suitable donor organs (7, 8).

Following the publication of brain death criteria (9-11), cardiac transplantation has been performed almost exclusively with organs procured from donors that have been declared dead based on neurologic criteria (donation after brain death (DBD), Figure 2.1A). In this context, there is an opportunity to evaluate donor heart suitability for transplantation prior to organ procurement using echocardiography and coronary angiography. Acceptable organs are then arrested using a cold, hyperkalemic cardioplegic solution and stored on ice (cold static storage)

until they are transplanted (Figure 2.2A). This approach minimizes myocardial metabolic demands and generally provides adequate myocardial protection over preservation intervals of 4 – 6 hours (12, 13); however, anaerobic metabolism persists during cold static storage and causes ischemic injury (14). Cold ischemic time significantly impacts post-transplant outcomes; with 6-hour preservation intervals exhibiting 1-year mortality rates that are double those of preservation intervals of 3-hours or less (15). Cold static storage represents the current standard of care for donor heart preservation; however, *ex vivo* perfusion may provide an avenue to improve donor heart preservation and expand the donor pool in the future (16).

A contributing factor to the current organ shortage is a high non-utilization rate of donor hearts that are offered for transplantation. In the United States, only 34 – 43% of donor hearts are accepted for transplantation and the utilization rate appears to be decreasing (7, 17). Donor characteristics associated with non-utilization include older age, female sex, stroke as a cause of death, a history of smoking, hypertension, diabetes mellitus, or coronary artery disease, a positive troponin assay, a lower ejection fraction or regional wall motion abnormalities, and left ventricular hypertrophy (7). The utilization rate in Canada is similar, with only 39% of donor hearts being accepted for transplantation (18). While cardiac transplantation remains the treatment of choice for eligible patients with advanced heart failure and is associated with excellent post-transplant outcomes, there is a decline in the use of available DBD hearts in the face of a severe organ donor shortage and a growing number of patients awaiting transplantation (17). In addition to improving the utilization rate of DBD hearts offered for transplantation, exploration of alternative donor sources is warranted to mitigate the growing disparity between the number of eligible transplant recipients and available organs.

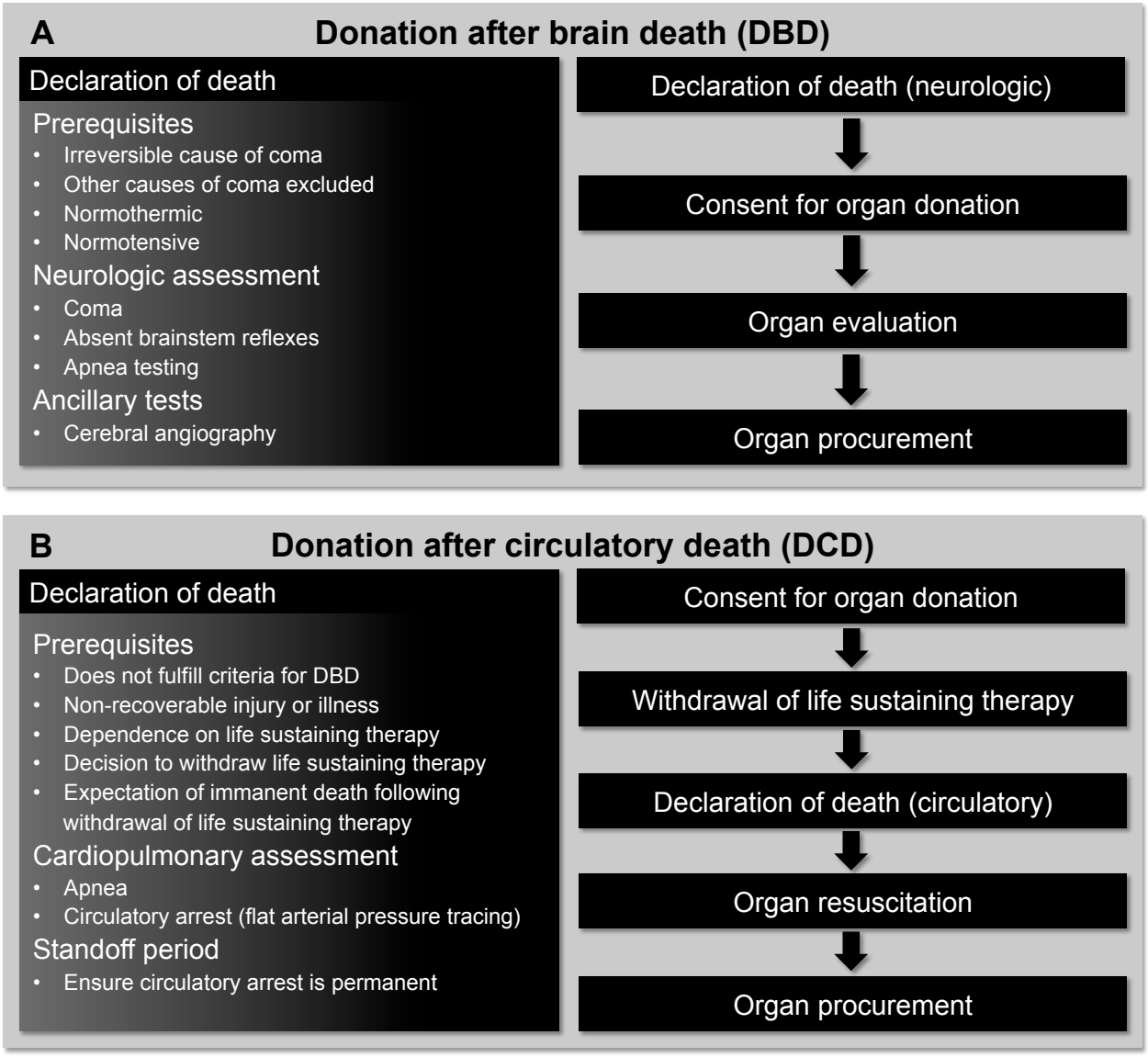

Figure 2.1. Pathways for deceased organ donation. A) Patients donating organs after brain death have intact cardiorespiratory function that allows donor heart evaluation to be undertaken before organ procurement. B) Patients donating organs after circulatory death have suffered a hypoxemic cardiac arrest following withdrawal of life sustaining therapy and donor heart evaluation can only be undertaken after organ resuscitation has occurred.

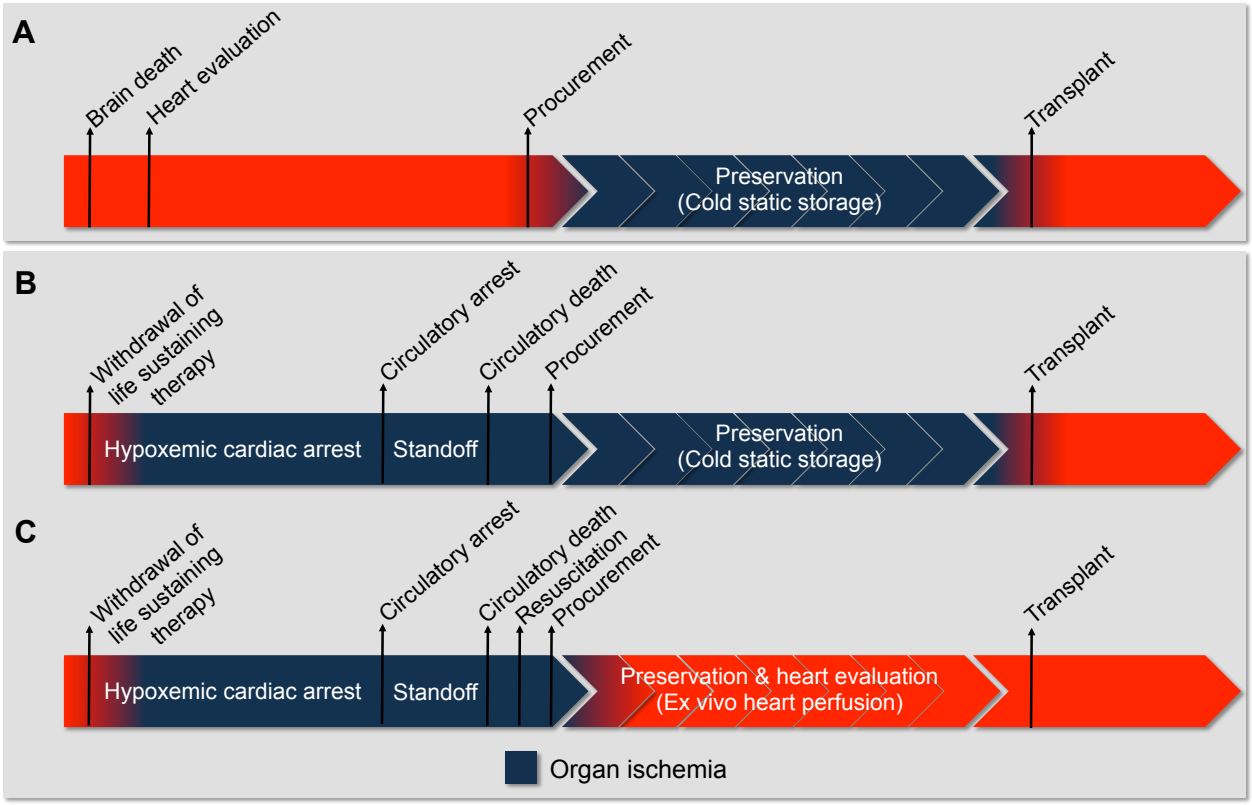

Figure 2.2. Process of heart transplantation. A) The traditional approach to transplantation of a heart procured from a donation after brain death donor. Donor heart evaluation is carried out in the donor with intact cardiorespiratory function. Viable organs are arrested with a cardioplegic solution and stored in a profoundly hypothermic state (cold static storage) until transplantation. Organ ischemia is limited to the time between procurement and transplantation (cold ischemic time). B) The traditional approach to transplantation of a heart procured from a donation after circulatory death donor. The donor progresses to circulatory arrest following withdrawal of life sustaining therapy. An ethically mandated standoff period must then be observed before circulatory death can be declared. Consequently, the heart has sustained a significant warm ischemic insult before organ procurement can proceed. Subsequent preservation using cold static storage subjects the heart to an additional cold ischemic injury and does not provide an opportunity for organ resuscitation and evaluation. The traditional approach is unlikely to facilitate successful transplantation of hearts donated after circulatory death. C) The tailored

approach to transplantation of a heart procured from a donation after circulatory death donor. Following withdrawal of life sustaining therapy and declaration of circulatory death, the heart is resuscitated using an approach tailored to minimize ischemia–reperfusion injury. The heart is then preserved using *ex vivo* heart perfusion, which minimizes exposure to cold ischemia and facilitates organ evaluation. Organ ischemia can be limited to the time between withdrawal of life sustaining therapy and organ resuscitation (warm ischemic time).

Donation after circulatory death

Donation after circulatory death (DCD) describes the procurement of organs from donors that have been declared dead based on circulatory criteria (Figure 2.1B) (19). Many early transplant programs utilized organs from DCD donors, including the first heart transplants performed by Christiaan Barnard (20, 21). This practice was largely abandoned following the acceptance of brain-death criteria (9-11); however, a critical shortage of suitable organs from DBD donors has promoted a renewed interest in DCD.

The Maastricht classification system is used to describe 4 different categories of DCD donors according to the circumstances of the donor's death (22), and was recently modified at the 6th International Conference on Organ Donation after Circulatory Death (Table 2.1) (23). Experimental and clinical transplantation of DCD hearts have been restricted to Maastricht Category III donors. These donors typically have a non-recoverable neurologic injury, are dependent on advanced life support therapies (mechanical ventilation and/or vasopressor medications), but do not meet brain-death criteria. If ongoing medical care is deemed futile and a decision to withdraw life-sustaining therapies (WLST) is made, consent for organ donation may be obtained. In this scenario, life-sustaining therapies are withdrawn and palliative care is provided by the critical care team according to institutional practices. Depending on institutional protocols, WLST may occur in the intensive care unit, an anesthetic room, or an operating room. The patient is monitored for progression to apnea and circulatory arrest, which is declared when

a pulse pressure is no longer present on an arterial pressure tracing (mechanical asystole) (23). An ethically mandated standoff period (2-20 minutes depending on institutional protocols) is then observed before circulatory death is declared and organ procurement can proceed (Figure 2.2 B-C). Therefore, in the DCD donor the diagnosis of death is based on the cessation of cardiorespiratory function (23). This differs in many respects from DBD, where the declaration of death is based on neurologic criteria in a donor that has intact cardiorespiratory function.

Following WLST hearts from DCD donors are forced to function in an increasingly hypoxemic environment, while attempting to maintain systemic oxygen delivery (24). Therefore, DCD hearts are exposed to a variable period of warm ischemia during the progression to circulatory arrest and declaration of death (Figure 2.2 B-C). The duration of this warm ischemic period is an important criterion in the selection of donor hearts for transplantation (25, 26). The total warm ischemic time refers to the time between WLST and organ reperfusion (23); however, some Maastricht category III donors maintain a stable cardiorespiratory status for some time before eventual progression to circulatory arrest. The functional warm ischemic time refers to the time from when the systolic blood pressure decreases below 50 mmHg for at least 2 minutes (irrespective of oxygen saturation) until organ reperfusion (23). The functional warm ischemic time is meant to provide a more accurate estimate of the ischemic injury sustained by donor organs following WLST; however, there is some evidence to suggest that oxygen desaturation should also be considered in this estimation (24).

Table 2.1. Modified Maastricht classification of donation after circulatory death[1-2]

Uncontrolled	Description
Category I	**Found dead**
	Sudden unexpected circulatory arrest without any attempt of resuscitation by a medical team
	Category IA: out-of-hospital, Category IB: in-hospital
Category II	**Witnessed cardiac arrest**
	Sudden unexpected irreversible circulatory arrest with unsuccessful resuscitation by a medical team
	Category IIA: out-of-hospital, Category IIB: in-hospital
Category IV	**Cardiac arrest while brain dead**
	Sudden circulatory arrest after brain-death diagnosis during donor management but prior to planned organ retrieval; donation proceeds after unsuccessful resuscitation by a medical team
Controlled	**Description**
Category III	**Withdrawal of life-sustaining therapy**
	Planned withdrawal of life sustaining therapy and expected circulatory arrest
Category IV	**Cardiac arrest while brain dead**
	In countries where legislation does not accept brain death criteria or patient will never meet the neurologic criteria for the diagnosis of brain death; the procedure for this potential DBD can be converted to a DCD

[1]Kootstra G, Daemen JH, Oomen AP. Categories of non-heart-beating donors. *Transplant Proc.* 1995;27(5):2893-2894.

[2]Thuong M, Ruiz A, Evrard P, et al. New Classification of Donation after Circulatory Death Donors Definitions and Terminology. *Transpl Int.* 2016.

DCD in solid organ transplantation

The DCD pathway for organ donation is increasing in many countries around the world and in some countries accounts for more than one-third of all deceased organ donation (27-30). In 2012, DCD accounted for 42% of kidney, 19% of liver, and 19% of lung transplants from deceased donors in the United Kingdom (31). Recent studies have demonstrated that outcomes using organs from DCD donors are comparable to those from DBD donors in kidney (32), pancreas (33), and lung (34) transplantation. In contrast, a recent meta-analysis of liver transplantation outcomes suggested that recipients of DCD organs demonstrate a higher incidence of ischemic cholangiopathy, graft loss, and mortality (35).

Potential impact of DCD in heart transplantation

Multiple authors have attempted to describe the potential impact of DCD on heart transplantation volume. Noterdaeme *et al* examined deceased donor data from a single center in

Belgium (36). Over a 6-year period there were 247 deceased donors, 70 (28%) of whom were Maastricht Category III donors. The authors applied the same inclusion criteria used for DBD heart donors, and required that the warm ischemic time not exceed 30 minutes. This approach identified 8 potential DCD heart donors, with a mean age of 35 ± 3 years and a warm ischemic time of 15.1 ± 0.5 minutes. During the same time period 82 patients were listed for heart transplantation, 53 were transplanted, 9 were still waiting, 11 were removed from the list, and 9 died while waiting. Therefore, the authors conclude that the transplantation of 8 additional hearts could have significantly reduced the number of deaths on the waiting list and increased heart transplant activity by 15%. Messer *et al* examined 3,073 DCD donors referred over a 3-year period in the United Kingdom and found 149 (5%) to be suitable potential heart donors (37). The authors concluded that an increase of 50 heart transplants per year could have grown the overall transplant activity by 30%. In the United States, 12 – 17% of DCD donors may be suitable heart donors, and transplantation of these hearts could increase the annual transplant volume by 4 – 16% (38, 39). In Australia, the transplantation of hearts from DCD donors could increase transplant activity by up to 17% (40). Overall, it appears that the transplantation of hearts from DCD donors has the potential to significantly increase the annual transplant volume in many countries.

DCD Heart transplantation

The approach to heart transplantation displayed in Figure 2.2 A represents the current standard of care for the procurement and preservation of DBD hearts. Following the declaration of brain death, heart function is evaluated to determine suitability for donation. At the time of organ procurement, the donor has intact cardiorespiratory function and the heart is not ischemic. Hearts are arrested using a cold, hyperkalemic cardioplegic solution and undergo cold static storage until they are transplanted. Myocardial metabolic demands are minimized during cold static storage; however, anaerobic metabolism persists and cold ischemic injury limits the safe

preservation interval to a period of 4 – 6 hours (12-14). The DBD heart is only exposed to ischemia in the time between organ procurement and transplantation.

Simply applying the standard DBD approach for heart procurement and preservation to the DCD context is unlikely to adequately resuscitate the DCD heart and provide a viable organ for transplantation (Figure 2.2 B). The DCD heart has already sustained significant warm ischemic injury following donor extubation and would not tolerate the additional ischemic injury occurring during cold static storage. Therefore, an approach tailored specifically to the DCD context is required to facilitate successful transplantation (Figure 2.2 C). Such an approach must include: 1) organ resuscitation at the time of procurement in order to minimize the detrimental effects of warm ischemia that occur following donor extubation, 2) a preservation strategy that minimizes exposure to additional ischemic injury and provides an opportunity for ongoing organ resuscitation, and 3) the ability to assess organ function and identify viable organs prior to transplantation (Figures 2.2 C – 2.3).

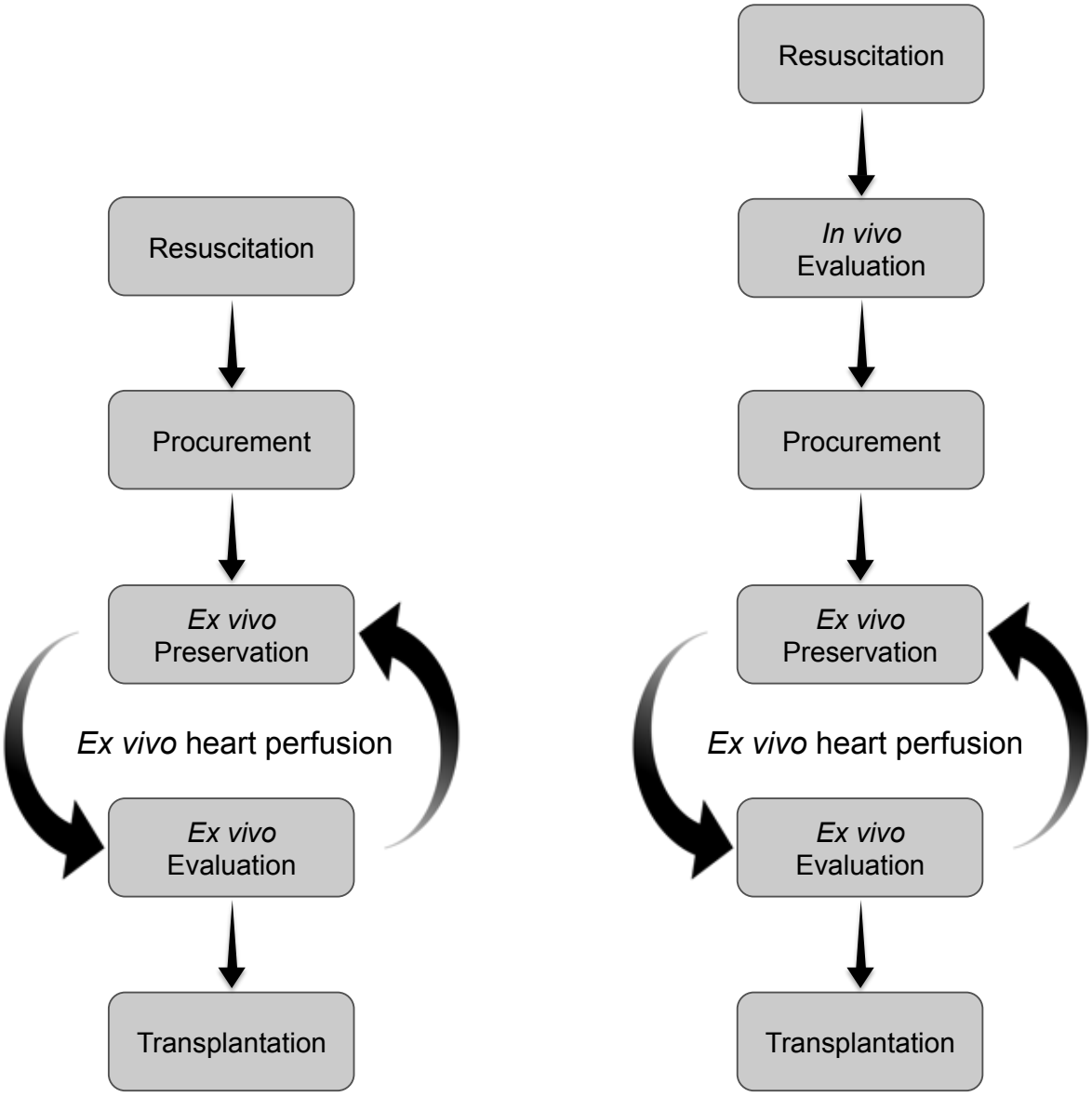

Figure 2.3. Alternative approaches to the resuscitation, preservation, and evaluation of hearts donated after circulatory arrest. A) Direct procurement and preservation. Hearts are resuscitated with a cardioplegic solution tailored to minimize ischemia–reperfusion injury, then preserved *ex vivo* in a normothermic beating state. Organ evaluation is carried out during *ex vivo* preservation to identify viable organs for transplant. B) Normothermic regional perfusion. Hearts are resuscitated *in vivo* on veno-arterial extracorporeal membrane oxygenation (ECMO). The donor is subsequently weaned from ECMO, *in vivo* assessments of heart function are carried out, and

then viable organs are procured and preserved *ex vivo* in a normothermic beating state until transplant. Supplementary organ evaluation can be carried out during *ex vivo* preservation.

DCD heart resuscitation

Ischemia–reperfusion injury

Understanding the physiologic impact of donor extubation and warm ischemia on the DCD heart is fundamental to developing a resuscitation strategy that can facilitate successful transplantation (24). Following donor extubation the DCD heart is forced to function in an increasingly hypoxemic environment while attempting to maintain systemic oxygen delivery (24). Progressive hypoxemia and hypercarbia cause constriction of the pulmonary vasculature and distention of the right ventricle. These changes prompt a massive catecholamine surge and a transient hyperdynamic circulatory phase; however, myocardial energy stores are rapidly depleted, cardiac output declines, and the donor progresses to circulatory arrest (24). The donor heart remains in a warm, distended, and ischemic state during the ethically mandated standoff period (24); therefore, at the time of organ procurement the DCD heart has withstood exposure to a massive catecholamine surge and sustained significant ischemic injury (Figure 2.2 B – C).

The ischemic injury sustained by the DCD heart following donor extubation results in the depletion of adenosine triphosphate (ATP) stores and anaerobic metabolism, which cause intracellular acidosis, activation of the sodium-hydrogen exchanger (NHE), and sodium influx into the myocyte (41, 42). The sodium-potassium ATPase normally functions to extrude sodium ions entering the myocyte via the NHE. In the DCD context; however, intracellular acidosis develops concurrently with the depletion of ATP stores. The combined effect of increased NHE activity and inhibition of the sodium-potassium ATPase produces a pathological accumulation of intracellular sodium (Figure 2.4 A) (42, 43). Subsequent reperfusion at the time of organ procurement rapidly normalizes the extracellular pH and creates a large hydrogen ion gradient across the plasma membrane that causes further sodium influx via the NHE (44). This increase

in intracellular sodium forces the sodium-calcium exchanger (NCX) to function in reverse mode and import calcium ions across the sarcolemma (Figure 2.4 B). The resultant intracellular calcium overload propagates myocyte death through the development of hypercontracture, activation of calcium-dependent proteases (I.E. calpain), generation of reactive oxygen species (ROS), uncoupling of mitochondrial ATP production, activation of the mitochondrial permeability transition pore, and initiation of apoptotic pathways (Figure 2.5) (41, 42, 44). Limiting the severity of this ischemia–reperfusion injury at the time of organ procurement represents the cornerstone of DCD heart resuscitation.

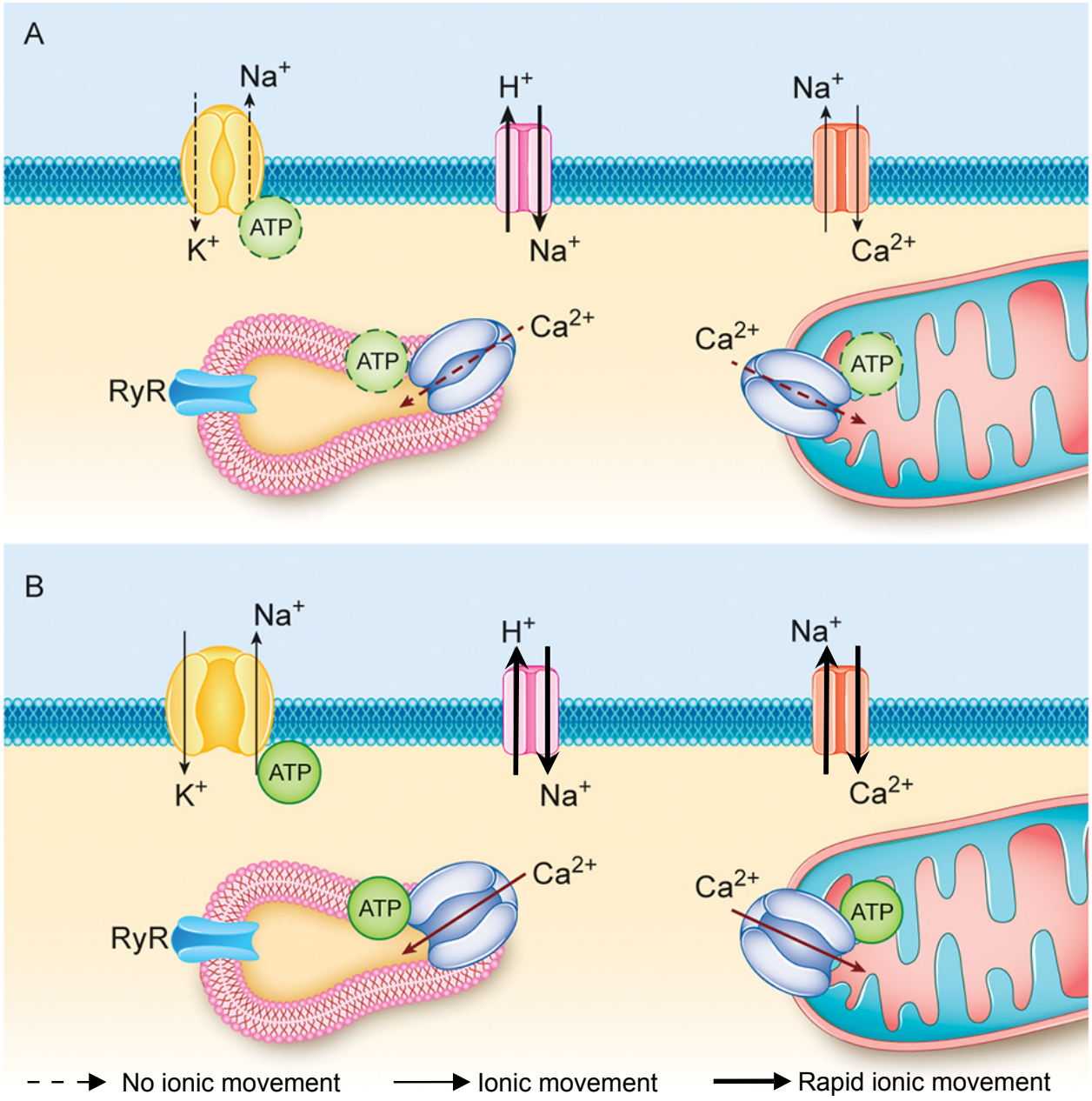

Figure 2.4. A) Ionic changes during ischemia. Anaerobic metabolism results in the production of hydrogen ions that activate the sodium-hydrogen exchanger and the accumulation of sodium ions inside the myocyte. The sodium-potassium ATPase is not able to extrude the excess sodium ions and maintain the normal membrane potential due to a lack of available ATP. Consequently, as ischemia progresses there is an accumulation of sodium and hydrogen ions inside the myocyte and depolarization of the membrane potential. B) Ionic changes during

reperfusion. Reperfusion washes out the hydrogen ions that have accumulated in the interstitial space and creates a large gradient for sodium-hydrogen exchange. The influx of sodium ions into the myocyte during early reperfusion forces the sodium-calcium exchanger to function in reverse mode and import calcium ions across the sarcolemma. Intracellular ionic homeostasis cannot be restored until the sodium-potassium ATPase is able to reestablish the resting membrane potential and normal intracellular sodium levels, which will allow the sodium-calcium exchanger to return to a forward mode of operation and extrude excess calcium from the cytoplasm.

Adapted with permission (87).

Approach to DCD heart resuscitation

With the aim of minimizing ischemia–reperfusion injury and optimizing functional recovery, two methods of DCD heart resuscitation have emerged and have been successfully utilized in the clinical context (45). The first approach has been termed Direct Procurement and Perfusion (DPP). This approach involves the delivery of a cardioplegic solution at the time of organ procurement to resuscitate the DCD heart. The composition of the solution is designed to promote ischemic postconditioning and limit the detrimental effects of ischemic-reperfusion injury. A rapid cardiectomy is then performed, the heart is connected to an *ex vivo* perfusion device, and the heart is preserved in a normothermic and beating state until transplantation (Figure 2.3 A) (25, 46). The second approach is Normothermic Regional Perfusion (NRP) (45). Following declaration of circulatory death, a median sternotomy is performed and the cerebral circulation is isolated. The donor is then placed on central veno-arterial extracorporeal membrane oxygenation (ECMO) and reperfused for a period of approximately 60 minutes. The donor is subsequently weaned from ECMO, which facilitates assessments of organ function to be carried out *in vivo*. Viable organs are then arrested with a traditional cardioplegic solution, connected to an *ex vivo* perfusion device, and preserved in a normothermic, beating state until

transplantation (Figure 2.3 B) (26, 45).

Direct Procurement and Perfusion

Initial reperfusion solution

At the time of organ procurement the DCD heart is energy deplete and vulnerable to the influx of sodium and calcium upon reperfusion. The reactivation of ATP production in the calcium-overloaded myocyte after prolonged ischemia can propagate calcium oscillations and the development of hypercontracture (Figure 2.5) (47, 48). However, initial reperfusion with a cardioplegic solution inhibits myocardial contraction at the onset of re-oxygenation. This facilitates the repletion of myocardial energy stores and the restoration of intracellular calcium homeostasis before activation of the myofibrillar contractile unit can occur, thereby preventing the development hypercontracture (49, 50). Previous research has demonstrated that hearts subjected to a period of ischemia exhibit better functional recovery when reperfused with a cardioplegic solution compared to reperfusion with unmodified blood (51-54). Therefore, initial reperfusion of the DCD heart should maintain cardiac arrest to provide an opportunity for the restoration of intracellular ion homeostasis and limit the propagation of hypercontracture.

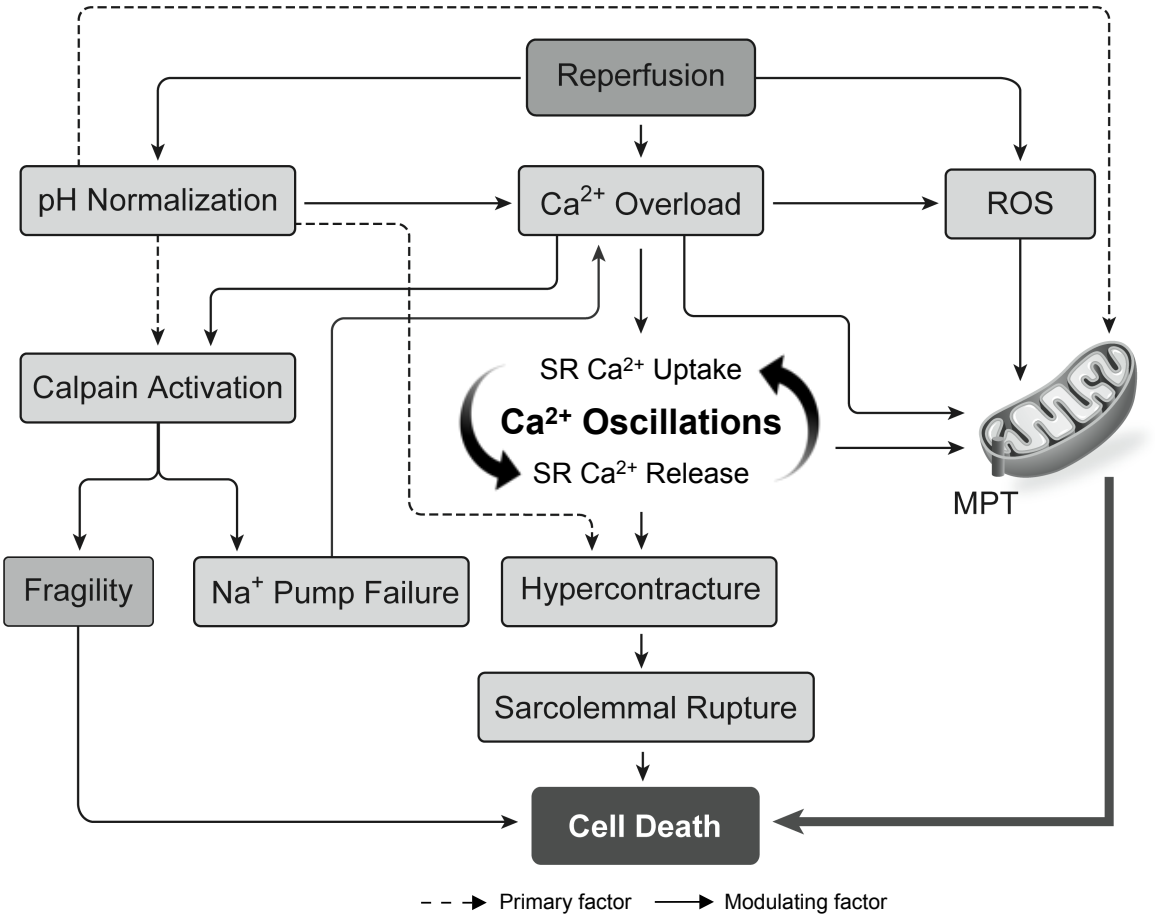

Figure 2.5. Pathogenesis of ischemia–reperfusion injury. Intracellular calcium overload and the production of reactive oxygen species cause opening of the mitochondrial permeability transition pore and the propagation of cell death. The normalization of intracellular pH during reperfusion is an important modulating factor in the pathogenesis of ischemia–reperfusion injury.

MPT; mitochondrial permeability transition, ROS; reactive oxygen species, SR; sarcoplasmic reticulum

Adapted with permission (42).

Cardioplegia composition

In 1986, Murry *et al* first demonstrated that repetitive periods of brief ischemia could

protect the myocardium from a subsequent period of prolonged ischemia, and established the concept of ischemic preconditioning (55). This research paved the way for decades of investigation into the molecular pathways involved in the pathogenesis of ischemia–reperfusion injury and the identification of therapeutic strategies to mitigate its' detrimental effects. In 2003, Zhao *et al* demonstrated that repeated cycles of ischemia and reperfusion following a prolonged index ischemic insult (ischemic postconditioning) could attenuate reperfusion induced myocardial injury to the same extent achieved with ischemic preconditioning (56). Subsequent research has demonstrated that common molecular pathways are involved in the processes of ischemic preconditioning and postconditioning (57). These landmark discoveries have tremendous implications for DCD heart transplantation because the delivery of therapeutics to the donor prior to the declaration of death (ischemic preconditioning) is ethically prohibited in the majority of cases. However, the activation of ischemic postconditioning pathways at the time of organ procurement provides an opportunity to mitigate reperfusion injury and resuscitate the DCD heart. The therapeutic window is narrow and optimal success is realized in the first minutes of organ reperfusion (58); therefore, enhancing the composition of the initial reperfusion cardioplegia to activate ischemic postconditioning pathways and inhibit mediators of ischemia–reperfusion injury is the primary focus of DCD heart resuscitation.

The activation of prosurvival kinases at the time of reperfusion confers powerful cardioprotection against ischemia–reperfusion injury through inhibition of the mitochondrial permeability transition pore, and activation of the reperfusion injury salvage kinase (RISK) and survivor activating factor enhancement (SAFE) pathways (57, 59, 60). The delivery of erythropoietin, adenosine, and insulin during reperfusion has been shown to activate the RISK pathway and mitigate myocardial ischemia–reperfusion injury (59, 61, 62). Erythropoietin supplemented cardioplegia has also been shown to improve the functional recovery of ischemic myocardium through activation the SAFE pathway and inhibition of mitochondrial permeability transition pore (63-65). Additionally, insulin causes vasodilation, inhibits apoptosis, limits the

inflammatory response, and reduces the production of reactive oxygen species (ROS) (66). Adenosine inhibits apoptosis through up-regulation of the anti-apoptotic protein Bcl-2, and has anti-inflammatory properties that attenuate neutrophil infiltration into endothelial cells and inhibit the generation and release of ROS (67-73). The nitric oxide donor glyceryl-trinitrate has also been shown to activate prosurvival kinases and Bcl-2 (74). Overall, a variety of pharmacologic activators of the RISK and SAFE pathways have been shown to confer protection against ischemia–reperfusion injury and hold great promise in optimizing DCD heart resuscitation through the activation of ischemic postconditioning pathways.

The influx of sodium and calcium into the myocyte play a central role in the pathogenesis of ischemia–reperfusion injury. Reperfusion at the time of organ procurement normalizes the extracellular pH, and creates a large hydrogen ion gradient across the plasma membrane that causes sodium influx via the NHE and calcium influx via reverse NCX (Figure 2.4 B). Inhibiting these ionic fluxes early in the reperfusion period represents an important opportunity to limit hypercontracture, the activation of calcium-dependent proteases, and activation of the mitochondrial permeability transition pore (Figure 2.5) (59).

The cardioprotective effects of NHE inhibition with amiloride was first reported in 1988 (75), and subsequently more NHE specific inhibitors have been developed. Cariporide has been shown to limit myocardial ischemia–reperfusion injury in animal models, including translational models of DCD heart transplantation (43, 76-78). Cariporide has also shown benefit in patients suffering an acute myocardial infarction and in high-risk patients undergoing coronary artery bypass grafting (79, 80). The benefits of cariporide in patients undergoing high risk coronary artery bypass grafting was examined in the Expedition trial, which confirmed a significant reduction in the risk of myocardial infarction in the treatment group but an increased risk of mortality that was driven by an increase in cerebrovascular events (81). Zoniporide is a recently developed NHE inhibitor that possesses greater potency and selectivity towards the cardiac NHE (82). Zoniporide has been shown to have potent cardioprotective effects through activation

of prosurvival kinase pathways and inhibition of apoptosis (83).

NHE inhibitors are no longer available for clinical use and enthusiasm for further development has been limited by the results of the Expedition trial; however, drug delivery in the context of DCD heart resuscitation is isolated to the heart in a deceased donor, and the potential detrimental impact of NHE inhibitors on other organ systems is irrelevant. Therefore, NHE inhibitors may still have an important role in the resuscitation of DCD hearts. Alternatively, delaying pH normalization at the onset of reperfusion by delivering an acidic cardioplegic solution inhibits NHE and calcium overload, prevents hypercontracture and the activation of calcium-dependent proteases, and limits activation of the mitochondrial permeability transition pore (Figure 2.5) (84-86). We have recently shown that initial reperfusion with a moderately acidic solution optimizes the functional recovery of DCD hearts (87). Therefore, modifying the cardioplegic solution composition may provide an avenue to realize the clinical benefit of NHE inhibition without relying on pharmacologic inhibitors that are not currently available in the clinical arena.

Pharmacologic inhibitors of reverse-mode NCX have also been investigated as a means of minimizing calcium influx into the myocyte during reperfusion (Figure 2.4). NCX inhibitors have been shown to minimize calcium overload, hypercontracture, infarct size, and contractile dysfunction following ischemia in animal models (88-92). Matsumoto *et al* have demonstrated that inhibition of reverse-mode NCX is more cardioprotective than NHE inhibition (93). While there are no clinically approved NCX inhibitors at present, calcium influx during reperfusion can be limited if the initial reperfusion solution is rendered hypocalcemic. This serves to minimize the calcium gradient that favors reverse NCX activity during early reperfusion, thereby limiting calcium overload and inhibiting ischemia–reperfusion injury (94-98). We have recently demonstrated in a large animal model that initial hypocalcemic reperfusion optimized the functional recovery of DCD hearts (87). Therefore, utilizing a hypocalcemic cardioplegic solution may provide a simple avenue achieve NCX inhibition and minimize ischemia–reperfusion injury

in the DCD heart.

Conventional cardioplegic solutions rely on high potassium concentrations to depolarize the membrane potential and achieve diastolic arrest; however, membrane depolarization is associated with an increase in intracellular sodium via a non-activating sodium current that may exacerbate calcium overload during reperfusion (83). Hyperkalemia also causes endothelial injury and coronary vasoconstriction that may compromise cardioplegia delivery (99-101). Alternatively, normokalemic adenosine-lidocaine cardioplegic solutions have been proposed, in which lidocaine blockade of sodium fast channels causes a diastolic arrest and adenosine maintains a polarized membrane potential (102-104). Rudd and Dobson have shown that ischemic rat hearts reperfused with an adenosine-lidocaine cardioplegia exhibited improved myocardial function compared to those reperfused with a traditional hyperkalemic cardioplegia (103). Similarly, polarized arrests using potassium channel openers have been shown to minimize calcium overload and improve myocardial function compared with depolarized arrests using hyperkalemic solutions (105-107). Mohri *et al* have applied this concept in a large animal model of DCD heart transplantation, and demonstrated improved post-transplant outcomes in hearts treated with a potassium channel opener (108). Further, we have utilized normokalemic adenosine-lidocaine based solutions in our translational models of DCD heart transplantation (87, 109, 110). Further research is required to determine if such alternative cardioplegic solutions are superior to traditional hyperkalemic solutions.

A wide variety of strategies have demonstrated efficacy in mitigating ischemia–reperfusion injury; however, it is likely that the delivery of a cardioplegic solution containing a cocktail of complementary pharmacologic postconditioning agents that target a variety of pathways involved in the pathogenesis of ischemia–reperfusion injury will further improve DCD heart resuscitation. For example, the work of Professor Macdonald has clearly demonstrated in large animal models of DBD and DCD transplantation, the synergistic beneficial effects when glyceryl-trinitrate, erythropoietin, and zoniporide are combined (111, 112). We have utilized a

cardioplegic solution containing the ischemic postconditioning agents adenosine and insulin, and rendered the solution acidic and hypocalcemic to minimize calcium overload during reperfusion (87). ROS scavengers, calpain inhibitors, and mitochondrial permeability transition pore inhibitors (I.E. cyclosporine) may represent other pharmacologic strategies that may further optimize DCD heart resuscitation (113-115). Further research in this area will undoubtedly contribute to improved post-transplant outcomes in the future.

Cardioplegia delivery

Optimizing the conditions of the initial reperfusion may further improve DCD heart resuscitation. Initial cardioplegic reperfusion provides an opportunity to restore myocardial energy stores and intracellular ion homeostasis prior to myocardial contraction; however, these reparative processes may be inhibited if the cardioplegia is delivered under profoundly hypothermic conditions. Hypothermia markedly lowers the activity of the ion pumps that are required to restore intracellular ion homeostasis in the DCD heart and may exacerbate ischemia–reperfusion injury (99, 116, 117). The delivery of a profoundly hypothermic cardioplegic solution at the time of organ procurement is standard practice when procuring hearts from DBD donors, to minimize metabolic demands during the subsequent period of cold static storage (Figure 2.2 A). In the context of DCD; however, organ preservation using normothermic *ex vivo* perfusion has been shown to provide superior outcomes in experimental models (118). In this context the potential benefits of cooling the DCD heart at the time of procurement only to rewarm it minutes later upon initiation of *ex vivo* heart perfusion (EVHP) seem questionable. The benefit of inducing a profoundly hypothermic state in the short period of time between procurement and initiation of EVHP may be outweighed by the negative impact of profound hypothermia on the reparative processes essential for resuscitation of the vulnerable DCD heart (110).

Previous work in animal models have demonstrated that delivery of warm cardioplegia

following ischemia reduces reperfusion arrhythmias, lowers troponin levels, and improves functional recovery (54, 119-121). These results have been confirmed in clinical trials of patients undergoing cardiac surgery, which demonstrated that terminal warm cardioplegia limited myocardial injury, reduced reperfusion arrhythmias, and improved function postoperatively (122, 123). Since DCD hearts have sustained ischemic injury during the anoxic arrest and warm ischemic standoff period prior to organ procurement, application of the terminal warm cardioplegia concept to the initial reperfusion of DCD hearts may optimize their resuscitation (51). Translational experiments in animal models of DCD have advocated for the avoidance of profound hypothermia during the initial reperfusion to facilitate donor heart resuscitation (51, 109, 114, 124-126). We recently confirmed in a large animal translational study that the avoidance of profound hypothermia during initial reperfusion minimizes troponin release, limits endothelial injury, and improves the functional recovery of DCD hearts (110).

It is clear that interventions aiming to limit ischemia–reperfusion injury must be administered at the onset of reperfusion to be effective; however, the duration of the initial cardioplegic reperfusion is another important variable that impacts DCD heart resuscitation. The initial reperfusion must be of sufficient duration to facilitate repletion of myocardial energy stores, restoration of ionic homeostasis, and activation of ischemic post-conditioning pathways that limit ischemia–reperfusion injury. Cohen *et al* have demonstrated that initial reperfusion with an acidic solution for 1 minute was not protective; however, when this acidic reperfusion was extended to 2 minutes it afforded protection against ischemia–reperfusion injury equivalent to that achieved with post-conditioning protocols (127). In contrast, initial reperfusion with an inhibitor of GSK-3β had to be extended over a period of 15 minutes to be effective (128). In a large animal translational model of DCD transplantation, Osaki *et al* investigated the optimal initial reperfusion duration in hearts subjected to 30 minutes of global ischemia following donor extubation (49). Post-transplant outcomes were optimized when initial reperfusion with a blood-based cardioplegia at 20°C was continued for 20 minutes, compared to durations of 5 and 60

minutes. These results suggest that the optimal initial reperfusion duration may depend to some degree on the composition of the reperfusion solution, the conditions of its delivery, and the means by which activation of the ischemic postconditioning pathways occur.

It is clear that the composition of the cardioplegic solution and the conditions of its delivery at the onset of reperfusion significantly impacts the resuscitation of DCD hearts. Future research in this area will undoubtedly delineate means to further optimize DCD heart resuscitation and improve outcomes in DCD heart transplantation.

Normothermic regional perfusion

Current protocols for NRP of the DCD donor do not involve initial reperfusion of the heart with a resuscitative cardioplegic solution. Instead the heart is reperfused with donor blood following initiation of veno-arterial ECMO (45). Donor blood at the time of reperfusion may have some beneficial properties that would facilitate DCD heart resuscitation. Following donor extubation a mixed respiratory and metabolic acidosis develops (24). As previously discussed, initial acidic reperfusion of ischemic myocardium has been shown to limit ischemia–reperfusion injury (Figure 2.5) (84-86). The DCD heart also has reduced anti-oxidant capacity in the early reperfusion period, and exposure to high oxygen partial pressures during the initial reperfusion may propagate the production of ROS and oxidative injury within the myocardium (129). Therefore, initial reperfusion with the hypoxemic and acidic blood of the DCD donor may actually be beneficial. Finally, the natural energy substrates and buffers that exist in donor blood may facilitate restoration of myocardial energy stores. In these regards, NRP may still serve to resuscitate the ischemic DCD heart, and proponents of this approach believe that it provides a more expeditious reperfusion and minimizes the warm ischemic time compared to the DPP method (45). A potential downside of the NRP method; however, is the high levels of circulating catecholamines and proinflammatory cytokines that are present within the DCD donor that are likely to have detrimental effects on myocardial resuscitation (130, 131).

Despite divergent approaches to DCD heart resuscitation, both DPP and NRP protocols have been successfully implemented in clinical programs with excellent results (25, 26). Further research is required to determine if one approach is superior to the other.

DCD heart preservation

Cold static storage represents the current standard method of DBD heart preservation prior to transplantation (Figure 2.2 A); however, the DCD heart has withstood a significant ischemic insult prior to declaration of death and exposure to incremental cold ischemia during the preservation interval is unlikely to facilitate successful organ resuscitation (Figure 2.2 B) (132). It is also necessary to confirm organ viability prior to transplantation given the severity of injury sustained by the DCD heart; however, static storage under hypothermic conditions prohibits such assessments from being undertaken (133). EVHP serves to minimize DCD heart exposure to additional ischemia following organ procurement, facilitate ongoing organ resuscitation, and provide an opportunity to perform assessments of organ viability prior to transplantation (Figure 2.2 C-2.3).

The potential impact of EVHP on heart transplantation was described in a review of organ preservation published in 1967, in which Dr. Humphries stated, "*If the surgeon could preserve the organ well, he could transplant it more deliberately, and perhaps even serotype it and so choose the best recipient. He can hope ultimately not only to preserve the damaged cadaver organ but to estimate its worth and even restore it towards normal* (134)." Nearly half a century later, this description still encompasses the ultimate goal of *ex vivo* organ perfusion. By minimizing exposure to cold ischemia and supporting aerobic metabolism during organ preservation, EVHP may extend safe organ preservation interval, optimize organ allocation and donor-recipient matching, and allow more time to perform the recipient cardiectomy in recipients that require explantation of a ventricular assist device (133, 135-144). *Ex vivo* perfusion also

facilitates the delivery of pharmaceuticals that can be used to resuscitate dysfunctional organs, including stem cell and gene therapies (145-149). Finally, EVHP provides an opportunity to perform metabolic and functional assessments in order to identify organs that are suitable for transplantation (16, 25, 150).

The Transmedics Organ Care System (OCS) is the only clinically approved EVHP device, and the results of the PROCEED II trial that describe the clinical outcomes of donor hearts preserved using the OCS prior to transplantation have been recently published (16). The OCS is primed with 1.5 L of leukocyte-depleted whole blood obtained from the donor at the time of organ procurement. This is combined with 0.5 L of proprietary priming solution containing a physiologic salt solution, mannitol, methylprednisolone, multivitamins, insulin, and antibiotics. During the perfusion interval a maintenance solution containing dextrose, amino acids, adenosine, and epinephrine, is titrated to achieve a desired aortic perfusion pressure and coronary blood flow. The perfusate solution circulates through an oxygenator and heat exchanger such that the coronaries are perfused with an oxygenated perfusate solution. The donor left ventricle is preserved in an unloaded and beating state; therefore, assessments of organ viability prior to transplant are limited to metabolic profiles (lactate metabolism) and perfusion parameters (16). The results of the PROCEED II trial demonstrated non-inferiority compared to cold static storage (16); however, 5 hearts randomized to the OCS group were not ultimately transplanted and were not included in the final analysis (151).

Donor heart preservation using EVHP has been utilized in numerous pre-clinical studies of DCD heart transplantation (49, 109, 112, 114, 118, 152, 153). Most notably, Iyer and colleagues have demonstrated the impact of EVHP in a large animal model of DCD heart transplantation (118). Donor hearts were assigned to conventional cold static storage or preservation using the OCS. None of the hearts preserved using cold static storage could be weaned from cardiopulmonary bypass, while all of the hearts deemed viable following perfusion on the OCS were successfully transplanted. These results underscore the importance of

minimizing DCD heart exposure to incremental cold ischemia during the preservation interval and the utility of EVHP in facilitating successful transplantation (Figure 2.2C-2.3). The OCS has been subsequently utilized in clinical DCD heart transplantation programs in Australia and United Kingdom (16, 26).

Despite the successful clinical application of donor heart preservation using the OCS, many questions regarding the optimal utilization of normothermic EVHP remain unanswered (151). The optimal perfusate composition (oncotic pressure, hematocrit, metabolic substrates, cardioprotective additives, beta-agonist, etc.), coronary perfusion pressure, and perfusion temperature are largely unknown. Much research is required to optimize the conduct of donor heart preservation using EVHP and realize the potential of this technology in clinical transplantation.

The OCS utilizes a whole blood-based perfusate to ensure adequate myocardial oxygen delivery during EVHP. Previous research has demonstrated that an oxygen carrier is required to meet the metabolic demands of a beating heart at normothermia (154). We have previously demonstrated that a hemoglobin concentration of only 40 g/L is sufficient to maintain myocardial energy stores during working-mode EVHP; however, further studies are required to determine if a higher hemoglobin concentration would be beneficial (154). Interestingly, preservation using a whole blood-based perfusate may improve donor heart preservation compared to a solution in which the plasma component has been removed. This observation may be related to the antioxidant and anti-inflammatory properties of albumin and other plasma proteins, and the metabolic substrates present in donor plasma (154).

The development of myocardial edema is common during EVHP, which may cause diastolic dysfunction in the donor heart and limit the safe preservation interval. This is exemplified in a recent case report describing donor heart preservation using the OCS over an 8.4-hour period. The heart became visibly edematous and following transplantation ECMO was required to the support the recipient's circulation (155). The OCS prime solution is supplemented

with methylprednisolone in order to minimize myocardial edema. Donor heart exposure to extracorporeal circulation during EVHP has been shown to elicit an inflammatory response, with a 60-fold increase in proinflammatory cytokines observed over a 5-hour preservation interval (156). The addition of methylprednisolone to the perfusate solution limits this inflammatory response, protects endothelial cells, and minimizes the development of myocardial edema (156). Oshima *et al* have also demonstrated that the suppression of tumor necrosis factor α and interleukin-1β during hypothermic EVHP improves post-transplant myocardial function (157). The limited data available suggests that the suppression of proinflammatory cytokines during EVHP is protective.

Another important variable impacting the development of myocardial edema during EVHP is the oncotic pressure of the perfusate solution. The OCS priming solution contains mannitol as an oncotic agent; however, the oncotic pressure of this solution has not been reported. We have utilized a perfusate solution comprised of STEEN Solution™ (XVIVO Perfusion, Goteborg, Sweden) and whole donor blood in our large animal studies (87, 110, 150, 154). STEEN solution™ is a buffered extracellular-type salt solution containing human serum albumin and dextran 40 for oncotic pressure, and when combined with whole donor blood the oncotic pressure of the perfusate solution is 33±1 mmHg (154). This oncotic pressure is supra-physiologic compared to the normal human oncotic pressure of 25 mmHg (158). Despite the oncotic pressure of the perfusate solution, we have observed, over a 6-hour preservation interval, that normal hearts may gain up to 12% of their initial heart weight and DCD hearts up to 24% (110). Sufficient oncotic pressure must be maintained to minimize the development of myocardial edema during EVHP (159); however, the optimal oncotic pressure and the ideal impermeant (albumin, mannitol, lactobionic acid, dextran, hydroxyethyl starch, succinylated gelatin) have not been established.

The coronary perfusion pressure may also impact the development of myocardial edema during EVHP. Inadequate perfusion pressures may compromise myocardial oxygen delivery,

while excessive pressures may damage endothelial cells and cause myocardial edema (160). The optimal coronary perfusion pressure has not been investigated. The target aortic pressure during EVHP on the OCS is 65-90 mmHg (161); however, we have observed that myocardial energy stores can be maintained with aortic pressures as low as 40 mmHg (154). Coronary perfusion pressure is delivered in a pulsatile fashion on the OCS, and previous authors have demonstrated that biologically variable perfusion may significantly reduce the development of myocardial edema (132). Further research is required to determine the optimal coronary perfusion pressure during EVHP.

Previous studies have demonstrated that myocardial function declines in a linear fashion during EVHP, a feature that is observed even when normal hearts undergo prolonged perfusion (110, 154). Such a functional decline limits the safe preservation time and the potential of resuscitating dysfunctional donor hearts. This functional decline may have a metabolic origin and research aiming to optimize myocardial energy metabolism may dramatically improve donor heart preservation. Fatty acids and carbohydrates represent the primary metabolic substrates for ATP production under normal physiologic conditions, and the respective contribution of each substrate to oxidative metabolism is tightly regulated (162). Pathological states can alter these precisely regulated pathways of substrate utilization, and optimizing metabolic substrate utilization during EVHP may dramatically improve the preservation of myocardial function. Current EVHP protocols include exogenous amino acids, glucose, and insulin as exogenous substrates (16); however, there is a paucity of research investigating the optimal substrate provision. Interestingly, Cobert *et al* have demonstrated that exogenous glucose does not contribute to oxidative metabolism during hypothermic machine perfusion, while pyruvate was rapidly incorporated into tricarboxylic acid cycle intermediates and was a significant contributor to anaplerosis (163). Segel *et al* have also demonstrated that the oxidation of glucose during EVHP is limited and the provision of pyruvate to the perfusate solution significantly improved post-transplant function (164). While amino acids are not generally used as primary substrates

for myocardial energy production, they play important roles in the intermediary metabolism of the cardiomyocytes and may play in important role in regulating substrate utilization (129). Free fatty acids are rapidly depleted during EVHP (165); however, the impact of exogenous supplementation on myocardial function has not been investigated. It appears that the substrate composition of the perfusate solution may impact myocardial energy metabolism during EVHP and the preservation of donor heart function. Further research in this area may dramatically improve donor heart preservation using EVHP.

DCD heart evaluation

The traditional approach to cardiac transplantation involves evaluation of heart structure and function within the donor following declaration of brain death (Figure 2.2 A). Similar assessments may be undertaken in the DCD context prior to WLST in order to identify unsuitable organs; however, the heart is subsequently exposed to a profound ischemic insult, ventricular distension, and a massive catecholamine surge following donor extubation (24). Consequently, it is necessary to evaluate the DCD heart prior to transplantation in order to identify viable organs and exclude those at high risk of primary graft dysfunction.

The DPP approach relies on *ex vivo* assessments of organ viability during the preservation interval (Figure 2.3 A). The Transmedics OCS preserves the heart in a non-working mode that prevents assessments of myocardial function to be undertaken; however, this device enables monitoring of arterial and venous oxygen saturation, lactate concentration, aortic pressure, and coronary blood flow. In addition, assessments of coronary anatomy using angiography during *ex vivo* perfusion on the OCS have been reported (166). In the PROCEED II randomized clinical trial, organs were deemed viable and transplanted if the venous lactate level was lower than the arterial level, and the lactate concentration at the completion of the EVHP interval was < 5 mmol/L (16). Similar criteria were utilized by Dhital *et al* to identify viable hearts from DCD donors for clinical transplantation (25). This protocol is based on an abstract

presented by Hamed and colleagues at the International Society for Heart and Lung Transplantation annual meeting, which sought to identify OCS parameters collected during the preservation of ideal DBD hearts that predicted post-transplant outcomes (167). The authors identified an ending lactate concentration < 4.96 mmol/L as the best predictor of 30-day graft failure (63% sensitivity and 98% specificity). These results suggest that a high lactate concentration could accurately identify hearts at risk of post-transplant graft failure; however, a low concentration does not necessarily rule out the possibility of a high-risk heart (151). This is exemplified by a recent case report describing the preservation of a DBD heart on the OCS over an 8.4-hour period (155). Despite a normal lactate profile and perfusion parameters, primary graft dysfunction occurred following transplantation that necessitated support with ECMO. Similarly, two-thirds of the DCD hearts that were preserved on the OCS and subsequently transplanted required mechanical circulatory support post-operatively, despite acceptable lactate profiles during the preservation interval (25). These outcomes emphasize the value of assessing myocardial function to confirm organ viability before transplantation, particularly when extended criteria or DCD hearts are being evaluated (26, 161); however, there are no clinically approved EVHP devices capable of evaluating myocardial function in a physiologic working mode.

Previous research in large animal models have demonstrated the feasibility of utilizing *ex vivo* assessments of myocardial function to predict post-transplant graft function (152). Reproducible, reliable, and easily acquired metrics are required to assess myocardial function prior to transplant. Conductance catheters have been used extensively and provide a broad range of myocardial functional parameters. We have previously examined the ability of various functional and metabolic parameters to predict myocardial performance and found that ejection fraction, stroke work, minimum dP/dt, and tau exhibited a high sensitivity and specificity for identifying nonviable organs (150). However, it is important that these parameters be acquired under equivalent preload, afterload, chronotropic, and inotropic conditions to ensure that serial assessments and those reported in different studies can be reliably compared (150).

The ideal index of contractility should be sensitive to the inotropic state of the heart, but insensitive to loading conditions, heart rate, and heart size (168). Preload-recruitable stroke work (PRSW) is one such parameter that provides a preload-independent assessment of myocardial function (169). Stroke work is most accurately determined by measuring the area confined within the pressure–volume loop; however, we observed that *ex vivo* assessments of PRSW were limited by inaccurate conductance-catheter assessments of ventricular volume in poorly functioning hearts (150). Further, the expensive, cumbersome, and invasive nature of conductance catheters limits their clinical translation. Alternatively, stroke work can be estimated from the product of stroke volume and developed pressure, parameters that can be measured non-invasively (170). Application of this concept to the *ex vivo* setting can be used to produce reliable, reproducible, automated, and non-invasive PRSW assessments of the donor heart in a physiologic working mode (171). Two-dimensional echocardiography has also been used to obtain non-invasive assessments of PRSW, fractional area change, and ejection fractions as markers of myocardial contraction (153, 172, 173); however, a standardized approach to echocardiography in the *ex vivo* perfused heart has not been developed. Overall, such non-invasive approaches could eliminate the need for conductance catheter assessments and facilitate translation of donor heart functional evaluation in the *ex vivo* setting to clinical practice.

It is intuitive that *ex vivo* assessments of donor heart function would be of tremendous value in assessing donor heart viability prior to transplantation. Early development of the Transmedics OCS was capable of assessing donor heart function in a physiologic working mode; however, the complexities associated with completing functional assessments and transitioning the heart between unloaded and loaded states led to the removal of this feature in the current clinically available platform (144, 161). Further advancements in EVHP technology may provide the ability to perform comprehensive functional and metabolic assessments of the donor heart in the future.

The NRP approach to donor heart evaluation involves weaning from veno-arterial ECMO

after a period of approximately 60 minutes. This facilitates assessments of organ function to be carried out within the donor by measuring cardiac output using a pulmonary artery catheter. A comprehensive transesophageal echocardiographic evaluation can also be carried out, which provides an opportunity to identify valvular lesions and other occult pathology. The NRP approach; therefore, provides an opportunity to directly assess myocardial function and suitability for transplantation following organ resuscitation, rather than relying on metabolic surrogates of organ viability during EVHP. Acceptability criteria for clinical transplantation following NRP include a cardiac index ≥ 2.5 L/min/m^2, central venous pressure ≤ 12 mmHg, pulmonary capillary wedge pressure ≤ 12 mmHg, and a left ventricular ejection fraction ≥ 50% on transesophageal echocardiography (26). Interestingly, 50% of the donor hearts that were accepted and successfully transplanted based on these *in vivo* functional criteria exhibited lactate profiles during EVHP that would have deemed the organ non-viable. Further, one third of the donor hearts that exhibited acceptable lactate profiles required mechanical circulatory support post-transplant (26). These results underscore the value incorporating metrics of myocardial function into pre-transplant viability assessment algorithms.

DCD heart transplantation

Experimental transplantation

Early investigators sought develop techniques for the resuscitation, preservation, and transplantation of hearts from donors that had suffered an anoxic arrest. These initial studies were undertaken in an era when little was known about ischemia-reperfusion injury, immunosuppression, or extracorporeal perfusion. Studies performed in the 1960's resuscitated donor hearts after anoxic arrest periods of up to 60 minutes by connecting the heart to an intermediate host animal to facilitate coronary perfusion. Hearts were preserved in this state before subsequent orthotopic transplantation (174, 175). Wuerflein and Shumway subsequently

resuscitated hearts subjected to 90 minutes of anoxia using a pump oxygenator (176). These hearts were then preserved using a functional heart-lung preparation for periods up to 30 hours before orthotopic transplantation was undertaken. These remarkable studies provided experimental evidence that hearts from DCD donors could be successfully resuscitated and transplanted (177). Christiaan Barnard confirmed the clinical impact of such research, by demonstrating that hearts from human DCD donors could be resuscitated *in vivo* on cardiopulmonary bypass and successfully transplanted (20, 21). However, enthusiasm for research seeking to optimize the technique of DCD heart resuscitation and transplantation waned over the next 20 years, following the acceptance of deceased organ donation from donors declared dead based on neurologic criteria (9-11).

The acceptance of heart transplantation as an established treatment for advanced heart failure resulted in a growing demand for organs from DBD donors. Heart transplantation soon became limited by a shortage of suitable donor organs and the number of transplants performed annually plateaued (178). This prompted a renewed interest in DCD heart transplantation as means to increase the number suitable donor organs. In 1992, Shirakura *et al* reported on the successful transplantation of 8 DCD dog hearts (179). The donor animals were partially exsanguinated to produce a 20-minute period of antemortem shock and then mechanical ventilation was stopped. During this time the donor was pre-treated with prostaglandin, verapamil, and propranolol. Ten minutes after donor extubation the hearts were reperfused with warm blood cardioplegia and then cold crystalloid cardioplegia, before undergoing 22 hours of cold static storage. Despite the prolonged cold-static storage period, all hearts were weaned from cardiopulmonary bypass without inotropic support.

A number of investigators subsequently published studies describing the successful resuscitation and transplantation of hearts subjected to 17 – 60 minutes of warm ischemia following donor exsanguination in a variety of large animal models (180-182). Martin *et al* demonstrated that a cariporide-supplemented blood cardioplegia improved post-transplant

outcomes in pig hearts subjected to 30 minutes of warm ischemia (78, 183). Suehiro *et al* demonstrated better post-transplant outcomes in dog hearts subjected to 60 minutes of warm ischemia that were reperfused with a warm blood cardioplegia containing a ROS scavenger compared to those reperfused with cold St. Thomas' solution (184). Using a similar protocol, Suehiro *et al* also demonstrated that *ex vivo* assessments of myocardial function could be used to predict successful transplantation (152). Finally, Martin *et al* demonstrated that controlled reperfusion of hearts subjection to 30 minutes of warm ischemia using a cariporide and adenosine-supplemented, leukocyte depleted, blood cardioplegia could facilitate successful transplantation, with myocardial function comparable to control animals 24-hours post-transplant (185, 186). These studies provided evidence that hearts from DCD donors could be successfully resuscitated and transplanted. In 2009, however; Osaki *et al* demonstrated that utilizing an exsanguination model to induce donor warm ischemia significantly reduced the severity of myocardial injury sustained by the heart compared to the more clinically relevant model of donor extubation and hypoxemic cardiac arrest (124). In this context, clinical translation of these early studies that employed an exsanguination model of donor warm ischemia is difficult.

Gundry *et al* utilized the a more clinically relevant model of hypoxemic cardiac arrest in lambs and demonstrated that donor pre-treatment with dextrose, methylprednisolone, prostaglandin E_1, and nifedipine could facilitate successful transplantation following 40 minutes of warm ischemia (187). Gundry *et al* employed the same protocol in baboons and reported post-transplant survival ranging from 1 to 34 days (188). The causes of death included acute rejection, stroke, and dehydration. Importantly, post-mortem histological analysis did not demonstrate any evidence of myocardial fibrosis or ischemic injury. Donor pretreatment with an endothelin-A receptor antagonist and an ATP-sensitive potassium channel opener have also been shown to facilitate successful DCD transplantation in dogs (108, 189). While these studies provided experimental evidence that hearts sustaining significant periods of warm ischemia following donor extubation could be successfully transplanted, donor pretreatment with

therapeutics aiming to minimize the detrimental effects of warm ischemia are ethically prohibited in most cases.

In 2006, Osaki *et al* utilized a clinically relevant model of DCD (donor extubation, hypoxemic cardiac arrest, no donor pre-treatments) and demonstrated that controlled reperfusion with a tepid blood cardioplegia could facilitate successful transplantation in pigs (49). To minimize donor heart exposure to additional ischemia following procurement, the hearts were continuously perfused in a beating state during preservation and implantation. The same group subsequently demonstrated that the addition of a ROS scavenger to the initial reperfusion solution minimized lipid peroxidation and edema formation, and improved post-transplant function (114). Iyer *et al* reported successful transplantation of DCD pig hearts exposed to 30-minutes of warm ischemia following donor extubation (118). These hearts were resuscitated with Celsior solution supplemented with erythropoietin, glyceryl trinitrate, and zoniporide, and then preserved in a normothermic beating state on the Transmedics OCS before transplantation. We have also demonstrated the importance employing an approach to donor heart resuscitation that limits ischemia–reperfusion injury, and utilizing EVHP to minimize incremental ischemic injury during preservation and transplantation can improve post-transplant outcomes (109). Finally, Ali *et al* demonstrated that DCD hearts resuscitated using NRP could be successfully transplanted, with outcomes comparable to hearts from DBD donors (190). These translational studies provided pre-clinical evidence to suggest that DCD heart transplantation could be successfully performed when an approach to donor heart resuscitation, preservation, evaluation, and transplantation is tailored to the DCD context (Figure 2.2C-2.3).

Clinical transplantation

Boucek *et al* published the first report of clinical DCD heart transplantation in the modern era, describing 3 pediatric transplants performed between 2004 and 2007 at Denver Children's Hospital (191). Each donor had suffered birth asphyxia and a decision to WLST was made

based on the futility of ongoing care. Informed consent was obtained for donation, anti-mortem heparin, and the insertion of central arterial and venous catheters. Donor extubation was undertaken in the operating room and the intensive care unit team provided palliative care. The donor was monitored for progression to cardiorespiratory arrest and mechanical asystole, after which time a standoff period was observed before circulatory death was declared. A 3-minute standoff period was observed in the first donor; however, this was shorted to 1.25 minutes in the subsequent donors based on the recommendations of the ethics committee. The mean time from extubation to declaration of death was 18 ± 8 minutes. Cold preservation fluid was then infused though a balloon catheter placed in the ascending aorta and venous exsanguination was undertaken. Simultaneously, a median sternotomy was performed and topical cooling of the donor heart was commenced. Post-transplant inotropic support, hospital length of stay, number of rejection episodes, ventricular function, and 6-month survival were comparable to control infants who underwent transplantation with hearts from DBD donors during the same period. This report prompted vigorous ethical debate in the transplant community regarding the definition of circulatory death, whether cardiac transplantation from DCD donors violates the "dead donor rule," and the minimum standoff period duration that must be observed before death can be declared (192-194).

In 2009, Ali *et al* reported on a DCD donor with a 23-minute warm ischemic time that was resuscitated using normothermic cardiopulmonary bypass, following exclusion of the cerebral circulation (195). After 5 minutes of reperfusion the heart spontaneously reverted to a sinus rhythm. The patient was re-intubated and 190 minutes later was weaned from cardiopulmonary bypass. Following insertion of a pulmonary artery catheter, a cardiac index of 2.4 L/min/m^2 was measured with a pulmonary artery capillary wedge pressure of 13 mmHg. The authors suggested that hearts from Maastricht category III donors might be suitable for use in clinical transplantation. This report again prompted ethical debate surrounding the post-mortem restoration of mechanical cardiac function using normothermic cardiopulmonary bypass in a

donor that had been declared dead based on cardiorespiratory criteria (196). However, the technique of NRP has become an accepted means of facilitating multi-organ DCD retrievals in some countries (197).

In 2014, Iyer *et al* utilized the DPP approach to DCD heart resuscitation in a donor that had suffered a 32-minute warm ischemic time (198). The heart was reperfused with a resuscitative cardioplegia and then transferred to a modified Transmedics OCS device for normothermic EVHP and functional assessment in a physiologic working mode. The heart produced a cardiac output of > 5 L/min with a left atrial pressure of 14 mmHg. Rosenfeldt *et al* also reported on a DCD donor heart that was reperfused with a resuscitative cardioplegia after 32 minutes of warm ischemia, and preserved using hypothermic EVHP during transport (199). The heart subsequently underwent normothermic EVHP and functional evaluation over a 12-hour period. Messer *et al* utilized the NRP approach in 3 DCD donors with a mean warm ischemic time of 28 minutes, and demonstrated that *in vivo* measurements of myocardial function correlated with assessments conducted *ex vivo* on a modified Transmedics OCS device (200). Finally, Osaki *et al* compared the myocardial function of 4 DCD hearts with 5 DBD hearts that had been declined for clinical transplantation due to the presence of coronary artery disease, advanced age, and donor social history (201). The DCD hearts sustained a warm ischemic time of 34 ± 3 minutes, were reperfused with a standard cardioplegic solution, and then underwent cold-static storage for 152 ± 55 minutes before evaluation in an *ex vivo* perfusion device. The DBD hearts were arrested with the same cardioplegic solution and underwent cold-static storage for 211 ± 31 minutes before *ex vivo* evaluation. Despite the additional cold ischemic insult sustained by the DCD hearts during the preservation interval, the recovery of myocardial function was not significantly different compared to the DBD hearts. These reports provided early clinical evidence that hearts from DCD donors could be successfully resuscitated, and formed a foundation on which clinical transplant programs could be developed.

Dhital *et al* reported the first clinical adult DCD heart transplants in the modern era in

2015 (25). These 3 transplants represented direct clinical translation of the groups' research in pharmacologic postconditioning and large animal models of DCD (43, 63, 74, 83, 111, 112, 118). Based these studies the authors considered Maastricht category III donors < 40 years of age with a warm ischemic time < 30 minutes (112). WLST occurred in the intensive care unit, an anesthetic bay, or an adjacent operating room, with standoff periods ranging from 2 – 5 minutes depending on the jurisdiction of donation within Australia. The warm ischemic times ranged from 22 – 28 minutes, with functional warm ischemic times ranging from 11 – 21 minutes (Table 2.2). Donor hearts were resuscitated using the DPP approach and St. Thomas' cardioplegia supplemented with erythropoietin and glyceryl trinitrate (112). Unfortunately, zoniporide is not approved for clinical use and could not be included in the resuscitative cardioplegia. Donor hearts were preserved on the Transmedics OCS for 245 – 260 minutes prior to transplantation. A perfusate lactate concentration < 5 mmol/L and evidence of myocardial lactate extraction were used as evidence of myocardial viability. Following completion of the preservation interval, the hearts were re-arrested and transplanted in the standard fashion. Two recipients required temporary mechanical circulatory support post-transplant; however, all patients were subsequently weaned from support and discharged from hospital after 21 – 28 days. Two patients experienced a rejection episode, but all patients demonstrated normal biventricular function at follow-up (Table 2.3). A fourth donor failed to progress to circulatory arrest in the pre-determined time frame and demonstrated a rising lactate concentration during preservation on the OCS; therefore, this heart was not considered for transplantation. This report represented the first clinical evidence that hearts from DCD donors could be resuscitated, preserved using EVHP while being transported from a distant site, and successfully transplanted. The authors have subsequently performed 6 additional DCD transplants, and all recipients are doing well with normal biventricular function at follow-up (personal communication).

Table 2.2. Donation and pre-transplant characteristics for clinical transplants performed with hearts donated following circulatory death

	Donation and pre-transplant characteristics												
	Messer et al. (26)									Dhital et al. (25)			
Age (years)	33	28	38	29	38	43	32	36	44	26	26	27	33±6
Sex (M/F)	M	M	M	M	M	M	F	M	F	M	M	M	83% M
BSA (m^2)	1.88	2.00	2.35	2.38	1.92	2.08	1.80	1.94	1.68	2.14	1.83	2.00	2.0±0.2
WIT (min)	60	18	29	17	28	24	21	146	23	28	25	22	37±36
FWIT (min)	17	12	25	16	24	18	19	16	13	21	20	11	18±4
NRP Duration (min)	52	52	190	61	34	27	45	29	40	-	-	-	59±50
In vivo CI (L/m^2)	3.2	2.8	2.9	4.1	3.6	3.5	2.9	4.5	2.8	-	-	-	3.4±0.6
In vivo HR (beats/min)	85	122	92	135	105	125	118	100	148	-	-	-	114±21
In vivo PCWP (mmHg)	12	9	11	8	11	8	10	6	8	-	-	-	9.2±1.9
In vivo EF (%)	58	66	70	60	68	70	59	67	66	-	-	-	65±5
OCS Duration (min)	170	173	0	170	184	166	139	428	209	257	260	245	200±99
Starting Lactate (mmol/L)	-	-	-	-	-	-	-	-	-	8.3	6.8	7.6	7.6±0.8
Ending Lactate (mmol/L)	-	-	-	-	-	-	-	-	-	3.6	2.8	2.7	3.0±0.5

BSA: body surface area, CI: cardiac index, EF: ejection fraction, FWIT: functional warm ischemic time, HR: heart rate, NRP: normothermic regional perfusion, OCS: organ care system, PCWP: pulmonary capillary wedge pressure, WIT: warm ischemic time.

Table 2.3. Recipient and post-transplant characteristics for clinical transplants performed with hearts donated following circulatory death

	Recipient and post-transplant characteristics												
	Messer et al. (26)									Dhital et al. (25)			
Age (years)	59	23	61	58	58	64	55	51	41	57	43	57	52±12
Sex (M/F)	M	M	M	M	F	M	M	M	F	M	F	M	M 75%
BSA (m^2)	2.08	2.03	1.89	2.00	1.73	1.94	1.84	1.98	1.72	1.77	1.86	1.91	1.9±0.1
Diagnosis	DCM	HCM	DCM	HCM	DCM	DCM	DCM	DCM	RVC	DCM	DCM	AVRD	DCM 67%
VAD	No	No	No	No	No	No	No	Yes	No	No	No	No	Yes 8%
TPG (mmHg)	7	4	7	8	8	5	8	8	7	7	5	8	6.8±1.4
PVR (Woods Units)	1.9	1.3	1.9	2.2	3.0	1.3	2.8	2.1	2.2	1.0	1.7	2.2	2.0±0.6
IABP (days)	1	0	0	9	0	0	0	0	0	1	0	2	1±3
VA ECMO (days)	0	0	0	7	0	0	0	0	0	4	0	0	1±2
ICU LOS (days)	4	5	4	29	5	5	7	5	4	7	9	6	8±7
Hospital LOS (days)	20	15	19	80	17	38	17	29	26	26	28	21	28±18
Rejection episodes	-	-	-	-	-	-	-	-	-	1	1	0	0.67±0.58
LV function	-	-	-	-	-	-	-	-	-	N	N	N	N (100%)
RV function	-	-	-	-	-	-	-	-	-	N	N	N	N (100%)

BSA: body surface area, IABP: intra-aortic balloon pump, ICU: intensive care unit, LOS: length of stay, LV: left ventricle, PVR: pulmonary vascular resistance, RV: right ventricle, TPG: transpulmonary gradient, VAD: ventricular assist device, VA ECMO: veno-arterial extracorporeal membrane oxygenation.

Most recently, Messer *et al* described 9 adult clinical DCD heart transplants performed following donor heart resuscitation using the NRP approach (26) These transplants also

represented clinical translation previous research in large animal models of DCD (190). The authors considered Maastricht category III donors < 50 years of age; however, donors with warm ischemic times extending beyond 30 minutes were still considered. Following WLST and progression to mechanical asystole, a 5-minute standoff period was observed before declaration of circulatory death. The warm ischemic times ranged from 17 – 146 minutes, with functional warm ischemic times ranging from 12 – 25 minutes (Table 2.2). Hearts were resuscitated using veno-arterial ECMO and subsequently weaned from support after 27 – 190 minutes. *In vivo* functional assessments demonstrated a cardiac index of 2.8 – 4.5 L/min/m^2 and an ejection fraction of 58-70%. A cardiac index ≥ 2.5 L/min/m^2, with a CVP ≤ 12 mmHg, pulmonary capillary wedge pressure ≤ 12 mmHg, and an ejection fraction ≥ 50% were used as evidence of myocardial viability. Donor hearts were then preserved on the Transmedics OCS for 0 – 428 minutes prior to transplantation (one local donor heart underwent a brief period of cold-static storage prior to transplant and was not preserved on the OCS). Hearts preserved on the OCS underwent continuous perfusion during transplantation to further minimize exposure to ischemic injury.

Two recipients required mechanical circulatory support post-transplant; however, all patients were subsequently weaned from support and discharged from hospital after 15 – 80 days (Table 2.3). Interestingly, 4 of the hearts displayed lactate profiles during preservation on the OCS that suggested non-viability (16, 25). However, these hearts were deemed viable based on the *in vivo* functional assessments and were successfully transplanted without need for mechanical circulatory support. Conversely, the heart that required veno-arterial ECMO following transplant displayed an acceptable lactate profile during preservation on the OCS. These results highlight the need for further research regarding assessments of organ viability prior to transplant, in order to minimize the risk of primary graft dysfunction. Importantly, the results of this study also demonstrate that DCD hearts with extended warm ischemic times may still be considered for transplantation, provided the functional warm ischemic time remains < 30

minutes and acceptable myocardial function is demonstrated. Finally, the authors utilized continuous myocardial perfusion during transplantation, a technique described by Christiaan Barnard in the first clinical transplants and in animal modes of DCD (20, 49, 109). In the PROCEED II trial, the donor heart was exposed to 83 minutes of additional cold ischemia during implantation (16). Therefore, utilizing continuous perfusion during implantation to eliminate DCD heart exposure to this additional ischemic injury may significantly improve post-transplant outcomes.

Conclusions

Over the last 50 years, a growing population of patients with advanced heart failure coupled with an ever improving transplant outcomes have resulted in a critical shortage of suitable donor organs. Advances in our understanding of ischemia–reperfusion injury and pharmacologic postconditioning have facilitated the development of controlled reperfusion strategies that can successfully resuscitate the DCD heart. The clinical availability of the Transmedics OCS now allow donor heart preservation in a beating state and a means to limit DCD heart exposure to additional cold ischemia prior to transplantation. As technology evolves the ability evaluate DCD hearts during EVHP will improve our ability to identify viable organs. This will allow donor organ utilization to be maximized and recipient risk of primary graft dysfunction to be minimizes. Finally, continuous perfusion during transplantation may represent an additional means to minimize organ injury and optimize post-transplant outcomes. Further research investigating ways to optimize the resuscitation, preservation, evaluation, and transplantation of DCD hearts is vital to ensure a broader application of DCD heart transplantation in the future.

Chapter 2 Summary

In Chapter 2: *Transplantation of hearts donated following circulatory death: a narrative review*, the current state of DCD heart transplantation was reviewed. This chapter highlights the need to expand the donor pool beyond those organs currently being accepted for transplantation. An approach to DCD heart transplantation that focuses on donor heart resuscitation at the time of organ procurement, preservation using EVHP, and organ assessment to identify viable organs is presented. Each aspect of this tailored approach to DCD heart transplantation will be explored in further detail in subsequent chapters (Chapter 4: application of this tailored approach in a 'proof-of-concept' large animal DCD transplant study, Chapter 5: optimizing donor heart preservation using EVHP, Chapter 6: optimizing DCD heart evaluation to identify viable organs, and Chapters 7-8: optimizing DCD heart resuscitation at the time of organ procurement).

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Chapter 3

Physiologic changes in the heart following cessation of mechanical ventilation in a porcine model of donation after circulatory death: implications for cardiac transplantation

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Chapter 3 Preface

In order to investigate the resuscitation, preservation, and evaluation of DCD hearts, it is first necessary to develop a reliable animal model of DCD. Chapter 3: *Physiologic changes in the heart following cessation of mechanical ventilation in a porcine model of donation after circulatory death: implications for cardiac transplantation*, describes a porcine model of DCD in which the extubation of an anesthetized donor animal is used to replicate the clinical scenario of life sustaining therapy withdrawal that is encountered in the intensive care unit. Further, a thorough understanding of the physiologic response to donor extubation is imperative to realize the potential of DCD heart transplantation. The hemodynamic data presented in this chapter suggests that the donor animal progresses through distinct physiologic phases following extubation that may have important implications for optimizing the resuscitation, preservation, and evaluation of DCD hearts for transplantation. Therefore, not only does this chapter describe a reliable animal model of DCD, but it also reveals specific physiologic aspects of the circulatory arrest process that have particular relevance in the context of heart transplantation.

Abstract

Hearts donated following circulatory death (DCD) may represent an additional source of organs for transplantation; however, the impact of donor extubation on the DCD heart has not been well characterized. We sought to describe the physiologic changes that occur following withdrawal of life sustaining therapy (WLST) in a porcine model of DCD. Physiologic changes were monitored continuously for 20 minutes following WLST. Ventricular pressure, volume, and function were recorded using a conductance catheter placed into the right (N=8) and left (N=8) ventricles, and using magnetic resonance imaging (MRI, N=3). Hypoxic pulmonary vasoconstriction occurred following WLST, and was associated with distension of the right ventricle (RV) and reduced cardiac output. A 120-fold increase in epinephrine was subsequently observed that produced a transient hyperdynamic phase; however, progressive RV distension developed during this time. Circulatory arrest occurred 7.6±0.3 minutes following WLST, at which time MRI demonstrated an 18±7% increase in RV volume and a 12±9% decrease in left ventricular volume compared to baseline. We conclude that hypoxic pulmonary vasoconstriction and a profound catecholamine surge occur following WLST that result in distension of the RV. These changes have important implications on the resuscitation, preservation, and evaluation of DCD hearts prior to transplantation.

Introduction

Cardiac transplantation remains the gold-standard treatment for eligible patients with advanced heart failure. Following the introduction of brain-death criteria in 1968 (1), cardiac transplantation has been performed almost exclusively with organs from brain-dead donors. A critical shortage of suitable organs from such donors has prompted a renewed interest in donation after circulatory death (DCD) to expand the donor pool. Clinical transplantation of DCD hearts has not been undertaken routinely because of concerns regarding the severity of injury sustained during the hypoxemic cardiac arrest and warm ischemic standoff period that ethically define death (2). However, translational experiments have demonstrated that successful transplantation of DCD hearts can be accomplished if ischemic postconditioning agents (i.e. erythropoietin, glyceryl trinitrate, sodium-hydrogen exchange inhibitors) are delivered at the time of procurement and organs are preserved using ex vivo perfusion (3-6). Clinical application of this approach has now resulted in the successful transplantation of human adult hearts donated after circulatory death (7).

Despite these recent successes, data describing the physiologic changes that occur following withdrawal of life sustaining therapy (WLST) are limited and a thorough understanding regarding the physiology of dying is lacking. This is exemplified by the variable definitions of donor circulatory arrest (electrical asystole, absent pulse, absent pulse pressure on an arterial pressure tracing, absence of cardiac output, etc.), standoff period durations that must be observed before death can be declared (2-20 minutes), and definitions of the functional warm ischemic time that are employed in the donation community (2, 8-11). An improved understanding regarding the natural history of the dying process following donor extubation is necessary to inform the development of standardized medical practices for DCD (12).

A more detailed description of the physiologic response to donor extubation is especially pertinent in the context of DCD heart transplantation. Following WLST the donor heart is forced to function in an increasingly hypoxic environment while attempting to maintain systemic oxygen

delivery. Previous studies have suggested that this process is associated with a catecholamine-mediated period of hyperdynamic circulation and ventricular distention that may exacerbate the severity of injury suffered by DCD hearts (13-16); however, a systematic description of the physiologic response to WLST in the donor heart has not been previously reported. A greater understanding of this process may inform the creation of resuscitation protocols tailored specifically to the DCD heart and evaluation strategies that identify organs suitable for transplantation. Therefore, the objective of this study is to characterize the physiologic changes that occur in the donor heart following WLST in a large animal model of DCD.

Materials and Methods

All animals received humane care in compliance with the National Institute of Health's *Guide for the Care of Laboratory Animals*. Institutional Animal Care Committees approved the experimental protocol. Physiologic changes following WLST were studied in 19 female domestic pigs (Figure 3.1).

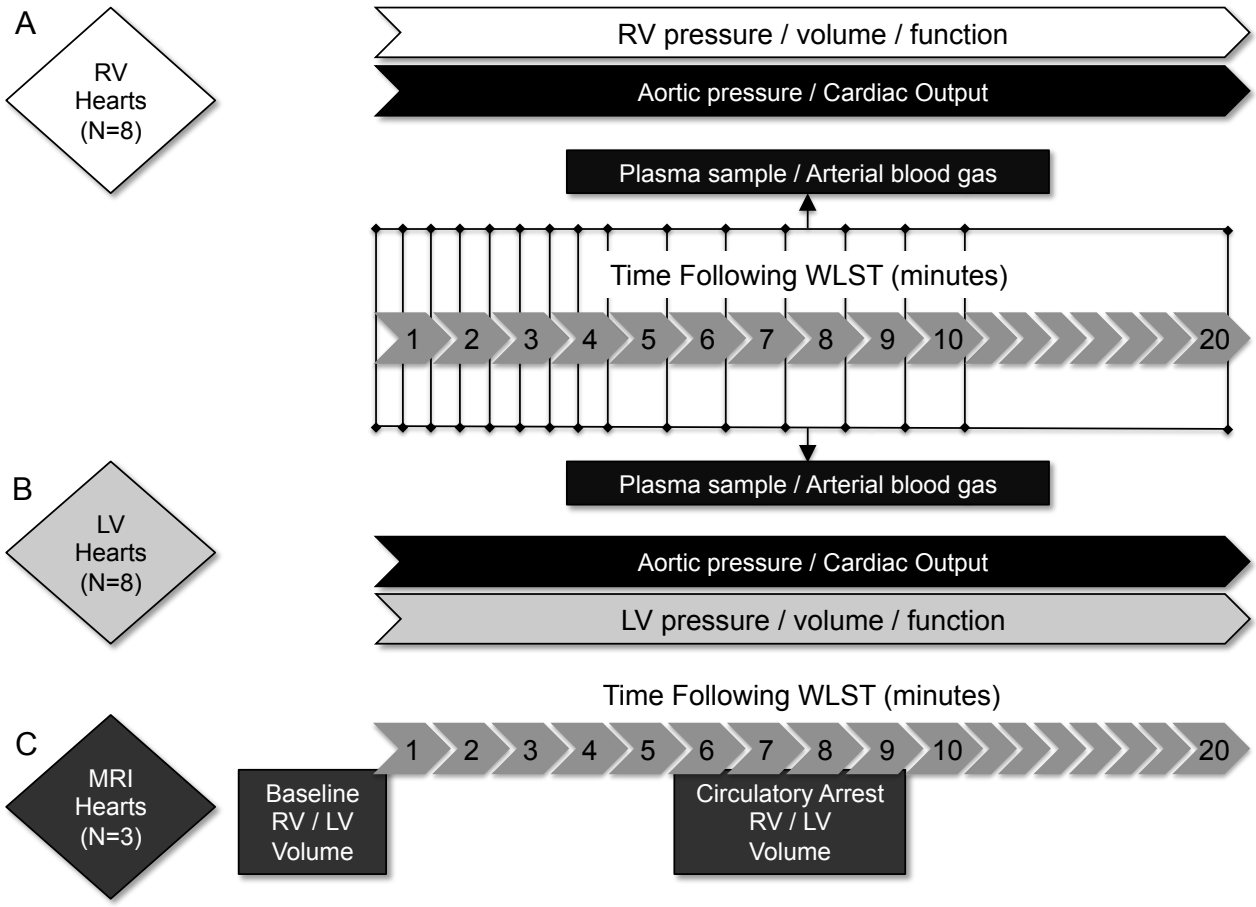

Figure 3.1. Experimental donation after circulatory death protocol describing the interventions performed during the 20-minute period following cessation of mechanical ventilation. A) RV hearts (N=8): aortic pressure, cardiac output, and right ventricular pressure, volume and function were recorded continuously. Arterial blood samples were obtained at regular intervals. B) LV hearts (N=8): aortic pressure, cardiac output, and left ventricular pressure, volume, and function were recorded continuously. Arterial blood samples were obtained at regular intervals. C) MRI

hearts (N=3): left and right ventricular chamber volumes were measured at baseline prior to cessation of mechanical ventilation, and following declaration of circulatory arrest.
LV: left ventricle, MRI: magnetic resonance imaging, RV: right ventricle, WLST: withdrawal of life sustaining therapy (cessation of mechanical ventilation)

Donor animal preparation

Pigs (40 kg) were sedated with an intramuscular injection of tiletamine (2.4mg/kg), zolazepam (2.4 mg/kg), and xylazine (0.9 mg/kg). Orotracheal intubation was established and general anesthesia was maintained with 2-3% isoflurane. A median sternotomy was performed and 1000 units/kg of heparin was delivered intravenously. 7F and 6F catheters were placed into the internal jugular vein and common carotid artery for blood sampling and continuous monitoring of central venous and aortic pressures (IntelliVue MP60, Philips Healthcare, Andover, USA).

Conductance catheter assessment

A 5F conductance catheter (Ventri-Cath 507, Millar Inc., Houston, USA) was placed retrogradely across the pulmonic valve into the RV (RV hearts, N=8), or across the aortic valve into the LV (LV hearts, N=8), for continuous recording of ventricular pressure, volume, and function data (Figure 3.1). Catheter pressure calibration was performed using a hand-held manometer and volume calibration was carried out following measurement of blood conductivity. The maximum (dP/dt maximum) and minimum (dP/dt minimum) rate of pressure change within the ventricle were used to assess systolic and diastolic function, respectively. Cardiac output was calculated using the stroke volume and heart rate measured with the conductance catheter. All parameters were recorded continuously and analyzed using LabChart 7 Pro Version 7.2.5 (ADInstruments Inc., Colorado Springs, USA).

Cine cardiac magnetic resonance imaging

RV and LV chamber volumes (MRI hearts, N=3) were evaluated using cine cardiac magnetic resonance imaging (MRI) with a 3-Tesla Siemens MR scanner (Sonata, Magnetom, Siemens, Erlangen, Germany) as previously described (17). Measurements were obtained at baseline prior to cessation of mechanical ventilation and upon declaration of circulatory arrest (Figure 3.1). A four-element phased array coil was used, with two coils placed on the anterior chest wall and two placed on the posterior chest wall. Images were analyzed using MASS version 4.2 software (MEDIS Medical Imaging Systems, Leiden, Netherlands). The contour of the ventricular wall endocardium at end-diastole was manually traced in all images containing the RV and LV, and the sum of the marked areas was used to calculate the end-diastolic volume.

Donation after circulatory death

Following acquisition of baseline conductance catheter and MRI assessments, the fraction of inspired oxygen was reduced to 25%, mechanical ventilation was discontinued, and the donor animal was extubated. A 10 mL blood sample was withdrawn from the ascending aorta at 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, and 20.0 minutes following cessation of mechanical ventilation (Figure 3.1). A 1 mL aliquot was analyzed on an ABL 800 Flex blood gas machine (Radiometer Medical ApS, Brønshøj, Denmark) to obtain pH, P_aO_2, P_aCO_2, hemoglobin concentration, % hemoglobin saturation, and lactate concentration. The remainder of the blood was centrifuged (4°C) at 1,500 g for 15 minutes and stored at -80°C for later analysis. Circulatory arrest was declared when pulsatility on the arterial pressure tracing was no longer evident. Animals were observed for a total of 20 minutes following cessation of mechanical ventilation (Figure 3.1).

Enzyme-linked immunosorbent assays

The concentrations of epinephrine and norepinephrine (Abnova, Walnut, USA), tumor necrosis factor alpha and interleukin-6 (R&D Systems, Minneapolis, USA), troponin-I (Life Diagnostics, West Chester, USA), and triiodothyronine (NeoBiolab, Massachusetts, USA) in plasma samples obtained following cessation of mechanical ventilation were measured using commercially available ELISA kits.

Adenosine release

The concentration of adenosine in plasma samples obtained following cessation of mechanical ventilation (N=4) was measured using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Plasma (500µL) was diluted with phosphate buffered saline (500µL) and 100ng/mL of 2-chloroadenosine (as an internal standard). Samples were subjected to solid phase extraction using Waters Oasis MCX 1cc cartridges, dried, and reconstituted in 100µL water containing 0.05% trifluoroacetic acid and 0.5% acetic acid. Quantification was performed on the Shimadzu Nexera ultra high performance liquid chromatograph (UHPLC) and LCMS-8040 triple quadrupole mass spectrometer. Chromatographic separation was performed using the Waters Acuity BEH C18 UHPLC column in isocratic conditions at 85% acetonitrile in water (0.05% trifluoroacetic acid, 0.5% acetic acid) with retention times of 1.9 min (adenosine) and 2.7 min (2-chloroadenosine). Mass detection was achieved with dual ion source atmospheric pressure chemical ionization/electrospray ionization using the multiple reaction monitoring for transitions 268.2>136.2 and 302.1>170.1 for adenosine and 2-chloroadenosine, respectively.

Calculations:

Systemic oxygen delivery was calculated as follows:

*O_2 delivery (mLO_2/minute) = C_aO_2 (mLO_2/100 mL) * CO (100mL/minute),*

where C_aO_2 represents the oxygen content in the arterial blood and CO represents the cardiac output. The C_aO_2 was calculated as follows:

*C_aO_2 (mL O_2/100 mL) = [1.34 (mLO_2/gram hemoglobin) * hemoglobin concentration (grams/100 mL) * hemoglobin oxygen saturation (%)] + [0.0031 (mLO_2/mmHg/100mL) * P_aO_2 (mmHg)],*

where P_aO_2 represents the partial pressure of oxygen in the aortic root.

Systemic vascular resistance was calculated as follows:

*Systemic vascular resistance (mmHg*minute/L) = [mean arterial pressure (mmHg) – right ventricular diastolic pressure (mmHg)] / cardiac output (L/minute).*

Statistical Analysis

Normally distributed continuous variables were reported as mean ± standard error of the mean (SEM) and were compared using the two-tailed, paired t-test. A *p*-value <0.05 was considered statistically significant. Analyses were performed using GraphPad Prism V6.0c (GraphPad Software Inc., La Jolla, USA).

Results

We identified 5 physiologic phases based on our observations of the hemodynamic changes that occurred following WLST: (1) pulmonary vasoconstriction (0–1.5 minutes), (2) hyperdynamic (1.5–4.0 minutes), (3) agonal (4.0–7.0 minutes), (4) circulatory arrest (7.0–8.0 minutes), and (5) standoff (8.0–20.0 minutes) phases. A summary of the study results is presented in Table 3.1.

Table 3.1. Hemodynamics, arterial blood gas parameters, hormones, and biomarkers following donor extubation.

	Time following donor extubation (minutes)				
Mean (SEM)	0	1.5	4	8	20
pH	7.45 (0.01)	7.34 (0.01)	7.27 (0.01)	7.18 (0.02)	7.13 (0.02)
PO_2 (mmHg)	107 (6)	23 (1)	13 (1)	12 (1)	13 (1)
PCO_2 (mmHg)	40.0 (1.2)	57.6 (1.3)	68.2 (2.1)	81.3 (4.2)	91.4 (5.0)
Lactate (mmol/L)	1.8 (0.1)	2.5 (0.3)	2.7 (0.2)	4.1 (0.3)	4.9 (0.4)
Troponin-I (ng/mL)	0.21 (0.13)	0.18 (0.11)	0.20 (0.12)	0.20 (0.11)	0.22 (0.13)
Norepinephrine (ng/mL)	1 (0)	2 (2)	204 (90)	377 (63)	591 (137)
Tumor necrosis factor-alpha (pg/mL)	153 (61)	145 (57)	116 (47)	116 (42)	104 (41)
Triiodothyronine (ng/mL)	334 (38)	390 (24)	367 (40)	361 (24)	365 (45)
Cardiac output (mL/min)	1702 (141)	1352 (131)	891 (131)	214 (39)	54 (15)
Systemic oxygen delivery (mL O2/min)	212 (4)	41 (4)	10 (1)	3 (0)	1 (0)
SVR (mmHg*min/L)	34 (3)	22 (2)	28 (4)	23 (4)	0 (0)
Heart rate (beats/min)	81 (3)	79 (3)	82 (5)	67 (5)	29 (6)
RV systolic pressure (mmHg)	27 (2)	28 (2)	29 (3)	15 (2)	9 (1)
RV diastolic pressure (mmHg)	7 (1)	8 (1)	10 (1)	12 (1)	9 (1)
LV systolic pressure (mmHg)	71 (3)	54 (4)	45 (7)	17 (6)	6 (1)
LV diastolic pressure (mmHg)	5 (1)	5 (1)	4 (1)	5 (1)	5 (1)
RV dP/dt maximum (mmHg/sec)	224 (16)	230 (14)	193 (26)	36 (11)	8 (0)
RV dP/dt minimum (mmHg/sec)	-157 (14)	-165 (10)	-139 (25)	-31 (7)	-9 (1)
LV dP/dt maximum (mmHg/sec)	780 (55)	627 (42)	654 (128)	157 (90)	8 (0)
LV dP/dt minimum (mmHg/sec)	-758 (42)	-389 (46)	-449 (102)	-128 (67)	-8 (0)
% Change RV diastolic volume	0 (0)	14 (3)	22 (3)	18 (4)	6 (4)
% Change LV diastolic volume	0 (0)	3 (1)	-8 (4)	-10 (2)	-21 (2)

LV, left ventricle; *RV*, right ventricle; *SEM,* standard error of the mean; *SVR,* systemic vascular resistance.

Pulmonary vasoconstriction phase (0 – 1.5 minutes)

The P_aO_2, cardiac output, and systemic oxygen delivery declined precipitously in the first 1.5 minutes following cessation of mechanical ventilation (Figures 3.2-3.3). A progressive decline in LV function and systemic vascular resistance was associated with a decline in aortic systolic and LV systolic pressures (Figures 3.2, 3.4, 3.5). In contrast, RV function remained unchanged during the pulmonary vasoconstriction phase, and the RV systolic and diastolic pressure, and the RV diastolic volume increased (Figures 3.4-3.6). During this time period the P_aCO_2 increased from 40±1 mmHg to 58±1 mmHg ($p<0.01$), the arterial lactate concentration increased from 1.8±0.1 to 2.5±0.3 mmol/L ($p=0.03$), the pH declined from 7.45±0.01 to 7.34±0.01 ($p<0.01$), and a 3.0±0.7 fold increase in the concentration of adenosine was observed (Figure 3.3).

Hyperdynamic phase (1.5 – 4 minutes)

The hyperdynamic phase was characterized by a 120-fold increase in the concentration of endogenous epinephrine and a 400-fold increase in the concentration of norepinephrine (Figure 3.7). This catecholamine surge was associated with a transient increase in heart rate (Figure 3.2), biventricular function (Figure 3.5), cardiac output (Figure 3.2), and systemic vascular resistance (Figure 3.2), which were associated with an increase in aortic systolic pressure (Figure 3.2). Despite the increase in RV contractility, the diastolic pressure and volume in the RV continued to increase over the duration of the hyperdynamic phase (Figure 3.4 and 3.6).

The increase in ventricular function and cardiac output observed during the hyperdynamic phase augmented the systemic oxygen delivery to a negligible degree (Figure 3.2), and the arterial lactate concentration increased significantly during this time (2.0±0.2 vs. 2.7±0.2 mmol/L, $p = 0.018$, Figure 3.3). The hemodynamic changes observed during this phase produced a logarithmic relationship between aortic systolic pressure and systemic oxygen

delivery following WLST (Figure 3.8). Consequently, aortic systolic pressure was maintained even after a precipitous decline in systemic oxygen delivery and the transition to anaerobic metabolism had occurred. In contrast, the relationship between arterial oxygen saturation and systemic oxygen delivery was linear (Figure 3.8).

Agonal phase (4 – 7 minutes)

Despite persistently elevated catecholamine levels, biventricular function (Figure 3.5) and cardiac output (Figure 3.2) declined rapidly during the agonal phase. During this time the RV continued to experience marked distention compared to the LV. The RV diastolic volume remained 22 ± 0% above baseline levels, while the LV diastolic volume remained 10 ± 0% below baseline levels (Figure 3.6). Similarly, the RV diastolic pressure was 2.5 times greater than that observed in the LV during the agonal phase (Figure 3.4). A progressive acidosis resulting from a rising lactate (2.7±0.2 vs. 4.1±0.3 mmol/L, $p<0.01$) and P_aCO_2 (68±2 vs. 81±4 mmHg, $p<0.01$) was also observed (Figure 3.3).

Circulatory arrest phase (7 – 8 minutes)

A pulse pressure on the arterial pressure tracing was no longer evident 7.6±0.3 minutes following WLST, and the time to circulatory arrest was comparable between RV and LV hearts (RV hearts: 7.8±0.4 vs. LV hearts: 7.4±0.6, $p=0.66$). At this point the central venous pressure was greater than or equal to the mean arterial pressure in all animals, confirming that cessation of antegrade circulation had occurred. No hearts had developed ventricular tachycardia or fibrillation at the time circulatory arrest was declared. During this phase the RV diastolic volume was 18±0% above baseline levels and the LV diastolic volume remained 10±0% below baseline levels (Figure 3.6). This difference in ventricular volume was confirmed using MRI, which demonstrated an RV volume 18±7% above baseline values and an LV volume 12±9% below baseline values (Figure 3.9).

Standoff phase (8 – 20 minutes)

The arterial lactate and P_aCO_2 continued to rise during the standoff period reaching values of 4.9±0.4 mmol/L and 91±5 mmHg, respectively, and resulting in a pH of 7.13±0.02 at 20 minutes following WLST (Figure 3.3). The right ventricle remained distended over the duration of the standoff period. Over the 20-minute period following WLST the net area under the RV volume curve was 3.5 fold greater than that of the LV (Figure 3.6). Similarly, the area under the RV pressure curve was 1.9 fold greater than that observed in the LV (Figure 3.4).

Ventricular fibrillation developed in 3 hearts at 15±1 minutes following WLST, 1 heart developed ventricular standstill despite persistent atrial activity 13.5 minutes following WLST, and the remaining hearts exhibited organized ventricular electrical activity over the duration of the 20-minute observation period. No mechanical autoresuscitation events were observed during the standoff phase.

In the time following donor extubation the concentrations of troponin-I, tumor necrosis factor alpha, interleukin-6, and triiodothyronine did not change over the duration of the 20-minute observation period (Table 3.1, Figure 3.7B).

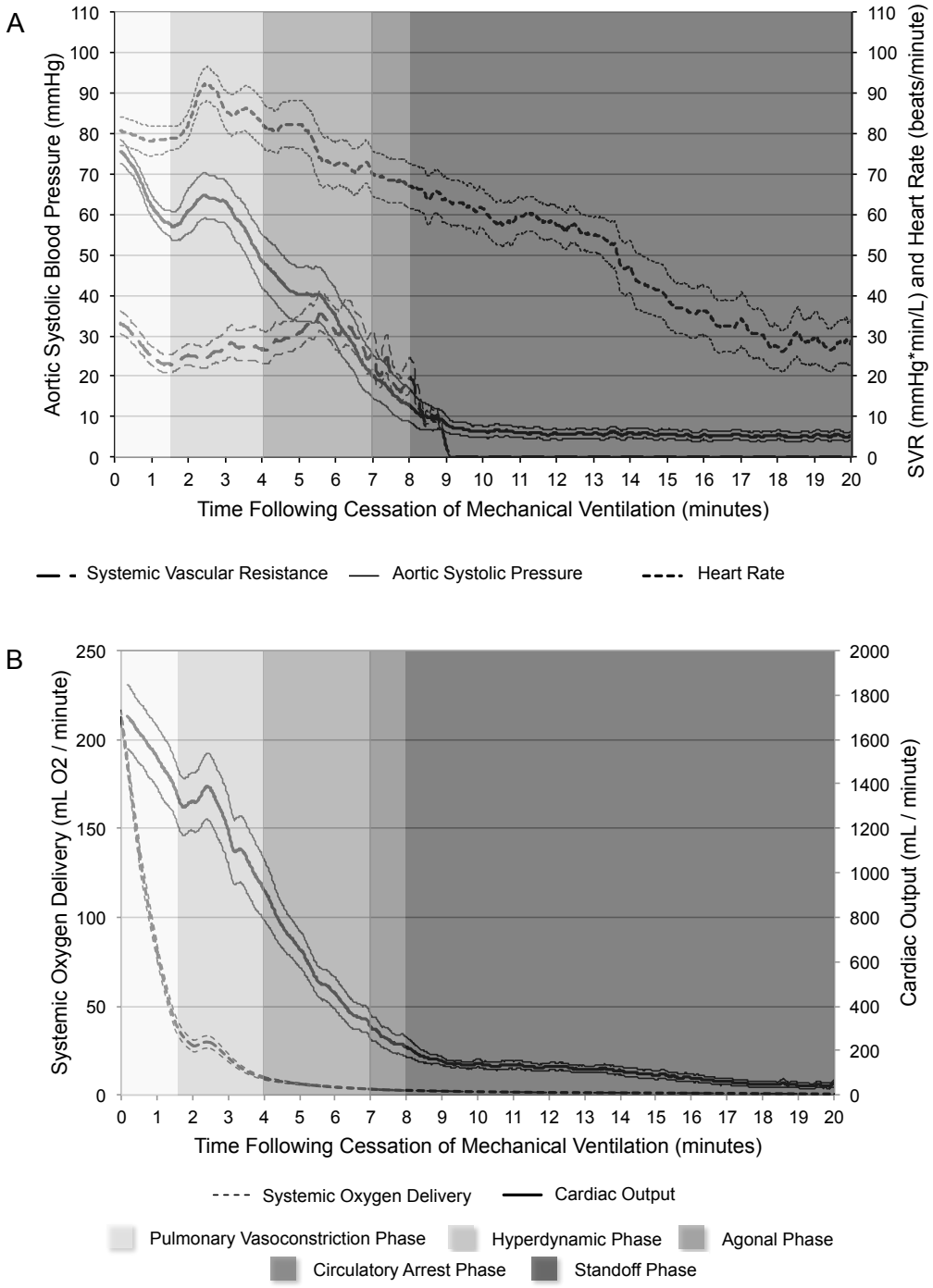

Figure 3.2. A) Aortic systolic pressure, systemic vascular resistance, heart rate, and B) cardiac output and systemic oxygen delivery categorized according to the physiologic changes occurring following cessation of mechanical ventilation. Values presented as mean ± standard error of the mean.

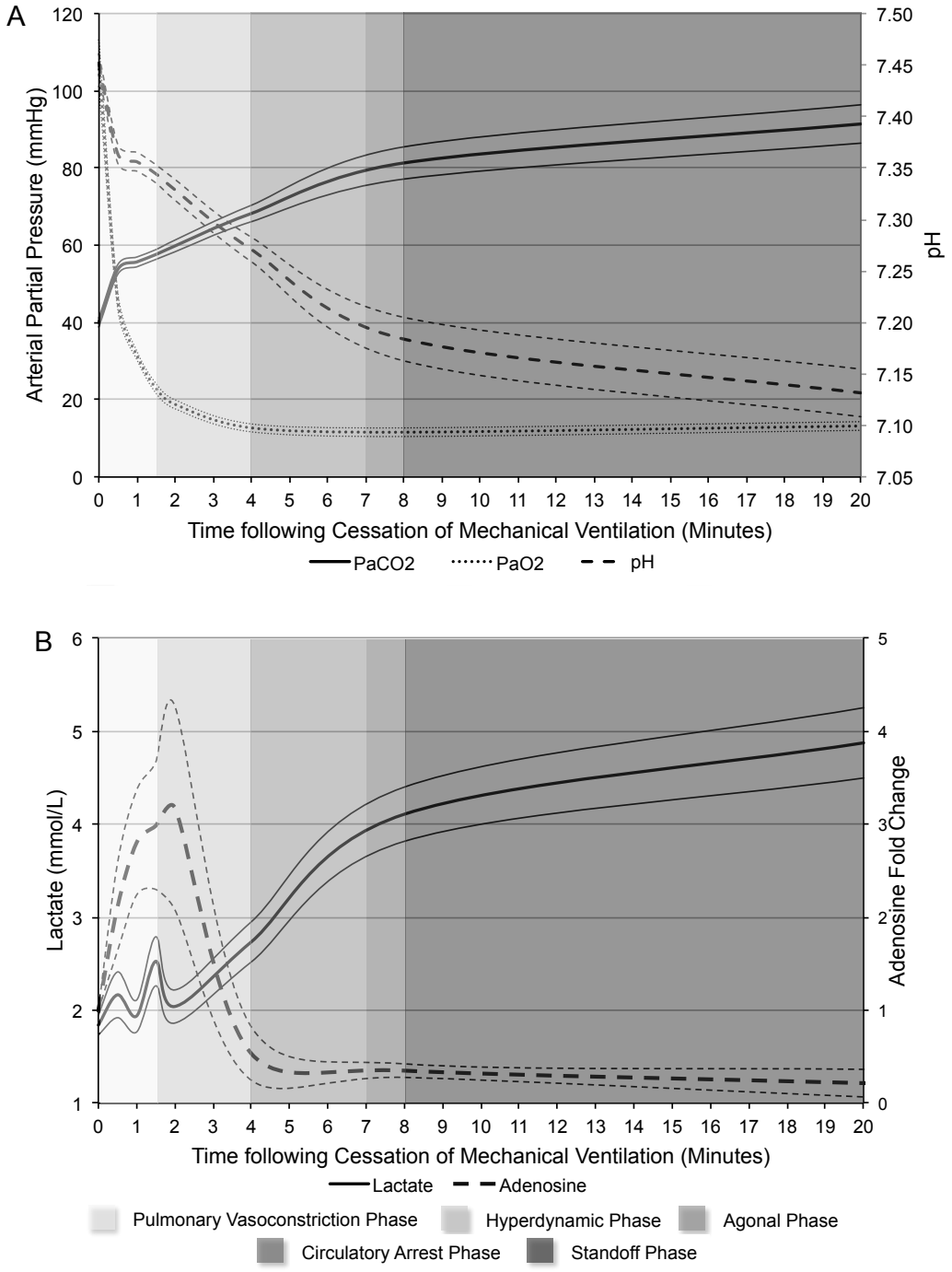

Figure 3.3. A) pH, partial pressure of oxygen (P_aO_2) and carbon dioxide (P_aCO_2), B) lactate and adenosine levels present in arterial blood samples, categorized according to the physiologic changes occurring following cessation of mechanical ventilation.

Values presented as mean ± standard error of the mean.

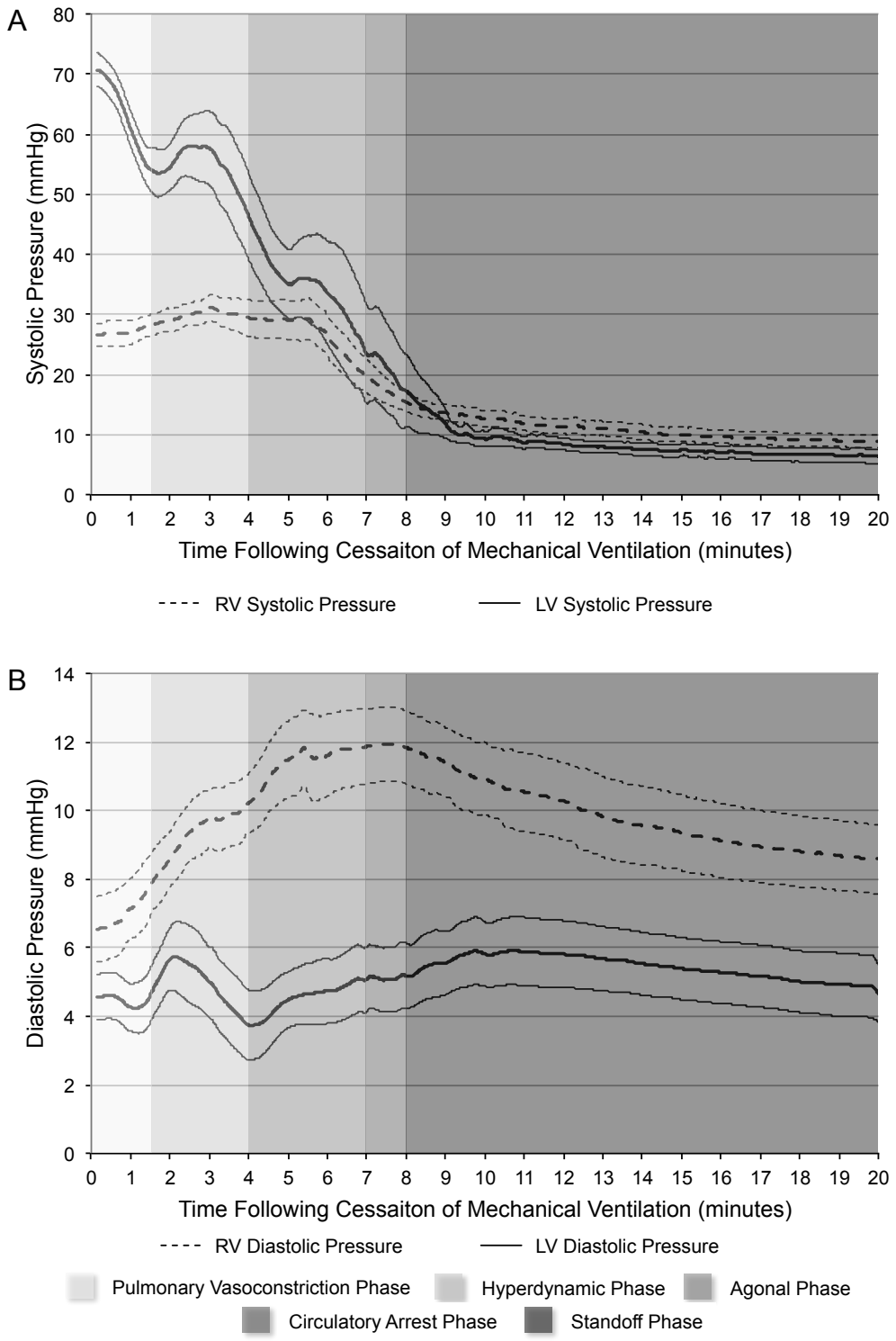

Figure 3.4. A) Systolic and B) diastolic pressures in the left and right ventricle categorized according to the physiologic changes occurring following cessation of mechanical ventilation. Values presented as mean ± standard error of the mean.

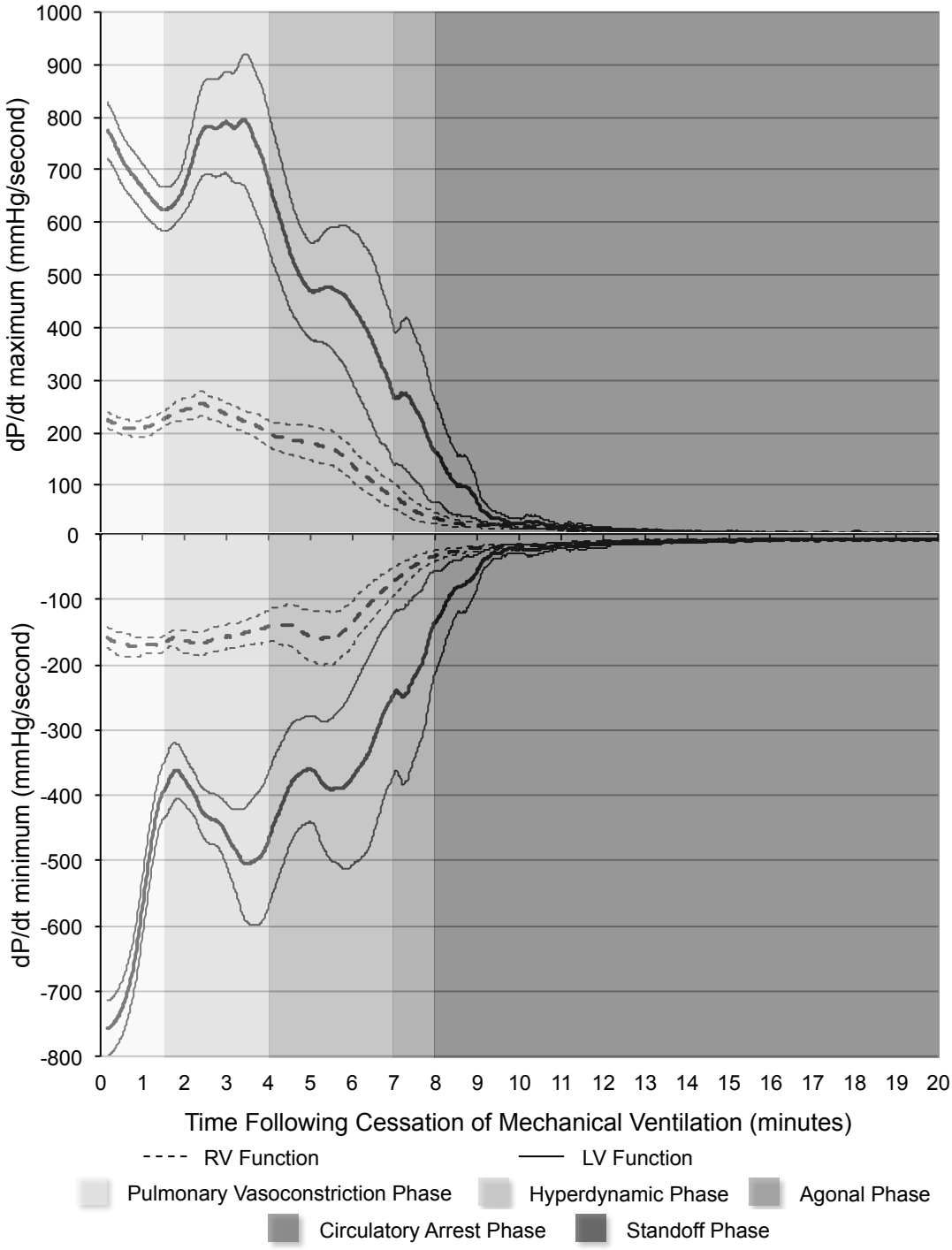

Figure 3.5. A) Systolic (dP/dt maximum) and B) diastolic (dP/dt minimum) function in the left and right ventricle categorized according to the physiologic changes occurring following cessation of mechanical ventilation. Values presented as mean ± standard error of the mean.

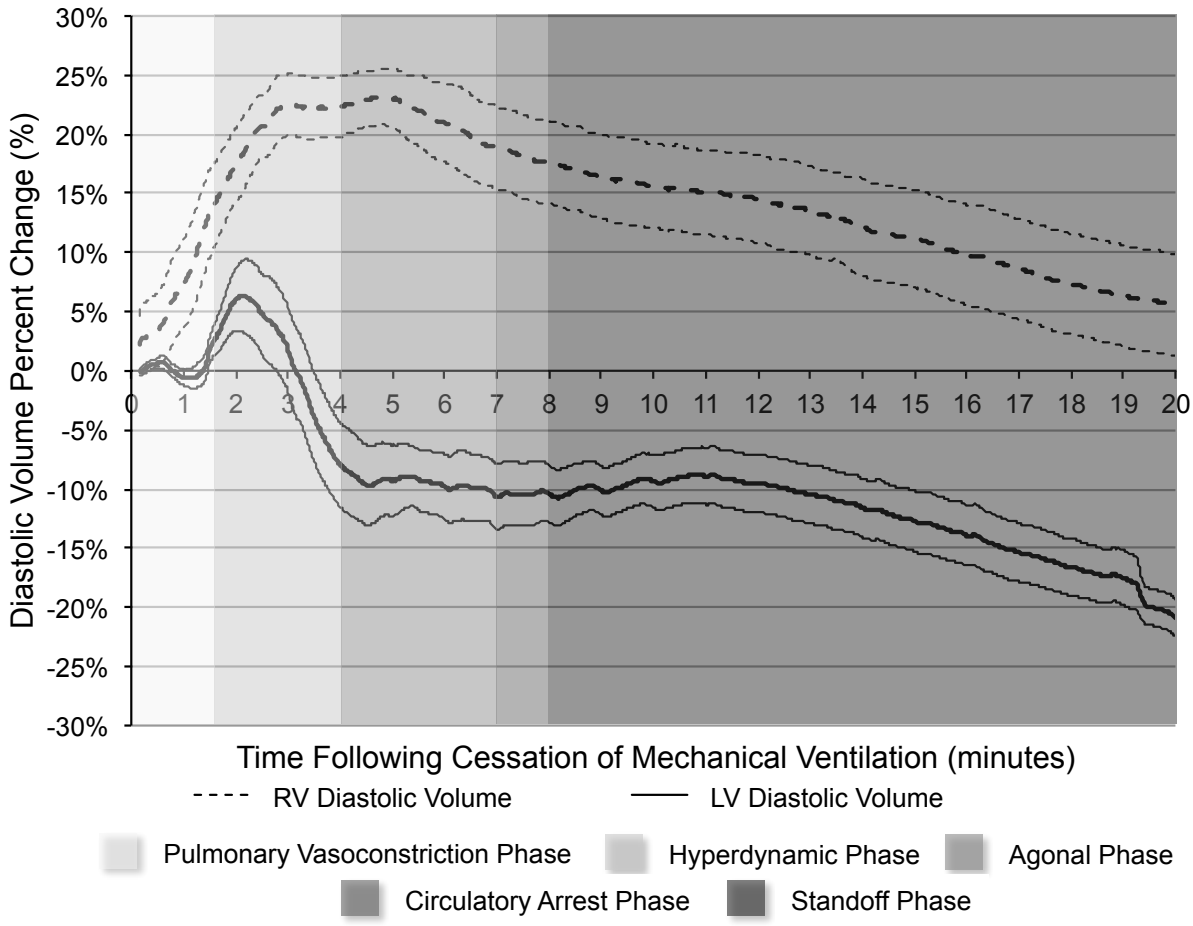

Figure 3.6. Percent change in right and left ventricular diastolic volume, categorized according to the physiologic changes occurring following cessation of mechanical ventilation. Values presented as mean ± standard error of the mean.

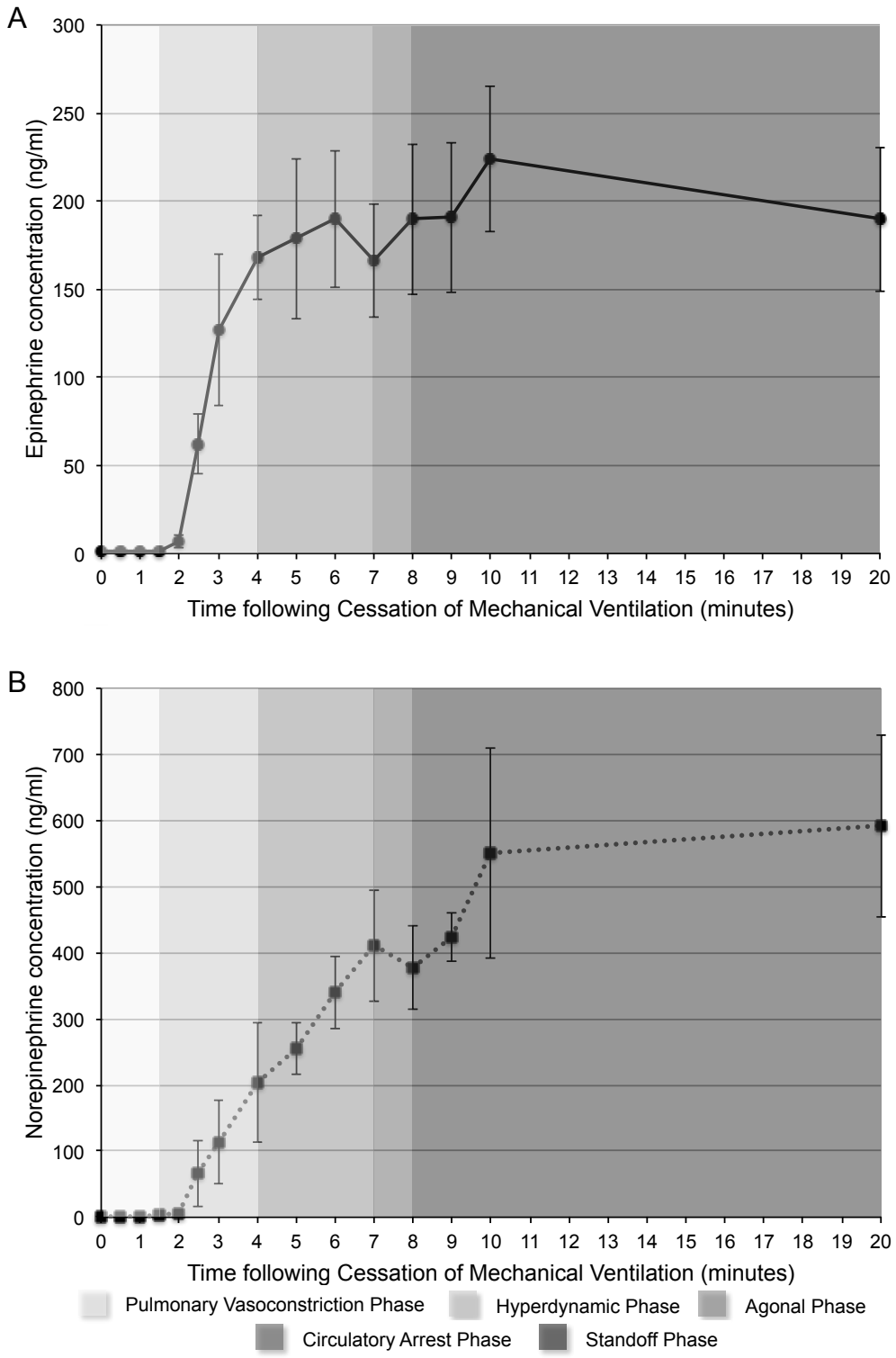

Figure 3.7. A) Epinephrine and B) norepinephrine in arterial blood samples, categorized according to the physiologic changes occurring following cessation of mechanical ventilation. Values presented as mean ± standard error of the mean.

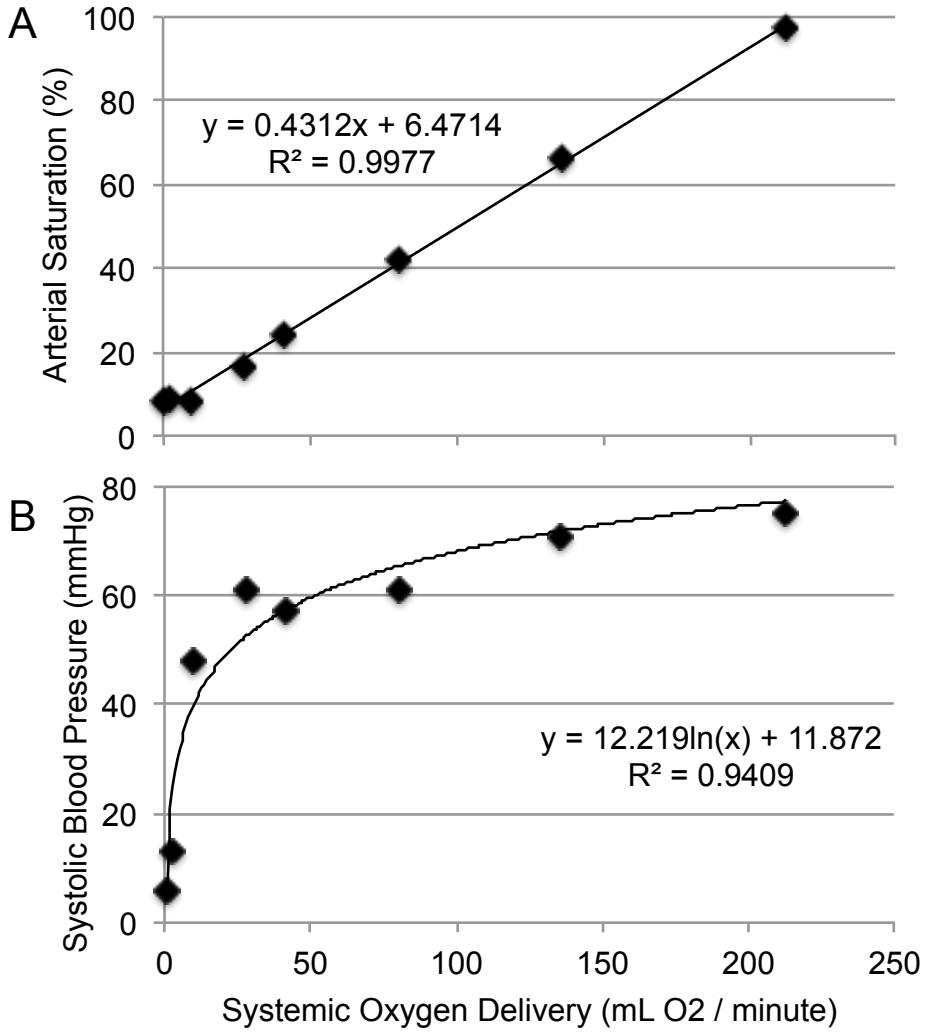

Figure 3.8. Relationship between systemic oxygen delivery and A) arterial oxygen saturation and B) systolic blood pressure following cessation of mechanical ventilation (N=20).

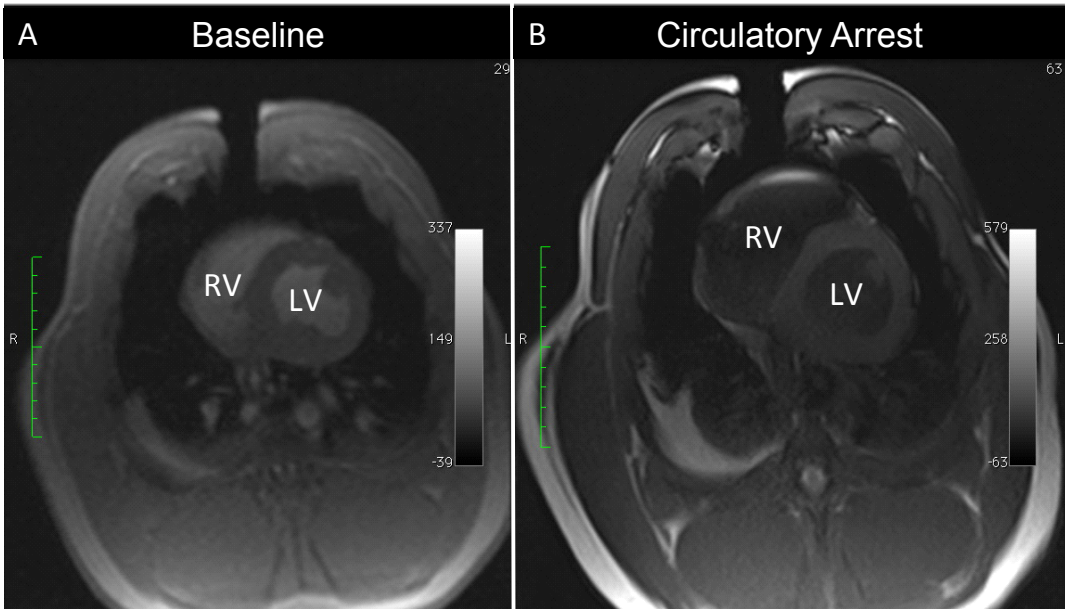

Figure 3.9. Magnetic resonance images demonstrating right and left ventricular volumes A) at baseline prior to cessation of mechanical ventilation and B) after declaration of circulatory arrest.

Discussion

The number of organs transplanted from DCD donors is increasing in many countries (10). A greater understanding regarding the physiologic processes that occur following WLST is important to establish accepted medical practices for DCD and optimize the resuscitation and evaluation of these organs prior to transplantation. Rhee et al. (18) have previously characterized the withdrawal phase in the context of abdominal transplantation; however, data regarding the impact of WLST on the donor heart is lacking. This is of particular relevance given the recent evidence suggesting that hearts donated after circulatory arrest can be resuscitated and successfully transplanted (3, 4, 7). We have now characterized the physiologic changes that occur following extubation in the DCD donor and have identified aspects that have relevance not

only in the context of heart transplantation, but also in the development of standardized medical practices for DCD.

An increase in pulmonary vascular resistance was observed following WLST (RV systolic pressure increased while cardiac output and LV diastolic pressure decreased). Precapillary pulmonary arterioles that supply blood to hypoxic regions of lung parenchyma have been shown to vasoconstrict to optimize ventilation-perfusion matching, a response that is augmented in the setting of concomitant hypercapnic acidosis (19, 20). Since we observed significant hypoxemia and hypercapnic acidosis following cessation of mechanical ventilation, it is not surprising that these changes were associated with an increase in pulmonary vascular resistance. Other authors have also observed an increase in pulmonary artery pressure following cessation of mechanical ventilation in canine and porcine models of DCD (14, 15).

The increase in pulmonary vascular resistance observed during the pulmonary vasoconstriction phase was associated with increased RV diastolic volume and pressure. These results suggest that the elevated RV afterload resulted in a decline in RV stroke volume, leading to the development of RV distension. The decrease in pulmonary blood flow resulting from these changes may account for the decline in cardiac output and systemic blood pressure observed initially following donor extubation. Obeid et al. (21) also demonstrated that once the arterial PO_2 decreased below 100 mmHg, the central venous pressure increased and the pulmonary artery pressure was maintained, while the left atrial pressure, pulmonary artery blood flow, and systemic blood pressure decreased. Their results combined with our observations suggest that hypoxic pulmonary vasoconstriction may contribute to the RV distension and decline in cardiac output we observed following WLST.

The hyperdynamic phase was initiated by a profound catecholamine surge that resulted in a transient increase in heart rate, biventricular function, cardiac output, and systemic vascular resistance, and produced an increase in RV, LV and aortic systolic pressure. In our study, activation of the sympathetic nervous system and catecholamine release appeared to be

associated with the decrease in systemic aortic pressure (baroreceptor response) and the worsening hypoxemia and hypercarbia (chemoreceptor response) that developed during the previous phase. Other authors employing a similar large animal model have noted a transient hyperdynamic phase following donor extubation; however, in these reports similar changes were observed without a preceding decrease in systemic blood pressure (15, 21, 22). This suggests that activation of the peripheral chemoreceptors in response to progressive hypoxemia and/or hypercarbia may be an important mediator of the catecholamine release that occurs following WLST.

Despite a positive inotropic, lusitropic, and chronotropic response during the hyperdynamic phase, RV diastolic volume and pressure continued to increase. This may reflect an increase in venous return to the RV. The majority of the total blood volume is contained within the venous capacitance system (23). A high density of alpha-adrenergic receptors distributed throughout the media of this system makes it highly sensitive to stimulation by the sympathetic nervous system, which would cause a decrease in venous capacitance and an increase in RV preload (23-25). However, the degree to which an increase in venous return can augment LV preload and systemic cardiac output in the DCD context may be limited by the presence of persistently elevated pulmonary vascular resistance. Therefore, following donor extubation the RV becomes progressively more distended due to increased venous return and elevated pulmonary vascular resistance, which are mediated by activation of the sympathetic nervous system and hypoxic pulmonary vasoconstriction responses, respectively.

RV distension following donor extubation may have a significant impact on post-transplant function. Kato et al. (14) have demonstrated that treatment with an endothelin-1 antagonist decreased the pulmonary artery pressure observed following donor extubation and improved the post-transplant function of DCD hearts, while some hearts from the control group demonstrated predominant RV failure following transplant and could not be weaned from cardiopulmonary bypass (14). Other authors have also observed predominant RV dysfunction in

some DCD hearts following experimental transplantation (3). This is not surprising since ventricular distension increases wall stress, decreases coronary perfusion pressure, and negatively impacts the recovery of myocardial function following ischemia (26). These results highlight the importance of assessing RV function prior to transplantation to minimize the risk of post-transplant graft failure; however, significant advancements in currently available ex vivo heart perfusion technology are required before such functional assessments could be undertaken in the clinical arena. Interestingly, Dhital et al. noted significant RV dysfunction in one human DCD heart upon initiation of ex vivo perfusion; however, RV function improved during the preservation interval and following transplantation extracorporeal membrane oxygenation was required only for LV failure (7). The other two human DCD hearts described in this case series exhibited good RV function post-transplant (7). Further studies are required to determine the clinical significance of RV distension following donor extubation.

The catecholamine surge associated with the hyperdynamic phase may have detrimental effects on the DCD heart. Myocardial dysfunction resulting from catecholamine release in the setting of elevated intracranial pressure and the progression to brain death is a well described phenomenon (27). Experimental exposure to catecholamines has been shown to cause ATP depletion, lactate accumulation, and contraction band necrosis within 30-minutes (28, 29). Catecholamine exposure following brain death is also associated with desensitization of the beta-receptor and myocardial dysfunction (30). Interestingly, the magnitude of catecholamine release in the DCD context may be greater than that observed following the progression to brain death (17). Therefore, the warm ischemic injury, ventricular distension, and profound catecholamine surge that occur following WLST make assessments of organ viability prior to transplant particularly important.

Ex vivo perfusion has been used clinically to minimize the exposure of DCD hearts to cold ischemic injury during organ preservation and facilitate successful transplantation (7). Current clinical protocols require the collection of 1.5 L of donor whole blood for priming of the

ex vivo heart perfusion circuit (7). While an oxygen carrier is required to meet myocardial metabolic demands during ex vivo perfusion (31), the current approach re-exposes the vulnerable DCD heart to significantly elevated catecholamine levels once it is connected to the ex vivo perfusion device. Previous work has suggested that ex vivo perfusion with a whole blood-based perfusate improves the preservation of donor heart function; however, the blood used in these experiments was not obtained from brain-dead or DCD donors and would likely not have contained elevated levels of catecholamines (31). The authors suggest that a washed donor red-blood cell concentrate combined with banked plasma may provide a whole blood equivalent that could be used during ex vivo heart perfusion (31). The efficacy of this approach for the prevention of catecholamine-mediated injury has not been confirmed experimentally.

The systemic oxygen delivery declined precipitously following WLST, owing to a significant decline in arterial oxygen saturation and cardiac output. These changes were associated with an increase in the concentration of adenosine, which has been previously associated with the oxygen supply/demand and [ATP]/[ADP]+[Pi] ratios (32). The increase in adenosine observed in the present study likely represents the hydrolysis of available ATP stores in the setting of critical hypoxemia and insufficient oxygen delivery to meet metabolic demands. Interestingly, the increase in cardiac output during the hyperdynamic phase did not appreciably increase systemic oxygen delivery because of the extremely low oxygen content present in the arterial blood. The increasing lactate concentration observed during this phase also suggests that the onset of functional warm ischemia was not delayed by the period of hyperdynamic circulation and recovery of systemic blood pressure. However, many transplant programs rely exclusively on hemodynamic criteria to define the onset of the functional warm ischemic time (American Society of Transplant Surgeons: mean arterial pressure <60 mmHg, United Kingdom: systolic blood pressure <50 mmHg) (11). The logarithmic relationship we observed between aortic systolic blood pressure and systemic oxygen delivery suggest that blood pressure can be maintained even after a critical decline in systemic oxygen delivery and the onset of organ

ischemia have occurred (systemic oxygen delivery declined to <10 mL O_2/minute by the time the systolic blood pressure declined to < 50 mmHg). In contrast, we observed a linear relationship between arterial oxygen saturation and systemic oxygen delivery. These data suggest that the arterial oxygen saturation provides useful information regarding end-organ oxygen delivery and should be included in the criteria that indicate the onset of warm ischemia following WLST.

The changes in systemic oxygen delivery following WLST have important ethical implications in the context of DCD. First, all hearts exhibited organized ventricular electrical activity at the time circulatory arrest was declared; however, it was clear that no antegrade circulation was present and no oxygen delivery was occurring. In an observational study of human patients undergoing WLST, Dhanani et al. (12) have also observed that electrocardiographic activity persists after declaration of circulatory arrest in the majority of patients. Our results support the opinion that the standoff period should commence when mechanical asystole (no pulse pressure on an arterial pressure tracing) has occurred, and that waiting for electrical asystole lacks a sound physiologic rationale. Second, we did not observe any autoresuscitation events following declaration of circulatory arrest. Dhanani et al. (12) observed a transient return of arterial blood pressure activity in 4 (13%) patients following declaration of circulatory arrest; however, the pulse pressure amplitude observed is unlikely to have actually been associated with cerebral perfusion (33). The observation of arterial blood pressure activity following declaration of circulatory arrest has generated extensive ethical debate regarding the minimum warm ischemic standoff period that must be observed before organ procurement can proceed. Autoresuscitation is defined as the spontaneous resumption of cardiopulmonary activity after circulatory arrest (34); therefore, the detection of arterial blood pressure activity alone does not signify an autoresuscitation event unless it produces clinically relevant cerebral oxygen delivery. This is highly unlikely unless a simultaneous resumption of respiratory activity occurs, given the extremely low oxygen content present in arterial blood during the standoff period (P_aO_2 = 11-13 mmHg, arterial saturation = 8-9%). There is a

desperate need for research investigating the clinical relevance of mechanical cardiac activity following declaration of circulatory arrest to clarify these ethical issues.

Study limitations

We have described the physiologic changes that occur following WLST in a large animal model of DCD; however, extrapolation of these findings to clinical practice may be limited by a number of variables. Our model of DCD utilized healthy donor animals, yet the majority of donors have suffered a devastating neurologic injury prior to WLST. The impact of donor trauma, cardiac arrest, and neurologic injury on the physiologic response to extubation is unknown. Following WLST in our model the animals experienced complete apnea and progressed to circulatory arrest in a short period of time. In contrast, DCD donors in the clinical context may exhibit persistent respiratory efforts for a variable period of time before progression to hypoxemic circulatory arrest. The impact of such respiratory efforts on the physiologic changes that occur following donor extubation requires further study. We quantified changes in various circulating factors following donor extubation; however, cessation of circulation may have limited our ability to detect changes in these factors at later time points. Finally, changes in pulmonary vascular resistance have been inferred based on changes in right ventricular systolic pressure, left ventricular diastolic pressure, and cardiac output; however, pulmonary vascular resistance was not directly calculated in this study.

Conclusions

Following donor extubation in a large animal model of DCD, the heart is forced to function in an increasingly hypoxemic environment while attempting to maintain systemic oxygen delivery. Hypoxic pulmonary vasoconstriction and a profound increase in circulating catecholamines cause marked distension of the RV and may exacerbate myocardial injury in the DCD heart. These changes should be considered when protocols for donor heart resuscitation,

preservation, and evaluation are being developed. The severity of ischemic injury sustained by the DCD heart prior to organ procurement necessitates that the composition of the cardioplegic solution and the conditions of its delivery be optimized to limit the severity of reperfusion injury.

Chapter 3 Summary

In Chapter 3: *Physiologic changes in the heart following cessation of mechanical ventilation in a porcine model of donation after circulatory death: implications for cardiac transplantation*, a porcine model of DCD was described. This chapter provides insight into aspects of the physiologic response to donor extubation that have particular relevance to the context of heart transplantation. The hemodynamic data suggests that the donor animal progresses through distinct physiologic phases following extubation that may have important implications for optimizing the resuscitation, preservation, and evaluation of DCD hearts for transplantation. The DCD heart is forced to function in an increasingly hypoxemic environment while attempting to maintain systemic oxygen delivery. During this time the heart is exposed to a profound catecholamine surge and the right ventricle experiences significant distension. Therefore, prior to organ procurement the DCD heart suffers significant injury, and the approach to donor heart resuscitation, preservation, and evaluation must be tailored specifically to the DCD context. For example, donor heart resuscitation should focus on methods to minimize ischemia-reperfusion injury at the time of organ procurement. Further, utilizing donor whole blood during *ex vivo* preservation may be detrimental due to the high levels of catecholamines that accumulate during the circulatory arrest process. Finally, evaluation of donor heart function prior to transplantation is essential to select viable organs for transplantation, given the significant ischemic insult and right ventricular distention experienced by the donor heart following donor extubation.

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Chapter 4

A cardioprotective preservation strategy employing *ex vivo* heart perfusion facilitates successful transplant of donor hearts after cardiocirculatory death

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Chapter 4 Preface

In Chapter 3, the physiologic injury sustained by the DCD heart following donor extubation was described. At the time of donor heart procurement, the DCD heart has been exposed to a massive catecholamine surge and sustained a significant ischemic insult. In contrast, DBD donors have an intact circulatory system and the heart has not experienced such injurious conditions. It is therefore likely that the approach employed in the preservation of DBD hearts will not sufficiently resuscitate and preserve hearts procured from DCD donors. Chapter 4: *A cardioprotective preservation strategy employing ex vivo heart perfusion facilitates successful transplant of donor hearts after cardiocirculatory death*, serves to establish DCD heart transplantation as a 'proof-of-concept'. In addition, data presented in this chapter suggests that employing an approach to donor heart resuscitation and preservation that is tailored specifically to the DCD context optimizes post-transplant outcomes.

Abstract

Background: *Ex vivo* heart perfusion (EVHP) has been proposed as a means to facilitate the resuscitation of donor hearts after cardiocirculatory death (DCD) and increase the donor pool. However, the current approach to clinical EVHP may exacerbate myocardial injury and impair post-transplant function. Therefore, we sought to determine if a cardioprotective EVHP strategy that eliminates myocardial exposure to hypothermic, hyperkalemia cardioplegia and minimizes cold-ischemia could facilitate successful DCD heart transplantation.

Methods: Anesthetized pigs sustained a hypoxic cardiac arrest and 15-minute warm-ischemic standoff period. Strategy 1 hearts (S1, N=9) underwent initial reperfusion with a cold hyperkalemic cardioplegia, normothermic EVHP, and transplantation following a cold hyperkalemic cardioplegic arrest (current EVHP strategy). Strategy 2 hearts (S2, N=8) underwent initial reperfusion with a tepid adenosine-lidocaine (A-L) cardioplegia, normothermic EVHP, and transplantation with continuous myocardial perfusion (cardioprotective EVHP strategy).

Results: At completion of EVHP, S2 hearts exhibited less weight gain (S2=9.7±6.7 vs. S1=21.2±6.7 grams/hour, p=0.008) and less troponin-I release into the coronary sinus effluent (S2=4.2±1.3 vs. S1=6.3±1.5 ng/mL, p=0.014). Analysis of oxidized phosphatidylcholines in post-transplant myocardium by mass spectrometry revealed less oxidative stress in S2 hearts. Thirty-minutes following wean from cardiopulmonary bypass, post-transplant systolic (PRSW: S2=33.5±1.3 vs. S1=19.7±10.9, p=0.043) and diastolic (Tau: S2=42.9±6.7 vs. S1=65.2±21.1, p=0.020) function were superior in S2 hearts.

Conclusion: In this experimental model of DCD, an EVHP strategy employing initial reperfusion with a tepid A-L cardioplegia and continuous myocardial perfusion minimizes myocardial injury and improves short-term post-transplant function compared to the current EVHP strategy employing cold hyperkalemic cardioplegia prior to organ procurement and transplantation.

Introduction

Despite a growing number of patients awaiting cardiac transplantation, the number of transplants being performed annually remains static (1). *Ex vivo* heart perfusion (EVHP) is currently under clinical investigation as a means to optimize utilization of donation after brain death hearts (2). EVHP also facilitates the delivery of pharmaceutical agents that support reparative processes in ischemic myocardium and the assessment of organ function prior to transplantation (3-5). This may permit the resuscitation of hearts from donation after cardiocirculatory death (DCD) donors and increase the number of organs available for transplantation (4-7).

The DCD heart sustains an obligatory hypoxic cardiac arrest and warm-ischemic standoff period prior to organ procurement (8). Subsequent reperfusion initiates a complex cascade of events that generate intracellular Ca^{2+} overload, reactive oxygen species (ROS), and an inflammatory response that result in myocardial injury (9-11). The presumed severity of this ischemia-reperfusion injury (IRI) has precluded clinical transplantation of DCD hearts (12). However, cardioprotective interventions delivered upon reperfusion can significantly reduce IRI (13). Therefore, an EVHP strategy that optimizes initial reperfusion conditions and limits myocardial exposure to additional ischemia prior to transplantation may facilitate successful resuscitation of DCD hearts (7, 14).

The current approach to EVHP employed clinically for the preservation of brain dead hearts involves administration of a hypothermic, hyperkalemic preservation solution at the time of organ procurement (15); however, hyperkalemia has no cardioprotective properties and may actually exacerbate IRI (16-18). This approach also involves a hypothermic, hyperkalemic arrest following completion of the preservation interval to facilitate transplantation, exposing the myocardium to additional cold-ischemia (19). While this approach may provide adequate myocardial protection for brain dead hearts that have experienced a negligible period of warm

ischemia prior to organ procurement, it may limit the probability of resuscitating vulnerable DCD hearts.

Alternatively, a normokalemic adenosine-lidocaine (A-L) cardioplegia has been developed that avoids the detrimental effects of hyperkalemia. It is effective at tepid temperatures and permits organ procurement and preservation without myocardial exposure to profound hypothermia (20-22). Additionally, the use of continuous myocardial perfusion during heart transplantation can eliminate exposure to a second period of cold-ischemia prior to transplantation (14). Therefore, we sought to determine if a novel EVHP strategy employing initial reperfusion with a tepid A-L cardioplegia and continuous myocardial perfusion during transplantation could minimize myocardial injury and improve the post-transplant function of DCD hearts, compared to the current EVHP strategy employed clinically.

Materials and Methods

Institutional Animal Care Committees approved the experimental protocol. Thirty-four female domestic pigs (52.2±6.7 kg) were divided into 17 donor-recipient pairs and allocated to 2 EVHP strategies: Strategy 1/Current EVHP (S1, N=9), Strategy 2/Cardioprotective EVHP (S2, N=8).

Circulatory Arrest, Initial Reperfusion, and Donor Heart Procurement

Premedication was undertaken with an intramuscular injection of midazolam, ketamine, and atropine. Orotracheal intubation and mechanical ventilation were established for maintenance of general anesthesia using isoflurane. A median sternotomy was performed and 400 units/kg of heparin were delivered intravenously. DCD was simulated as previously described (23). Briefly, animals were paralyzed with intravenous pancuronium, mechanical ventilation was discontinued, and hypoxic cardiac arrest ensued. Circulatory arrest was declared when there was no evidence of pulsation on the arterial pressure tracing (8) and central venous

pressure equaled the mean arterial pressure. Following observation of a 15-minute standoff period, a cross-clamp was placed across the ascending aorta and cardioplegic solutions were delivered antegrade into the aortic root (Figure 4.1).

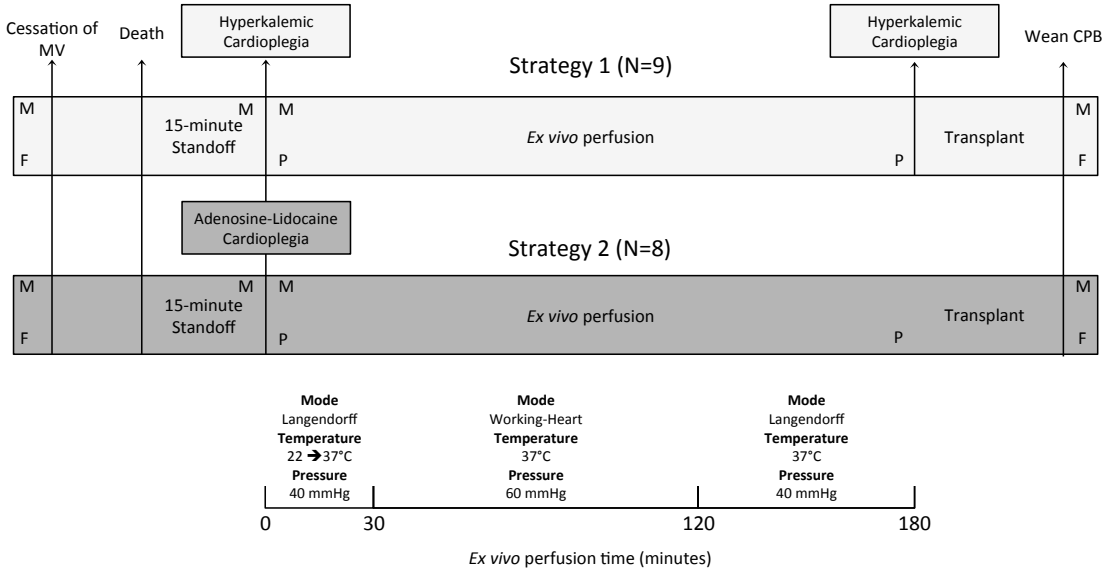

Figure 4.1. Experimental protocol.

CPB, cardiopulmonary bypass; F, myocardial functional assessment; MV, mechanical ventilation; M, myocardial biopsy; P, plasma sample.

Experimental groups

S1 (Current EVHP)

There were 9 donor-recipient pairs allocated to S1. Hearts were reperfused with hypothermic (4°C), hyperkalemic cardioplegia containing 100 mL of St Thomas' Hospital solution No. 2 (Plegisol, Hospira Inc., Illinois, USA) and 400 mL of blood. The final potassium concentration was 15 mmol/L. The donor heart was excised and submerged in cold saline while anastomosis of the aortic, left atrial, and pulmonary arterial cannulas were completed. EVHP was then established and myocardial biopsies and plasma samples were obtained for subsequent analysis. Following completion of the EVHP interval, hearts were arrested with

hypothermic (4°C), hyperkalemic cardioplegia and transplanted into the recipient animal in the standard fashion (Figure 4.1).

S2 (Cardioprotective EVHP)

There were 8 donor-recipient pairs allocated to S2. Hearts were reperfused with a tepid (22°C), normokalemic A-L cardioplegia (20) containing 200 µmol/L adenosine (Adenocard, Astellas Pharma Canada, Ontario, Canada) and 500 µmol/L lidocaine (Stevens Drugs, New Jersey, USA) in 500 mL of STEEN solution (Vitrolife Inc., Colorado, USA). The final potassium concentration was 5 mmol/L. The donor heart was excised and maintained at 22°C while anastomosis of the aortic cannula was completed. Anastomosis of the left atrial and pulmonary arterial cannulas were accomplished following initiation of EVHP. Myocardial biopsies and plasma samples were obtained for subsequent analysis. Hearts were maintained in a beating state with continuous EVHP during transplantation (Figure 4.1).

Ex vivo heart perfusion

All hearts underwent EVHP with a perfusate consisting of 1.5 L of STEEN solution and 0.5 L of autologous blood. STEEN solution is a buffered extracellular-type salt solution with human serum albumin and dextran 40, producing an osmotic pressure optimized for *ex vivo* perfusion (24). FiO_2 and gas flow through the membrane oxygenator were titrated to maintain a pH of 7.35-7.45, PaO_2 of 300-400 mmHg, and $PaCO_2$ of 35-45 mmHg. The hemoglobin concentration was 4.0-4.5 g/dL. A Medtronic Affinity NT oxygenator, venous reservoir, and BPX-80 BIO centrifugal pumps (Medtronic, Ontario, Canada) were used to construct EVHP circuit (Figure 4.2). Hearts were initially perfused in Langendorff mode at 22°C and a perfusion pressure of 40 mmHg. Over a 30-minute period, hearts were warmed to 37°C and the perfusion pressure was increased to 60 mmHg (14). Hearts were transitioned into working mode for 90 minutes and then reverted to Langendorff mode for the remainder of the EVHP interval (Figure 4.1).

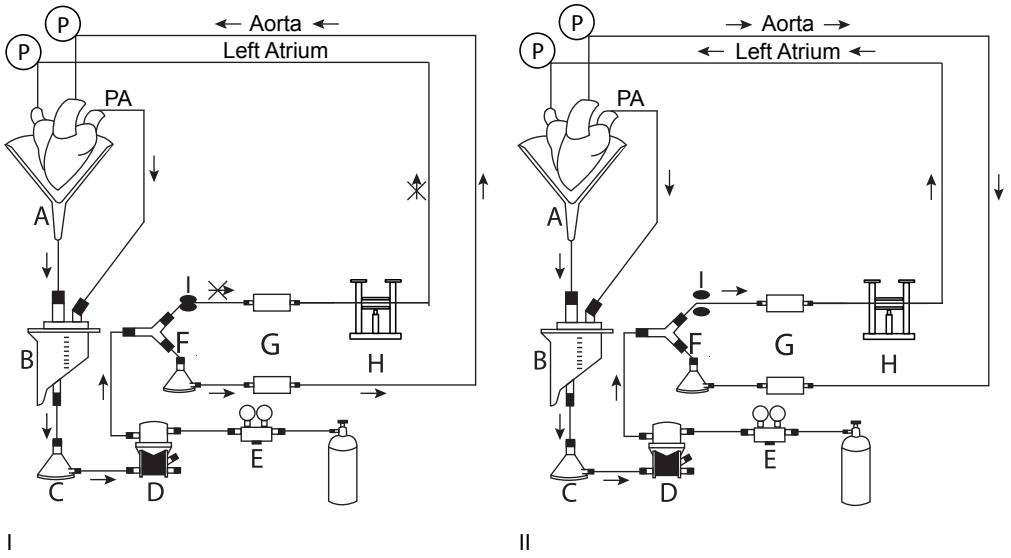

Figure 4.2. *Ex vivo* perfusion circuit. I) Langendorff perfusion. II) Working heart perfusion.

A, funnel to scavenge blood from inferior and superior vena cava; B, venous reservoir; C, centrifugal pump; D, oxygenator; E, oxygen supply; F, centrifugal pump; G, flow probe; H, left-atrial pressure control; I, occlusion at this point converts circuit from working heart to Langendorff mode; P, pressure transducer.

Orthotopic heart transplantation

Heart transplantation was performed as previously described (23). Briefly, the recipient animal was anesthetized, mechanically ventilated, heparinized, and placed on CPB. The recipient heart was excised and the donor heart was implanted using the standard bi-atrial technique. The donor heart was reperfused for 30 min, dobutamine and norepinephrine infusions were initiated, and animals were separated from CPB.

Assessment of myocardial injury

Myocardial edema

Hearts were emptied of blood and weighed at the beginning and end of EVHP. Total weight gained was normalized to the duration of the EVHP interval.

Troponin-I

Plasma samples were obtained from coronary sinus effluent at the beginning and end of EVHP. The concentration of troponin-I was determined using a Pig Cardiac Troponin-I ELISA Kit (Life Diagnostics, Pennsylvania, USA).

Oxidative stress

Oxidized phosphatidylcholine (OxPC) has been identified as a marker of oxidative stress in IR injury (25), and can be quantified using liquid chromatography and mass spectrometry. Lipid extraction from myocardial biopsies obtained at initiation of EVHP and post-transplant (Figure 4.1) was performed using a protocol adapted from Folch et al. (26) OxPC analysis was performed as previously described (27). Briefly, a normal-phase high-performance liquid chromatography (HPLC) of phospholipids was performed on a 2.7µm Supelco column in a SIL-20ACHT Prominence UFLC (Shimadzu, Kyoto, Japan) and eluted into a 4000 Q-TRAP® quadrupole linear ion trap hybrid mass spectrometer (Applied Biosystem/MDS Sciex, Ontario, Canada). The fold-change in 82 distinct OxPC molecules between myocardial biopsies obtained at initiation of EVHP and post-transplant were compared between groups using the Students *t*-test.

Histology

Hearts were fixed in 10% buffered formalin and transmural horizontal tissue specimens were obtained from the anterior and lateral walls of the left ventricle and embedded in paraffin. Histologic sections were stained with hematoxylin and eosin, and examined by one clinical pathologist (JK) for evidence of contraction bands, hypereosinophilic myocytes, interstitial neutrophils, and intravascular leukostasis. Sections of the left anterior descending coronary artery and vein were assessed for endothelial neutrophil infiltration.

Assessment of ventricular function

Ventricular function was assessed at baseline prior to cessation of mechanical ventilation and post-transplant (Figure 4.1) using a conductance catheter placed in the left ventricle (23). Load dependent measures were based on steady state data, with systolic and diastolic function assessed by maximum (dP/dt_{max}) and minimum (dP/dt_{min}) rate of pressure change in the ventricle, respectively. Load independent measurements were based on data collected following transient occlusion of the inferior vena cava. Systolic function was assessed by the end-systolic pressure volume relationship (ESPVR) and preload recruitable stroke work (PRSW). Diastolic function was assessed by the end-diastolic pressure volume relationship (EDPVR) and isovolumic relaxation constant (Tau).

Statistical Analysis

Normally distributed continuous variables were reported as mean ± standard deviation (SD) and compared using the Students *t*-test. Categorical variables were expressed as frequencies and compared using the Freeman-Halton extension of the Fisher exact test. A *p*-value <0.05 was considered statistically significant.

Results

Cardiocirculatory death, initial reperfusion, and EVHP

Time intervals during withdrawal of life sustaining therapy and EVHP are displayed in Table 4.1. The time from cessation of mechanical ventilation to determination of death and the warm ischemic standoff period observed prior to reperfusion were similar between S1 and S2 hearts. Following reperfusion, S1 hearts were placed in cold storage for 16.0±3.9 minutes while the heart was being prepared for EVHP, whereas S2 hearts sustained a 9.3±2.1 minute warm-ischemic period during preparation for EVHP (p<0.001). Total *ex vivo*, Langendorff, and working

mode perfusion times were similar between groups. There was no significant difference in perfusate pH, PaO_2, $PaCO_2$, or hemoglobin concentration between groups (data not shown).

Table 4.1. Time periods for withdrawal of life sustaining therapy and *ex vivo* heart perfusion

Time periods (minutes)	Strategy 1	Strategy 2	*p value*
Cessation of MV to death	9.9 (5.0)	7.2 (3.0)	0.182
Death to initial reperfusion (Standoff period)	16.4 (1.7)	16.0 (0.9)	0.488
Initial reperfusion to *ex vivo* heart perfusion	16.0 (3.9)	9.3 (2.1)	<0.001
Total *ex vivo* heart perfusion	156 (47)	190 (34)	0.100
Langendorff perfusion	86 (30)	98 (41)	0.510
Working mode perfusion	70 (27)	87 (38)	0.267

MV, mechanical ventilation

Myocardial Injury

Myocardial Edema

S2 hearts demonstrated less weight gain over the EVHP interval compared to S1 hearts (9.7±6.7 vs. 21.2±6.7 grams/hour, p=0.008; Figure 4.3), suggesting the development of less myocardial edema.

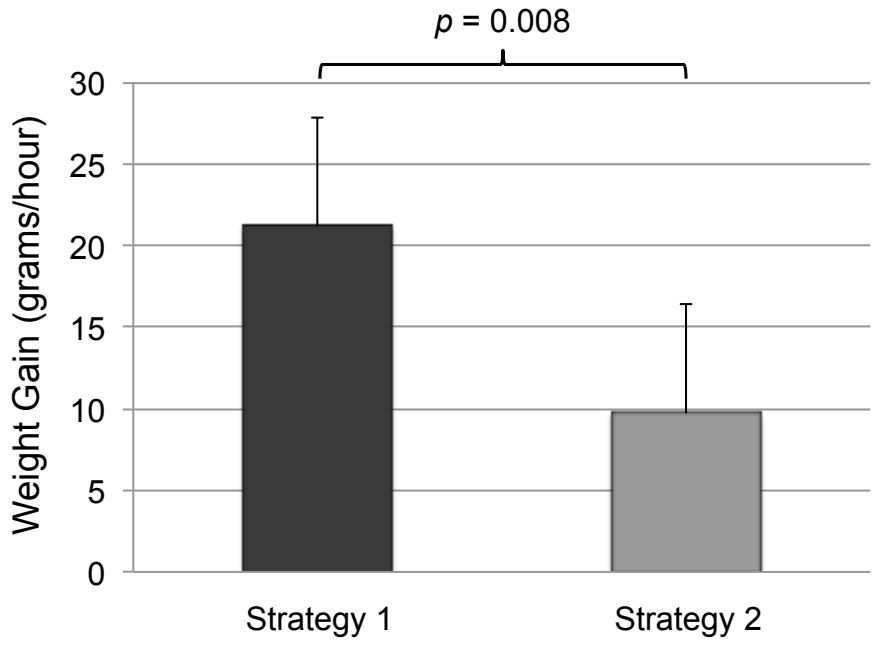

Figure 4.3. Development of myocardial edema over the *ex vivo* perfusion interval as evidenced by myocardial weight gain.

Troponin-I

S2 hearts exhibited less troponin-I in coronary sinus effluent at the start (0.7±0.6 vs. 2.1±1.7 ng/mL, p=0.047) and end (4.2±1.3 vs. 6.3±1.5 ng/mL, p=0.014) of EVHP (Figure 4.4).

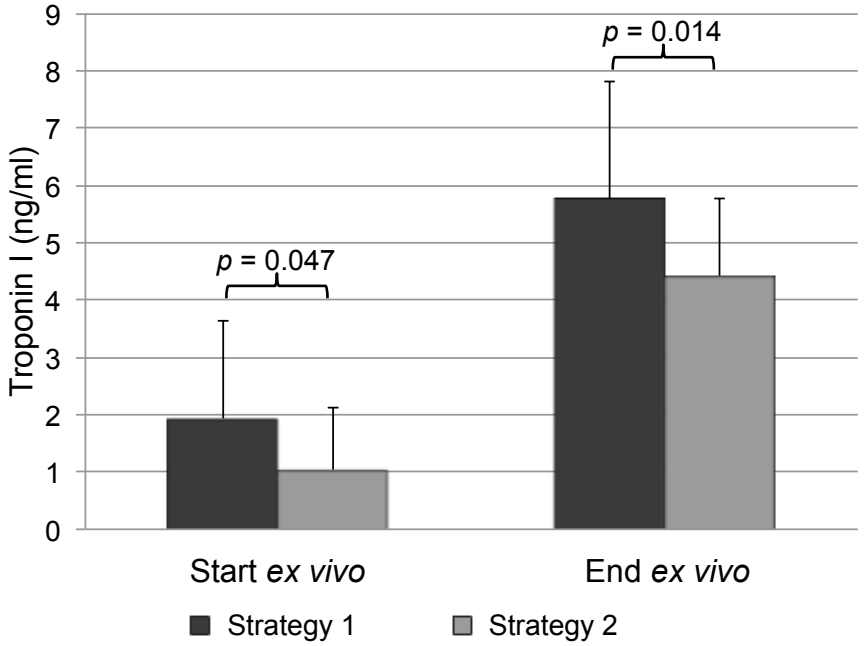

Figure 4.4. Concentration of troponin-I in coronary sinus effluent obtained at initiation and end of the *ex vivo* perfusion interval.

Oxidative Stress

The degree of oxidative stress was determined in a subset of 6 animals (S1, N=3; S2, N=3) by quantification of 82 OxPCs using mass spectrometry. Overall S1 hearts demonstrated a greater fold-increase in OxPCs between post-reperfusion and post-transplant myocardium compared to S2 hearts (S1=6.6±4.4 vs. S2=1.6±1.0 fold-increase, p<0.001). S1 hearts

demonstrated a significantly greater fold-increase in 70 (85%) of the distinct OxPC molecules examined (Figure 4.5).

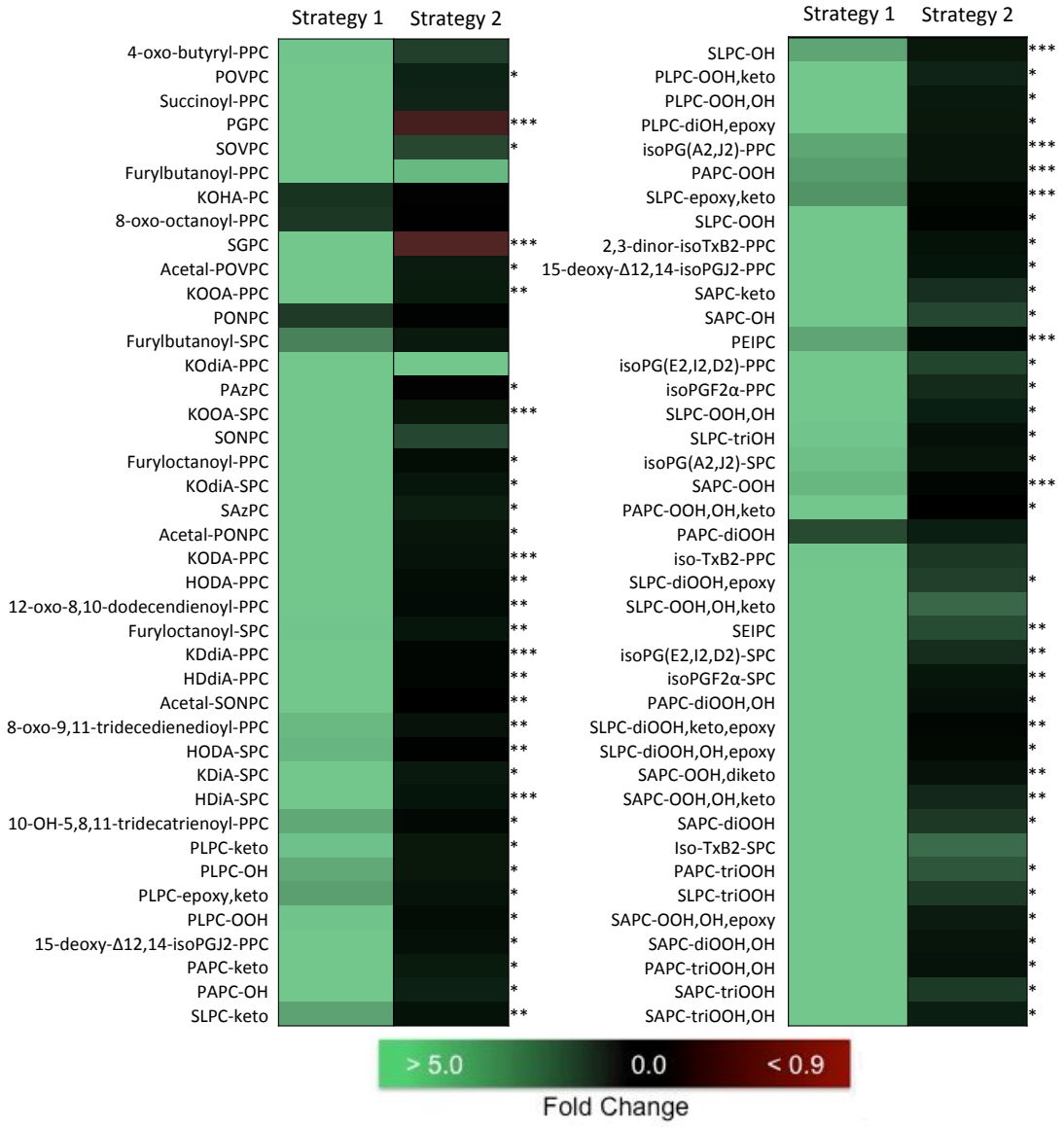

Figure 4.5. Heat map illustrating fold-change between post-reperfusion and post-transplant myocardium in 82 oxidized phosphatidylcholine compounds. The intensity of green or red represents the magnitude of increase or decrease in oxidized phosphatidylcholine compounds, respectively. $p<0.05$ *, $p<0.01$ **, $p<0.005$ ***

Histology

Hematoxylin and eosin stained myocardial specimens from representative animals obtained following completion of the post-transplant functional assessments are shown in Figure 4.6. Histological examination revealed greater evidence of cardiomyocyte and endothelial injury in S1 hearts.

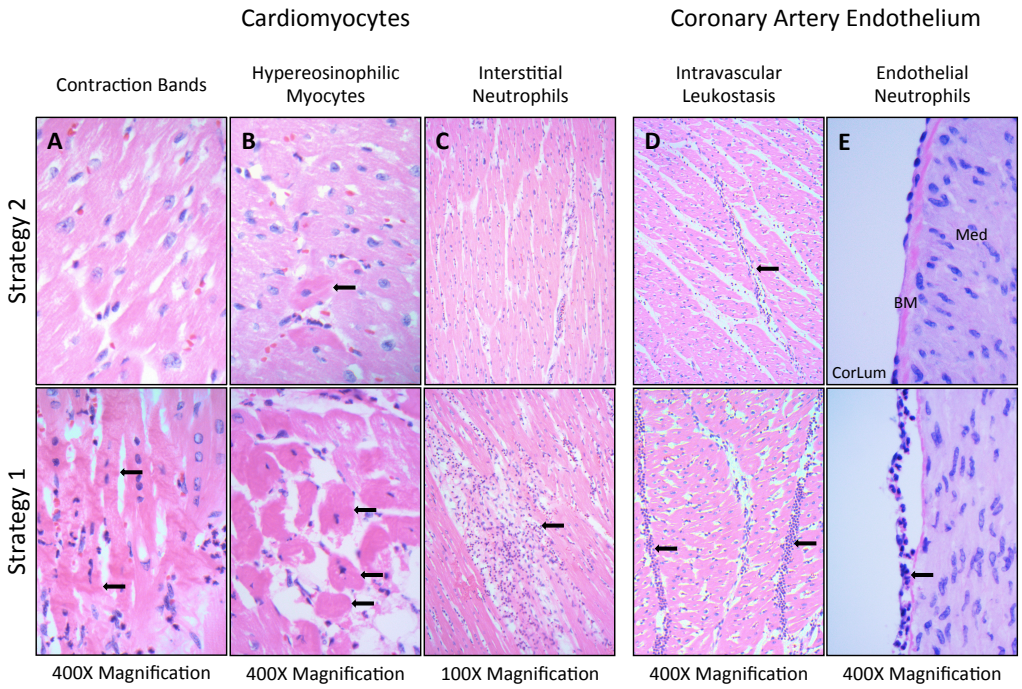

Figure 4.6. Hematoxylin and eosin stained histological sections from representative animals by EVHP strategy. A) Contraction bands: a primary type of cardiomyocyte death that occurs when a calcium overloaded cell rapidly undergoes repletion of its energy stores B) Hypereosinophilic myocytes: evidence of ischemic injury to the myocardium C) Interstitial neutrophil infiltration D) Intravascular leukostasis: neutrophil adherence to endothelial cells that leads to an increase in capillary blood flow resistance and the no-reflow phenomenon E) Coronary endothelial neutrophil infiltration: adherence and subsequent migration of neutrophils into coronary endothelial cells.

BM, basement membrane; CorLum, coronary artery lumen; Med, coronary artery media.

Post-transplant function

Pressure-volume loop analysis

At baseline there was no significant difference in systolic or diastolic function between treatment groups. Post-transplant function was assessed following wean from cardiopulmonary bypass on infusions of dobutamine and norepinephrine. S2 hearts demonstrated superior post-transplant systolic and diastolic function compared to S1 hearts (Table 4.2, Figure 4.7).

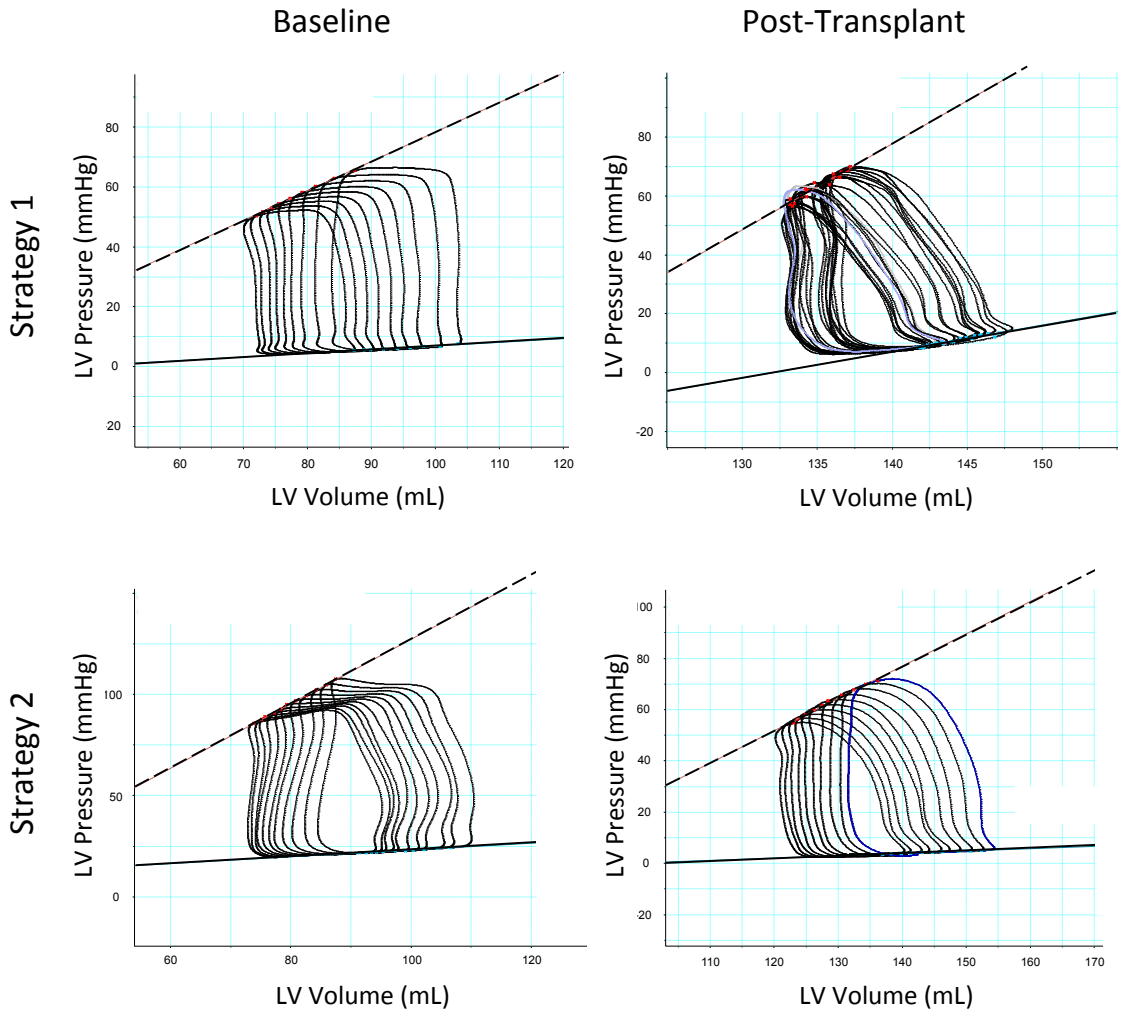

Figure 4.7. Left ventricular pressure-volume loops from representative animals by preservation strategy, obtained during transient inflow occlusion.

− − − end-systolic pressure volume relationship; ── end-diastolic pressure volume relationship

Table 4.2. Pressure-volume loop assessment of left ventricular function

	Baseline			Post-Transplant		
	Strategy 1	Strategy 2	*p value*	Strategy 1	Strategy 2	*p* value
Systolic Function						
dP/dt max (mmHg/s)	845 (120)	925 (126)	0.181	718 (156)	998 (180)	0.009
ESPVR	1.36 (0.38)	1.35 (0.34)	0.981	4.50 (5.93)	3.04 (3.01)	0.580
PRSW	36.7 (11.0)	37.6 (17.5)	0.892	19.7 (10.9)	33.5 (10.8)	0.043
Diastolic Function						
dP/dt min (mmHg/s)	-817 (162)	-955 (154)	0.084	-475 (201)	-671 (120)	0.047
EDPVR	0.14 (0.03)	0.15 (0.04)	0.575	0.60 (0.40)	0.19 (0.08)	0.022
Tau (ms)	38.6 (4.4)	42.7 (5.8)	0.108	65.2 (21.1)	42.9 (6.7)	0.020

EDPVR, end-diastolic pressure volume relationship; *ESPVR,* end-systolic pressure volume relationship; *PRSW,* preload recruitable stroke work.

Load independent assessments of post-transplant function in S2 hearts were not significantly different from baseline values (PRSW: 33.5±10.8 vs. 37.6±17.5, p=0.591; EDPVR: 0.19±0.08 vs. 0.15±0.04, p=0.180; Tau: 42.9±6.7 vs. 42.7±5.8, p=0.946). In contrast, S1 hearts demonstrated worse post-transplant function compared to baseline (PRSW: 19.7±10.9 vs. 36.7±11.0, p=0.012; EDPVR: 0.60±0.40 vs. 0.14±0.03, p=0.004; Tau: 65.2±21.1 vs. 38.6±4.4, p=0.002).

Clinical outcomes

Post-transplant clinical outcomes are displayed in Figure 4.8. All S2 hearts recovered sufficient function during EVHP to facilitate transplantation and wean from CPB, and the majority (63%) demonstrated stable post-transplant hemodynamic status. Conversely, 22% of S1 hearts demonstrated extremely poor function during EVHP and could not be transplanted, 67% were unstable following separation from CPB and required boluses of vasoactive medications to

maintain adequate cardiovascular status, and only 11% exhibited stable post-transplant hemodynamic status (p = 0.059).

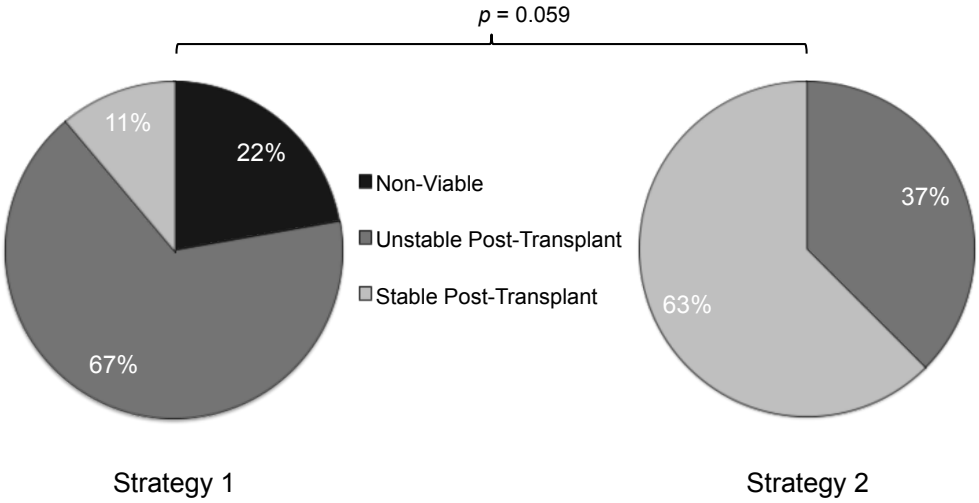

Figure 4.8. Post-transplant clinical outcomes

Non-viable: insufficient function during *ex vivo* perfusion to facilitate transplantation; Unstable: required repeated boluses of vasoactive medications to maintain hemodynamic status post-transplantation; stable: demonstrated stable hemodynamic status post-transplantation.

Discussion

EVHP has been proposed as a means to resuscitate hearts from DCD donors and increase the number of organs available for transplantation (4-6); however, the current approach to clinical EVHP exposes the heart to hypothermic, hyperkalemic cardioplegia and additional ischemia that may exacerbate myocardial injury and limit the likelihood of successful transplantation. We have shown for the first time that a novel cardioprotective EVHP strategy employing initial reperfusion with a tepid normokalemic A-L cardioplegia and continuous normothermic EVHP minimizes myocardial injury and permits recovery of sufficient myocardial function to facilitate successful transplantation of DCD hearts.

Ischemia-reperfusion injury in the DCD heart

A review of IRI is beyond the scope of this study and has been reported previously (9, 10, 13, 28). The obligatory hypoxic cardiac arrest and warm-ischemic standoff period that ethically define death in the DCD context expose the heart to a significant period of ischemia (8) that leads to a depletion of myocardial energy stores (23), anaerobic metabolism, and the development of intracellular acidosis (9). Reperfusion at the time of organ procurement causes a rapid normalization of the extracellular pH, creating a large H^+ gradient across the plasma membrane that activates the Na^+/H^+ exchanger and causes Na^+ influx (10). The Na^+/Ca^{2+} exchanger works in reverse mode to eliminate the excess intracellular Na^+ in exchange for Ca^{2+}, causing intracellular Ca^{2+} overload and the propagation of myocyte death through the development of hypercontracture, activation of calcium-dependent proteases, generation of ROS, activation of mitochondrial permeability transition pores, and initiation of apoptotic pathways (9, 10, 13).

Limitations of the current approach to EVHP

At the time of organ procurement the DCD heart is energy depleted, Na^+ overloaded, and vulnerable to the accumulation of intracellular Ca^{2+}. Therefore, initial reperfusion must maintain cardiac arrest to provide an opportunity for restoration of myocardial energy stores that will facilitate recovery of ionic homeostasis and minimize IRI (14). The current approach to clinical EVHP involves perfusion with hypothermic, hyperkalemic cardioplegia at the time of organ procurement (15). This may exacerbate intracellular Ca^{2+} overload, propagate IRI, and limit the probability of successful resuscitation of DCD hearts.

Restoration of ionic homeostasis requires the activity of ATP-dependent ion pumps (17-18), but profound hypothermia decreases the efficiency of oxidative phosphorylation and limits the synthesis of ATP (29). Hypothermia has also been shown to increase intracellular Ca^{2+} during reperfusion of hypoxic myocytes (16), cause endothelial injury (17), and enhance the

generation of reactive oxygen species in isolated hearts (30). Therefore, exposure of the ischemic myocardium to hypothermia upon initial reperfusion may retard restoration of normal transmembrane ion gradients and promote IRI.

Hyperkalemic cardioplegia achieves electromechanical arrest by depolarizing the cardiomyocyte membrane potential (31), which increases intracellular Ca^{2+} and is associated with a reduction in myocyte contractile function, beta-adrenergic responsiveness, and active relaxation (32, 33). Hyperkalemia also causes endothelial injury (17) and coronary vasoconstriction that may compromise cardioplegia delivery (34, 35). Therefore, hyperkalemia has no intrinsic cardioprotective properties (20) and may exacerbate intracellular Ca^{2+} overload and IRI (13, 28, 36).

The current approach to clinical EVHP involves cardioplegic arrests at the time of organ procurement and upon completion of the preservation interval that result in 60-80 minutes of cold-ischemia prior to transplant (19). The brain dead heart has experienced negligible ischemia prior to organ procurement and may tolerate the additional ischemia associated with this approach; however, the DCD heart has already sustained a significant ischemic insult and minimizing further ischemia during the preservation interval is critical to facilitate resuscitation (4, 5).

Benefits of a cardioprotective approach to EVHP

Optimizing initial reperfusion conditions at the time of organ procurement may minimize IRI and increase the probability of successful DCD heart resuscitation. We have demonstrated for the first time in a clinically relevant model of DCD that initial reperfusion with tepid A-L cardioplegia significantly reduces troponin release and the development of myocardial edema during EVHP. These benefits may be due to a number of factors.

First, A-L cardioplegia achieves diastolic arrest by lidocaine-induced blockade of sodium fast channels, while adenosine maintains a polarized membrane potential via the A1 receptor

(22). Maintaining a polarized membrane potential during initial reperfusion may minimize intracellular Ca^{2+} overload and protect the heart from IR injury (21, 22). Rudd and Dobson (37) have shown that rat hearts arrested with A-L cardioplegia experience less troponin release and improved myocardial function compared to those arrested with hyperkalemic cardioplegia (37, 38). Similarly, polarized arrests using potassium channel openers minimize the accumulation of intracellular Ca^{2+} (36, 39) and improve myocardial function compared to depolarized arrests with hyperkalemic cardioplegia (31, 33).

Second, A-L cardioplegia is effective at warmer temperatures and permits reperfusion and reanimation without myocardial exposure to profound hypothermia (20). This may avoid the detrimental effects of profound hypothermia mentioned above. Additionally, the tepid reperfusion strategy employed in this study may have facilitated restoration of myocardial energy stores and ionic homeostasis prior to contraction, and may partially explain why we observed less hypercontracture and troponin release in S2 hearts. The efficacy of a tepid initial reperfusion strategy during the procurement of DCD hearts has been demonstrated previously (14).

Third, A-L cardioplegia has intrinsic cardioprotective properties that may minimize IRI. Adenosine has been shown to inhibit apoptosis through up-regulation of the anti-apoptotic protein Bcl-2 (50). Adenosine and lidocaine have anti-inflammatory properties that attenuate neutrophil adhesion and infiltration into endothelial cells (40-42) and inhibit the generation and release of ROS (43-49). This may explain why we observed less neutrophil infiltration into coronary endothelial cells and less OxPCs in post-transplant myocardial specimens from S2 hearts. This protective effect on coronary endothelial cells may also have minimized the development of myocardial edema and improved post-transplant function.

Finally, minimizing myocardial exposure to additional ischemia prior to transplantation may have minimized oxidative stress and improved post-transplant function in S2 hearts. The results of this study and others (14, 51) have demonstrated that employing continuous EVHP during the

transplantation procedure can eliminate myocardial exposure to a second period of cold-ischemia and improve the likelihood of successful DCD heart transplantation.

Limitations

The treatment of S1 and S2 hearts differed with respect to the initial reperfusion strategy employed as well as the approach to transplantation. It is clear that the initial reperfusion strategy utilized in S2 hearts minimized myocardial injury (less troponin release) and myocardial edema (less weight gain) during EVHP; however, it is difficult to conclusively determine if the approach to transplantation provided an incremental benefit with regards to the superior post-transplant function observed in S2 hearts. The relative contribution of each component of this strategy requires further investigation. The limitations of an intact animal model precluded direct measurements of intracellular Ca^{2+} and myocyte membrane potentials. While our results demonstrate that a cardioprotective EVHP strategy improves post-transplant function, the impact on long-term graft survival is unknown.

Conclusions

A cardioprotective EVHP strategy employing initial reperfusion with a tepid A-L cardioplegia and continuous normothermic EVHP minimizes myocardial injury and improves the post-transplant function of DCD hearts compared to the current EVHP strategy employed clinically.

Chapter 4 Summary

In Chapter 4: *A cardioprotective preservation strategy employing ex vivo heart perfusion facilitates successful transplant of donor hearts after cardiocirculatory death*, DCD heart transplantation was established as a 'proof-of-concept'. In addition, the data presented suggests that post-transplant outcomes can be improved if the approach to donor heart resuscitation and preservation is tailored specifically to the DCD context. First, DCD heart resuscitation can be optimized if the cardioplegic solution delivered at the time of organ procurement is tailored to minimize ischemia-reperfusion injury. Second, preserving the vulnerable DCD heart in a normothermic beating state using *ex vivo* perfusion, limits incremental ischemic injury following organ procurement and optimizes post-transplant outcomes. Finally, *ex vivo* evaluation of DCD heart function provides an opportunity to identify non-viable organs prior to transplantation. This ensures that organ utilization is maximized, while recipient risk of receiving an organ at high risk of primary graft dysfunction is minimized. The data suggests that post-transplant outcomes can be optimized if a structured approach to DCD heart resuscitation, preservation, and evaluation is employed. Each component of this approach will be explored in greater detail in subsequent chapters.

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Chapter 5

A whole blood-based perfusate provides superior preservation of myocardial function during ex vivo heart perfusion

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Chapter 5 Preface

Optimizing donor heart preservation is a crucial component of a successful DCD transplant protocol (Figure 1.1). *Ex vivo* preservation a normothermic beating state provides an opportunity to minimize incremental ischemic injury sustained by the DCD heart in the time between organ procurement and transplantation, and evaluate heart function to ensure organ viability prior to transplantation. It is therefore necessary to ensure that the perfusate solution is capable of providing sufficient oxygen delivery to meet myocardial metabolic demands during *ex vivo* preservation and evaluation in a physiologic working mode. In Chapter 5: *A whole blood-based perfusate provides superior preservation of myocardial function during ex vivo heart perfusion*, the impact of different perfusate solution compositions on the preservation of myocardial energy stores and donor heart function during EVHP is explored. The data presented in this chapter suggests that a donor whole blood-based perfusate solution maintains myocardial energy stores, minimizes oxidative stress, and optimizes the preservation of donor heart function during EVHP.

Abstract

Background

Ex vivo heart perfusion (EVHP) provides the opportunity to resuscitate unutilized donor organs and facilitates assessments of myocardial function that are required to demonstrate organ viability prior to transplantation. We sought to evaluate the impact of different oxygen carriers on the preservation of myocardial function during EVHP.

Methods

Twenty-seven pig hearts were perfused *ex vivo* in a normothermic beating state for 6 hours, and were transitioned into working mode for assessments after 1 (T1), 3 (T3), and 5 (T5) hours. Hearts were allocated to 4 groups according to the perfusate composition. Red blood cell concentrate (RBC, N=6), whole blood (RBC+Plasma, N=6), an acellular hemoglobin based oxygen carrier (HBOC, N=8), or HBOC plus plasma (HBOC+Plasma, N=7) were added to STEEN solution to achieve a perfusate hemoglobin concentration of 40 g/L.

Results

Perfusate composition impacted the preservation of systolic (T5 dP/dt_{max}: RBC+Plasma=903±99, RBC=771±77, HBOC+Plasma=691±82, HBOC=563±52 mmHg/second, p=0.047) and diastolic (T5 dP/dt_{min}: RBC+Plasma=-574±48, RBC=-492±63, HBOC+Plasma=-326±32, HBOC=-268±22 mmHg/second, p<0.001) function, and the development of myocardial edema (Weight gain: RBC+Plasma=6.6±0.9, RBC=6.6±1.2, HBOC+Plasma=9.8±1.7, HBOC=16.3±1.9 grams/hour, p<0.001) during EVHP. RBC+Plasma hearts exhibited less histologic evidence of myocyte damage (Injury score: RBC+Plasma=0.0±0.0, RBC=0.8±0.3, HBOC+Plasma=2.6±0.2, HBOC=1.75±0.4, p<0.001) and less troponin-I release (Troponin-I fold change T1-T5: RBC+Plasma=7.0±1.7, RBC=13.1±1.6, HBOC+Plasma=20.5±1.1, HBOC=16.7±5.8, p<0.001). Oxidative stress was minimized by the addition of plasma to RBC and HBOC hearts (Oxidized phosphatidylcholine compound fold change T1-T5: RBC+Plasma=1.83±0.20 vs. RBC=2.31±0.20, p<0.001; HBOC+Plasma=1.23±0.17 vs. HBOC=2.80±0.28, p<0.001).

Conclusion

A whole blood-based perfusate (RBC+Plasma) minimizes injury and provides superior preservation of myocardial function during EVHP. The beneficial effect of plasma on the preservation of myocardial function requires further investigation.

Introduction

Ex vivo heart perfusion (EVHP) has been proposed as a means of resuscitating unutilized donor hearts and increasing the number and quality of donor organs available for transplant. EVHP provides an opportunity to deliver optimal concentrations of pharmaceutical agents that target pathways involved in ischemia-reperfusion injury and support reparative processes in the myocardium (1). EVHP also facilitates dynamic assessments of organ function that are required to confirm donor heart viability prior to transplantation (2); however, such assessments require the presence of an oxygen carrier in the EVHP perfusate to meet the metabolic demands of a normothermic heart in working mode.

A donor blood-based perfusate offers the oxygen carrying capacity of red blood cells, and the metabolic substrates, free radical scavengers, buffers, and oncotic properties of plasma (3). However, donor blood also contains immune cells that can propagate ischemia-reperfusion injury (4) and promote a proinflammatory cytokine milieu following exposure to the *ex vivo* circuit. Additionally, logistical barriers during organ procurement may limit the feasibility of obtaining a sufficient quantity of donor blood to prime the *ex vivo* circuit (3).

Hemoglobin-based oxygen carriers (HBOC) have been proposed as an innovative alternative to facilitate oxygen delivery during EVHP (5). HBOC-201 (Hemopure, OPK Biotech, Cambridge, MA) is an acellular glutaraldehyde-polymerized bovine hemoglobin solution with a lower oxygen affinity than human hemoglobin, thereby enhancing oxygen unloading at the tissue level (6). HBOC-201 has oncotic properties comparable to blood, does not contain any immune cells, does not require a cross-match prior to administration, is readily available, and has a shelf-

life at room temperature of 3 years (7). However, HBOC solutions are prone to the spontaneous oxidation of ferrous heme iron, resulting in the production of methemoglobin and the generation of reactive oxygen species (ROS) (8, 9).

Previous studies have suggested that the composition of the perfusate solution is an important factor impacting donor heart viability during EVHP (10), yet the optimal means of achieving myocardial oxygen delivery to meet the metabolic demands of a working heart has not been determined. Therefore, we sought to investigate the impact of different oxygen carriers on the preservation of myocardial function during EVHP.

Materials and Methods

Institutional Animal Care Committees approved the experimental protocol. Twenty-seven female domestic pigs (40.5±0.5 kg) were allocated to 4 treatment groups according to the composition of the *ex vivo* perfusate solution.

Donor Heart Procurement and Preparation for ex vivo heart perfusion

Pigs were induced with an intramuscular injection of tiletamine (2.4mg/kg), zolazepam (2.4mg/kg), and xylazine (0.9mg/kg). Orotracheal intubation was established and general anesthesia was maintained with 1-2% isoflurane. A median sternotomy was performed and 400 units/kg of heparin was delivered intravenously. A two-stage venous cannula was placed into the right atrium and the animal was exsanguinated into a Brat2 cell saver (Sorin Group Canada Inc., Burnaby, Canada), which was used to separate donor blood into red blood cell concentrate (RBC) and plasma components. A cross-clamp was placed across the ascending aorta and the heart was arrested with 1000 mL of cold, reverse 4:1 blood cardioplegia (St. Thomas Hospital Solution No. 2). The heart was excised and placed on ice while cannulas were placed into the aorta, left atrium, and pulmonary artery, and the inferior and superior vena cava were oversewn.

Experimental groups

A sufficient quantity of oxygen carrier was added to STEEN solution (XVIVO Perfusion Inc., Englewood, USA) in each group to produce a perfusate with a hemoglobin concentration of 40 g/L. STEEN solution is a buffered extracellular-type salt solution with a colloid osmotic pressure optimized for *ex vivo* organ perfusion (11).

RBC group (N=6)*:*

Following separation of donor whole blood into RBC and plasma components, the RBC was washed with 2 L of normal saline to produce the oxygen carrier for the RBC group.

RBC+Plasma group (N=6):

Donor whole blood was used as the oxygen carrier for the RBC+Plasma group.

HBOC group (N=8)*:*

HBOC-201 was used as the oxygen carrier for the HBOC group.

HBOC+Plasma group (N=7):

Donor plasma was added to HBOC-201 to produce the oxygen carrier for the HBOC+Plasma group.

Ex vivo heart perfusion

A Medtronic Affinity NT oxygenator, venous reservoir, 2 BPX-80 Bio-Medicus centrifugal pumps (Medtronic, Minneapolis, USA), and a leukocyte arterial blood filter (LeukoGuard LG, PALL Medical, Port Washington, USA) were used to construct the EVHP circuit (Figure 5.1). Computer control of the centrifugal pumps was used to maintain the desired aortic diastolic and left atrial pressures. Oxygen and gas flow through the membrane oxygenator were titrated to maintain a pH of 7.35-7.45, PaO_2 of 300-400 mmHg, and $PaCO_2$ of 35-45 mmHg.

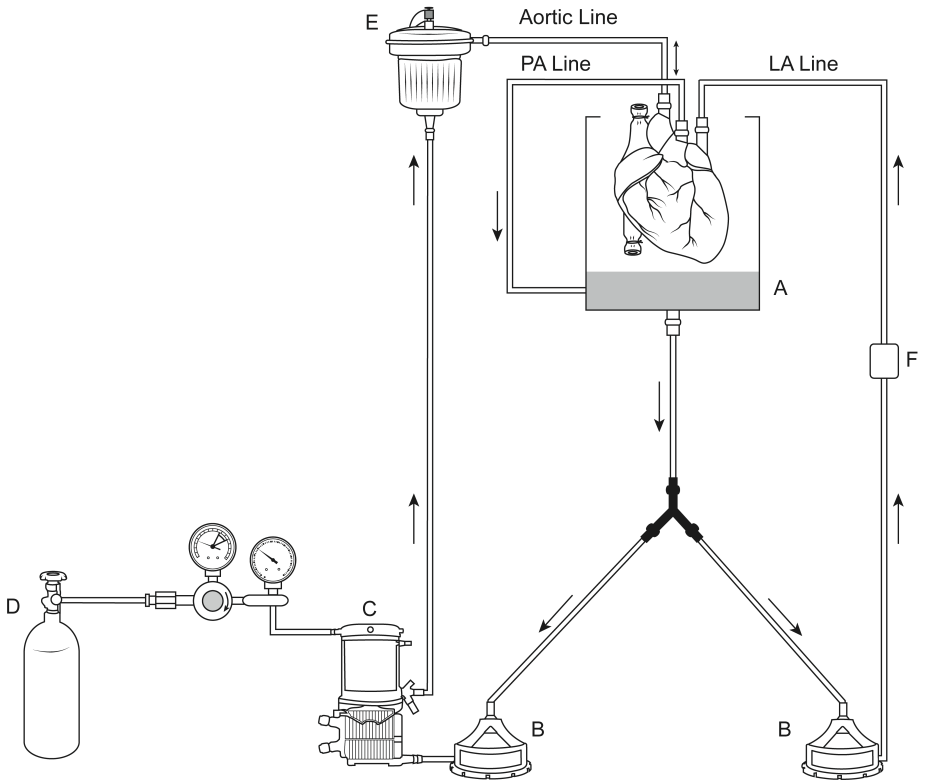

Figure 5.1. *Ex vivo* heart perfusion circuit.

A, venous reservoir; *B,* centrifugal pumps; *C,* oxygenator/heat exchanger; *D,* oxygen/medical air source; *E,* leukocyte filter

Hearts were initially perfused via the aortic root at 22˚C and were then rewarmed to 37˚C over a 30-minute period. Automated computer control of the aortic centrifugal pump (Figure 5.1) was used to maintain the aortic diastolic pressure at 30 mmHg. At 1 (T1), 3 (T3), and 5 (T5) hours of *ex vivo* perfusion, perfusate samples were obtained for subsequent analysis and hearts were transitioned into a working mode for assessments of myocardial function, energy metabolism, and coronary blood flow. The transition from an empty, non-working state (left atrial pressure = 0 mmHg) into a physiologic working mode (left atrial pressure = 8 mmHg) was accomplished by increasing the revolutions per minute on the left atrial centrifugal pump (Figure 5.1).

Perfusate composition

Electrolyte, hemoglobin and methemoglobin concentrations, and perfusate pH, PO_2 and PCO_2 were measured using the ABL800 Flex Analyzer (Radiometer Medical ApS, Brønshøj, Denmark). Cytokine profiles were assessed using a MILLIPLEX MAP Porcine Cytokine/Chemokine Magnetic Bead Panel (EMD Millipore Corporation, Billerica, USA) on a Luminex platform (Bio-Plex 200, BIO-RAD Laboratories Ltd., Mississauga, Canada). Perfusate osmotic pressure was assessed using a vapor pressure osmometer (VAPRO 5520, Wescor Inc., Logan, USA). Perfusate oncotic pressure was assessed using a colloid oncometer (Osmomat 050, Gonotec GmbH, Berlin, Germany).

Myocardial energy metabolism

To determine if a hemoglobin concentration of 40 g/L was sufficient to preserve myocardial energetics during EVHP, hearts (N=6, RBC+Plasma; N=6, HBOC) underwent magnetic resonance spectroscopy (MRS) to measure high-energy phosphate compounds at T1, T3, and T5. In addition, spectra from normal hearts *in vivo* (N=6) served as a positive control and spectra from a heart perfused with no oxygen carrier (STEEN solution only, N=1) as a negative control. Hearts were placed into a 3T Magnetom Sonata magnetic resonance scanner (Siemens, Erlangen, Germany). A purpose-built surface coil was positioned over the anterolateral wall of the left ventricle and transmural ^{31}P spectra were obtained using ECG gating.[12] Myocardial energy metabolism was assessed by measuring the ratio of inorganic phosphate to phosphocreatine (Pi/PCr).

Myocardial injury

Myocardial edema

Hearts were emptied of blood and weighed at the beginning and end of EVHP. Total weight gained was normalized to the duration of the EVHP interval.

Troponin-I

Perfusate samples were obtained from the coronary sinus effluent at T1 and T5. The amount of troponin-I was determined using a Pig Cardiac Troponin-I ELISA Kit (Life Diagnostics, Pennsylvania, USA) and normalized to the heart weight. The fold-change in troponin-I between T1 and T5 was compared between groups.

Oxidative stress

Lipid extraction and quantification of oxidized phosphatidylcholines (OxPC) were performed as previously described (13). Briefly, a normal-phase high-performance liquid chromatography of phospholipids was performed on a 2.7 µm Supelco column in a SIL-20ACHT Prominence UFLC (Shimadzu, Kyoto, Japan) and eluted into a 4000 Q-TRAP® quadrupole linear ion trap hybrid mass spectrometer (Applied Biosystem/MDS Sciex, Ontario, Canada). The fold-change in 82 OxPC molecules between T1 and T5 perfusate samples were compared between groups.

Histology

Hearts were fixed in 10% buffered formalin and transmural tissue specimens were obtained from the anterolateral wall of the left ventricle, embedded in paraffin, stained, and examined in a blinded fashion by one clinical pathologist (JK) who assigned a semi-quantitative injury score (0=none, 1=mild, 2=moderate, 3=severe). Sections stained with hematoxylin and eosin were assigned a myocyte injury score based on the presence of contraction bands and hypereosinophilic myocytes. Sections stained with Masson's trichrome were assigned a score based on the presence of extravascular fluid.

Coronary vascular resistance

Coronary blood flow (CBF) was determined by averaging 2 timed collections of pulmonary arterial blood flow (14). Indexed coronary vascular resistance (CVR) was calculated as follows:

CVR (mmHg•min/L/gram) = diastolic pressure (mmHg) / CBF (L/min) / heart weight (grams).

Ventricular function

Left ventricular function was assessed in working mode at T1, T3, and T5 using a 5F conductance catheter (Ventri-Cath 507, Millar Inc., Houston, USA). Load dependent measures were based on steady state data, with systolic and diastolic function assessed by the maximum (dP/dt_{max}) and minimum (dP/dt_{min}) rate of pressure change in the ventricle, respectively. Diastolic function was further assessed using the isovolumic relaxation constant (Tau). Load independent measurements were collected during a computer-controlled reduction in left atrial flow. Systolic function was assessed by the end-systolic pressure volume relationship (ESPVR) and preload recruitable stroke work (PRSW). Diastolic function was assessed by the end-diastolic pressure volume relationship (EDPVR) (13).

Statistical Analysis

Normally distributed continuous variables were reported as mean ± standard error (SE) and compared using the Students *t*-test or the analysis of variance. Non-normally distributed continuous variables were reported as median [interquartile range (IQR)] and compared using the Mann-Whitney test or the Kruskal-Wallis test. A p-value <0.05 was considered statistically significant. All analyses were performed using Prism V6.0C (GraphPad Software Inc., La Jolla, USA).

Results

Hearts sustained comparable periods of cold-ischemia prior to initiation of EVHP (RBC+Plasma=36±3, RBC=32±1, HBOC+Plasma=33±1, HBOC=37±1 minutes, p=0.176) and there were no differences between groups in *ex vivo* perfusion times or the defibrillation requirements to achieve sinus rhythm following reperfusion (Supplement Table 5.1).

Perfusate characteristics

The electrolyte composition, osmotic, and oncotic pressures of the perfusate solutions are displayed in Supplement Table 5.2. Despite the presence of a leukocyte filter in the EVHP circuit, the addition of plasma to RBC (RBC+Plasma 33±9, RBC 3±1, fold-change (T1-T5) in proinflammatory cytokines/IL-10, $p<0.001$) and HBOC (HBOC+Plasma 19±10, HBOC 8±3, fold-change (T1-T5) in proinflammatory cytokines/IL-10, $p=0.190$) hearts resulted in the production of proinflammatory cytokines during EVHP; Figure 5.2A, Supplement Figure 5.1).

Perfusate methemoglobin content remained at baseline levels in RBC+Plasma and RBC hearts over the 6-hour EVHP interval; however, spontaneous oxidation of the ferrous heme iron resulted in a significant increase in perfusate methemoglobin content over time in HBOC+Plasma and HBOC hearts (Figure 5.3).

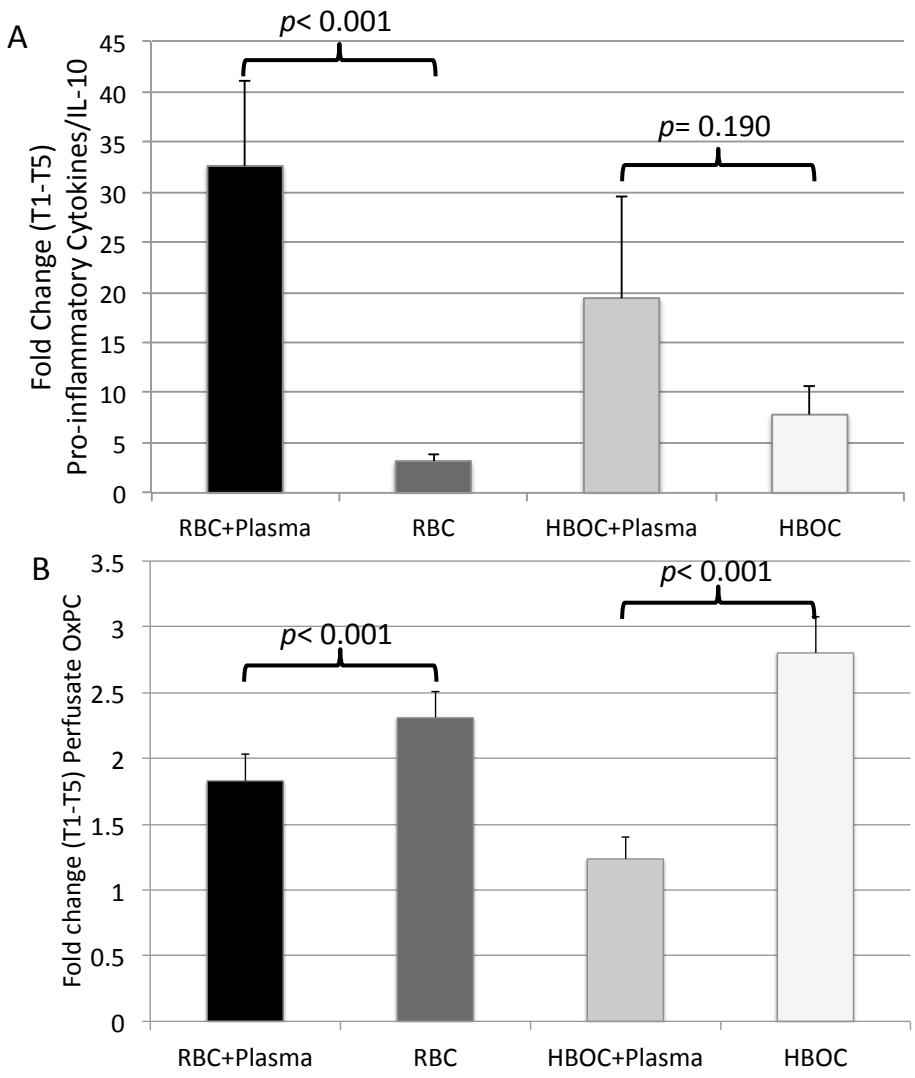

Figure 5.2. A) Fold change (T1-T5) in the ratio of proinflammatory cytokines (TNF-α, INF-γ, IL-1a, IL-1b, IL-6, IL-8) to the anti-inflammatory cytokine IL-10. B) Fold change (T1-T5) in perfusate oxidized phosphatidylcholine compounds.

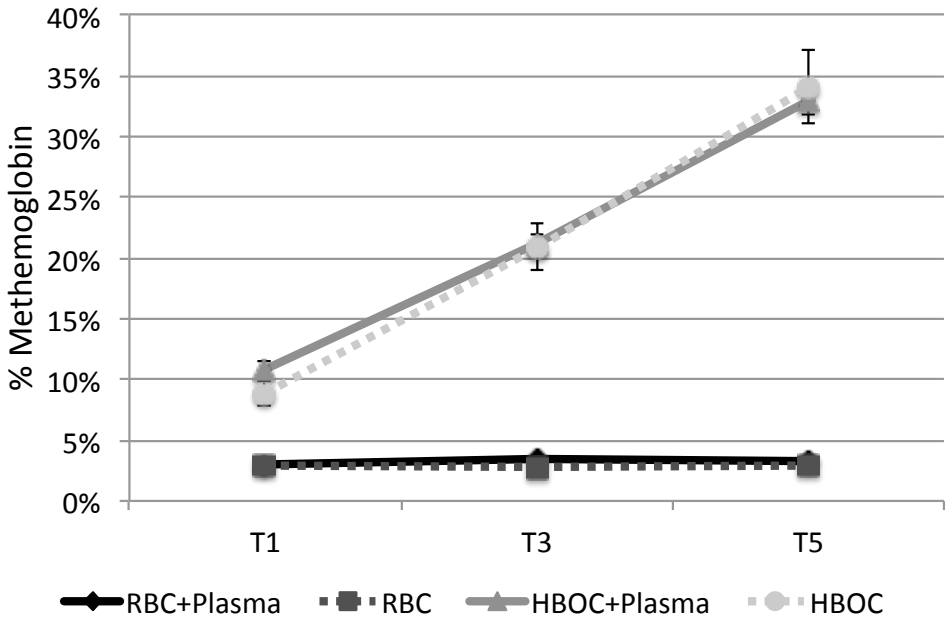

Figure 5.3. Perfusate percent methemoglobin at 1 (T1), 3 (T3), and 5 (T5) hours of *ex vivo* heart perfusion.

* indicates *p* value for comparison of percent methemoglobin at T1, T3, and T5 within the same treatment group.

Myocardial energy metabolism

Recovery of myocardial energy stores following cold ischemia occurred more quickly in HBOC hearts compared to RBC+Plasma hearts. HBOC hearts exhibited significantly better Pi/PCr ratios compared to RBC+Plasma hearts at T1, resulting in values that were not significantly different from normal hearts *in vivo* (Table 5.1). At T3 and T5 the spectra of HBOC and RBC+Plasma hearts were not significantly different from normal hearts *in vivo,* indicating the preservation of myocardial energy stores (Table 5.1, Figure 5.4). In contrast, perfusion without an oxygen carrier (STEEN solution only) resulted in a depletion of myocardial energy stores (increasing Pi, decreasing PCr, decreasing ATP) over time (Table 5.1, Figure 5.4).

Table 5.1. Magnetic resonance spectroscopy data obtained from normal hearts *in vivo,* and RBC+Plasma, HBOC, and STEEN only hearts at 1, 3, and 5 hours of *ex vivo* perfusion

	Pi/PCr	*p value*
In vivo (N=6), mean (SEM)	0.38 (0.05)	-
T1, mean (SEM)		
STEEN only (N=1)	0.52 (0.04)	-
RBC+Plasma (N=6)	0.49 (0.03)	0.067[#]
HBOC (N=6)	0.32 (0.05)	0.410[#]
p value	-	0.004[@]
T3, mean (SEM)		
STEEN only (N=1)	1.28 (0.15)	-
RBC+Plasma (N=6)	0.42 (0.02)	0.398[#]
HBOC (N=6)	0.37 (0.03)	0.926[#]
p value	-	0.279[@]
T5, mean (SEM)		
STEEN only (N=1)	3.78 (0.10)	-
RBC+Plasma (N=6)	0.48 (0.03)	0.100[#]
HBOC (N=6)	0.43 (0.04)	0.423[#]
p value	-	0.455[@]

ATP, adenosine triphosphate; *PCr,* phosphocreatinine; *Pi,* inorganic phosphate; *T1*, 1-hour of *ex vivo* perfusion; *T3*, 3-hours of *ex vivo* perfusion; *T5*, 5-hours of *ex vivo* perfusion

[#] RBC+Plasma vs. *in vivo* and HBOC vs*. in vivo,* [@] RBC+Plasma vs. HBOC.

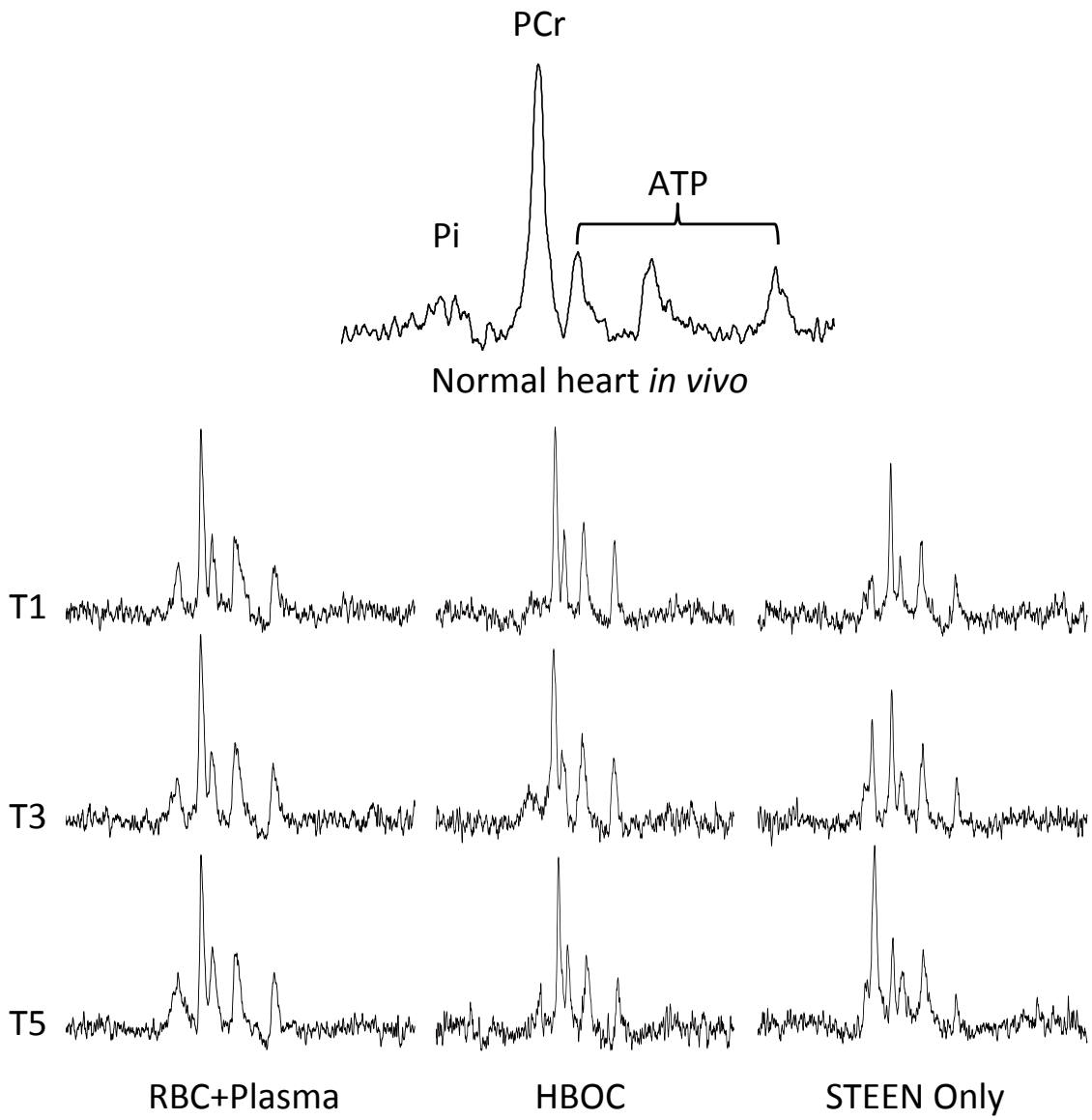

Figure 5.4. Magnetic resonance spectra obtained from normal hearts *in vivo* and hearts perfused with RBC+Plasma, HBOC, and STEEN solution at 1 (T1), 3 (T3), and 5 (T5) hours of *ex vivo* heart perfusion.

ATP, adenosine triphosphate; *PCr,* phosphocreatine; *Pi,* inorganic phosphate.

Myocardial injury

The development of myocardial edema during EVHP varied according to treatment group (RBC+Plasma=6.6±0.9, RBC=6.6±1.2, HBOC+Plasma=9.8±1.7, HBOC=16.3±1.9 grams/hour,

p<0.001), and was evident on histological examination (Figure 5.5). The change in CVR over the preservation interval is displayed in Figure 5.6. HBOC hearts experienced a linear increase in CVR over time (p=0.036); however, the increase in CVR did not occur until after T3 in HBOC+Plasma hearts (p=0.058). There was no significant change in CVR over time in RBC+Plasma and RBC hearts.

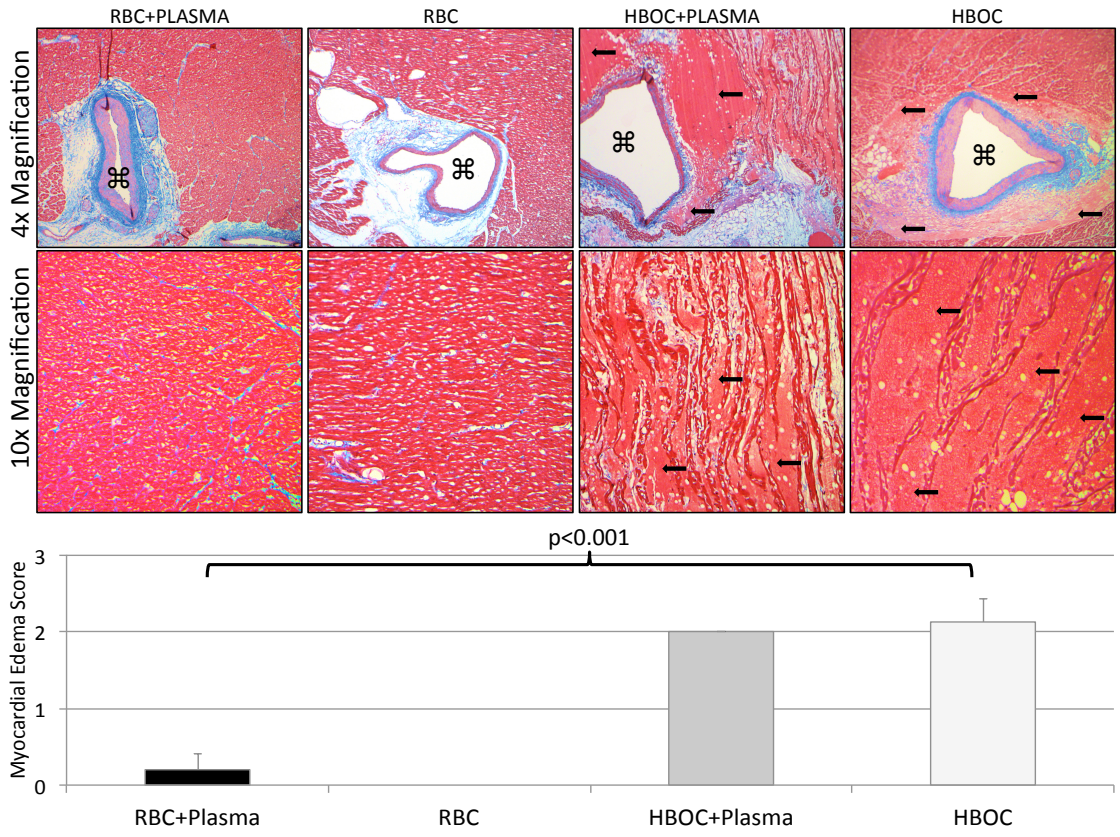

Figure 5.5. Mason's trichrome stained histologic sections from representative animals according to treatment group with corresponding myocardial edema scores.

⌘, coronary artery lumen; arrows, extravascular perfusate solution separating myofilbrils.

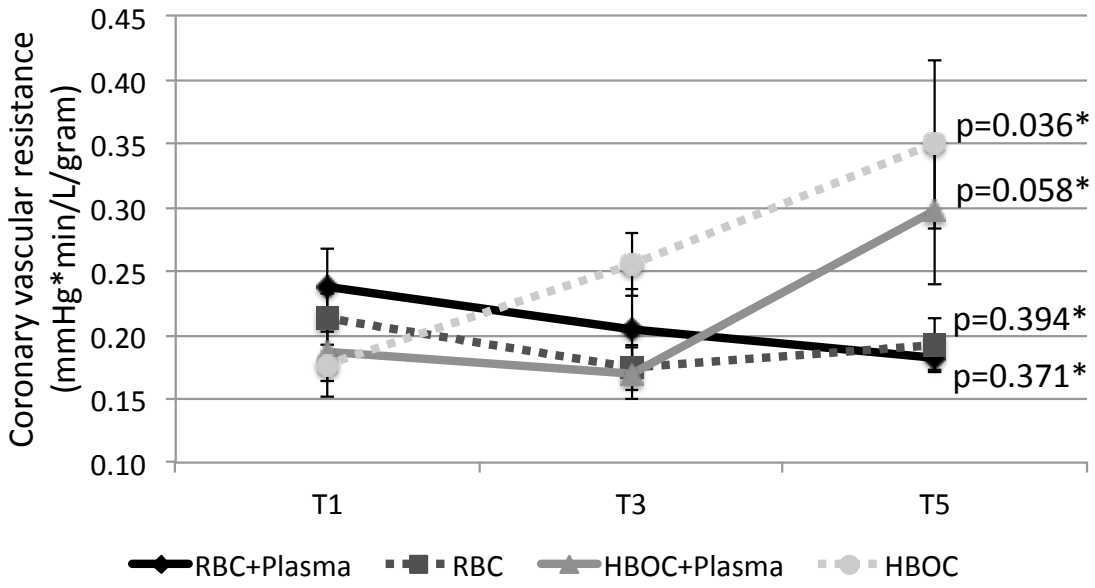

Figure 5.6. Coronary vascular resistance at 1 (T1), 3 (T3), and 5 (T5) hours of *ex vivo* heart perfusion.

* indicates *p* value for comparison of coronary vascular resistance at T1, T3, and T5 within the same treatment group.

RBC+Plasma hearts demonstrated less troponin-I release into the perfusate during EVHP (RBC+Plasma=7.0±1.7, RBC=13.1±1.6, HBOC+Plasma=20.5±1.1, HBOC=16.7±5.8 fold change T1-T5, $p<0.001$), and less histological evidence of myocardial injury (myocardial injury score: RBC+Plasma=0.0±0.0, RBC=0.8±0.3, HBOC+Plasma=2.6±0.2, HBOC=1.75±0.4, $p<0.001$, Figure 5.7).

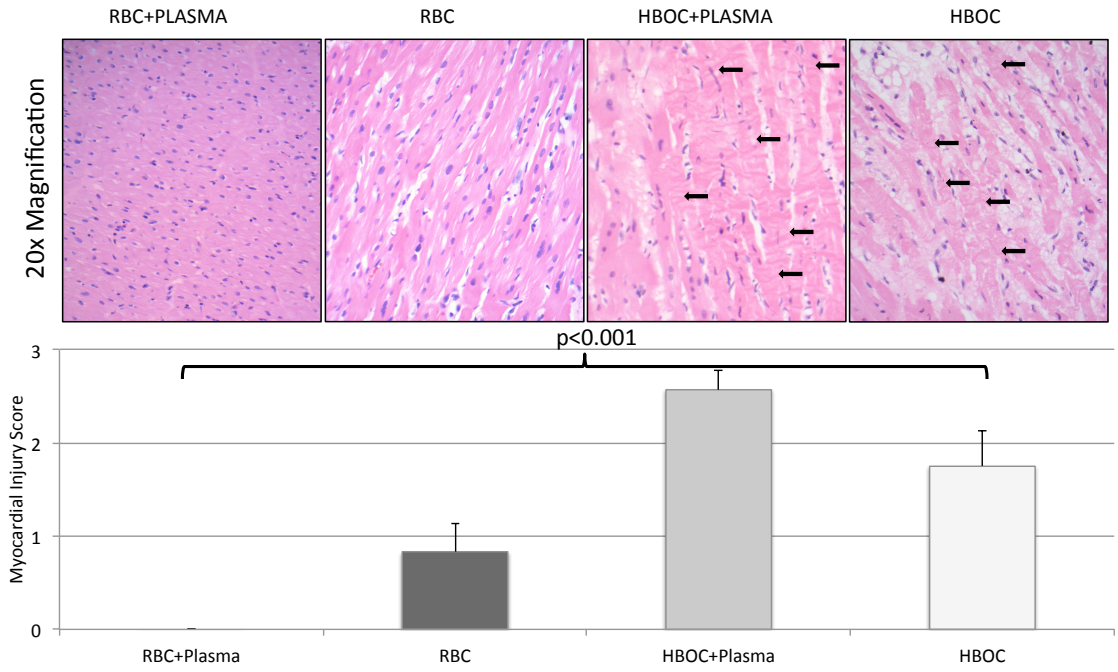

Figure 5.7. Hematoxylin and eosin-stained histologic sections from representative hearts according to treatment group with corresponding myocardial injury scores (arrows indicate contraction bands).

To evaluate the impact of plasma on the development of oxidative stress during EVHP, the fold change in perfusate OxPC compounds was measured (Figure 5.2B). The addition of plasma to RBC and HBOC hearts significantly reduced the generation of OxPC compounds in RBC+Plasma (RBC+Plasma=1.83±0.20 vs. RBC=2.31±0.20 fold change T1-T5, $p<0.001$) and HBOC+Plasma hearts (HBOC+Plasma=1.23±0.17 vs. HBOC=2.80±0.28 fold change T1-T5, $p<0.001$).

Myocardial function

Perfusate composition impacted the preservation of myocardial function during EVHP. Steady-state assessments of systolic and diastolic function were statistically different between treatment groups, with superior preservation of function observed in RBC+Plasma hearts (Figure

4.8). The impact of perfusate composition on Tau, EDPVR, ESPVR, and PRSW are displayed in Supplement Figure 5.2-5.5. RBC+Plasma hearts exhibited superior diastolic function as assessed by Tau and the EDPVR. Interestingly, the removal of plasma from RBC+Plasma hearts resulted in a trend towards worse diastolic function, while the addition of plasma to HBOC hearts resulted in a trend towards improved diastolic function. The ESPVR and PRSW were not significantly different between treatment groups.

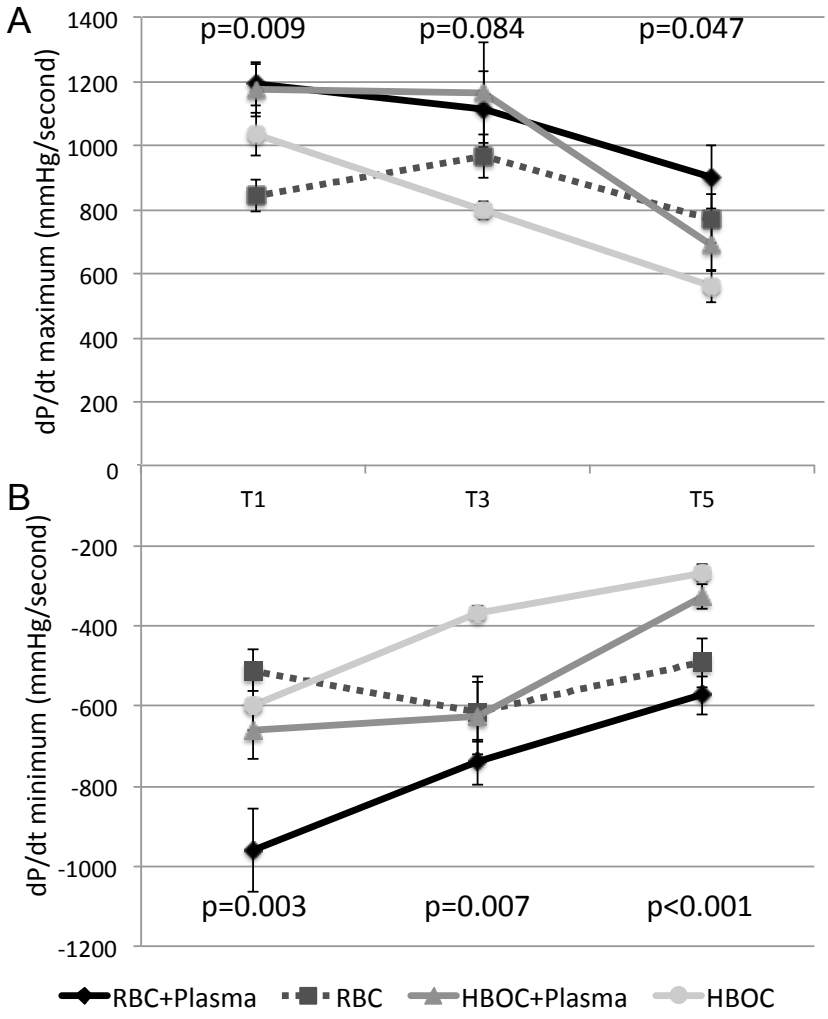

Figure 5.8. Systolic (A) and diastolic (B) function at 1 (T1), 3 (T3), and 5 (T5) hours of ex vivo heart perfusion.

Discussion

EVHP provides an opportunity to resuscitate donor hearts that might otherwise be discarded and therefore could expand donor pool; however, to minimize the risk of primary graft dysfunction it is necessary to assess myocardial function and demonstrate organ viability before transplantation (1). *Ex vivo* assessments of myocardial function require the presence of an oxygen carrier to meet the metabolic demands of a normothermic working heart. Previous studies have utilized whole blood- (15-19), RBC- (20), and HBOC-based (5, 21) solutions for this purpose; however, whether the type of oxygen carrier impacts the preservation of myocardial function during EVHP is unknown. We have now shown that a whole blood-based perfusate minimizes injury and provides superior preservation of myocardial function during EVHP.

Oxygenated crystalloid-based perfusates have been employed successfully for hypothermic *ex vivo* preservation of arrested hearts (22). In this state, the content of dissolved oxygen is sufficient since myocardial metabolic demands are reduced by approximately 95% compared to that of a normothermic working heart (23). While such an approach to EVHP preserves myocardial integrity, functional assessments are not possible and evaluation of organ viability is limited. Conversely, Podesser *et al*. have demonstrated that working mode EVHP with a crystalloid-based perfusate results in a depletion of myocardial energy stores, the development of myocardial edema, and impaired myocardial function (20). These results are in agreement with our magnetic resonance spectroscopy data, indicating that high-energy phosphate compounds are depleted during working mode EVHP with an oxygenated crystalloid perfusate, confirming the need for an oxygen carrier. We have also shown that a hemoglobin concentration of 40 g/L is sufficient to preserve myocardial energetics over a 6-hour EVHP interval. Interestingly, following cold-ischemia, the HBOC-based perfusate facilitated faster restoration of myocardial energy stores than the blood-based perfusate. This may be explained by the higher P_{50} (40 mmHg) of HBOC-201 compared to unmodified hemoglobin contained inside red blood cells, favoring oxygen unloading at the tissue level (7).

The development of myocardial edema and diastolic dysfunction during EVHP was prominent in HBOC hearts, despite comparable perfusion pressures and perfusate oncotic properties among treatment groups. This may be explained by the generation of ROS leading to endothelial cell injury and increased microvascular permeability in HBOC hearts. Under physiologic conditions, ferrous iron (Fe^{2+}) undergoes spontaneous oxidation to ferric iron (Fe^{3+}) containing methemoglobin and a superoxide free radical. Additionally, ferrous and ferric iron react with hydrogen peroxide (H_2O_2) to produce ferryl iron (Fe^{4+}) that is capable of causing oxidative injury to endothelial cells (8, 24). Hemoglobin contained within red blood cells exists in an environment with abundant catalase, superoxide dismutase, and methemoglobin reductase that catalyze the neutralization of superoxide free radicals and H_2O_2, and the reduction of ferric heme iron back to the ferrous state (25). Conversely, HBOCs exist outside the protective environment of the red blood cell and are vulnerable to the process of spontaneous oxidation and production of toxic ferryl iron species. This is exemplified in our study by the progressive increase in methemoglobin content in HBOC hearts during EVHP.

Ferryl iron species mediate oxidative injury to endothelial cells and increase microvascular permeability. *In vitro* evidence suggests that exposure of HBOC solutions to physiologic levels of H_2O_2 result in the production of ferryl iron species and promotes apoptosis in endothelial cells (26). Further, Dull *et al.* (27) demonstrated that HBOC solutions can cause the formation of actin stress fibers and intracellular gaps that result in increased endothelial permeability to albumin and polymerized hemoglobin molecules. The rate of ferryl iron production and endothelial injury may be exacerbated during ischemia and reperfusion since the exposure of HBOC molecules to H_2O_2 would be increased by several fold (28). Therefore, the experimental conditions of this study may have promoted the spontaneous oxidation of HBOC molecules and the production of ROS capable of causing significant oxidative injury to endothelial cells, increasing microvascular permeability and vascular resistance. This might explain the development of myocardial edema and increase in coronary vascular resistance

observed in HBOC hearts.

The development of myocardial edema in HBOC hearts was significantly reduced by the addition of plasma to the perfusate solution. Further, the increase in CVR and decline in diastolic function observed in HBOC+Plasma hearts were retarded until after T3. This suggests that plasma may have mediated protection from the oxidative stress experienced by HBOC hearts; however, this protective effect may have been exhausted by T5. Plasma contains reducing agents (reduced glutathione, ascorbic acid, uric acid, alpha-tocopherol, haptoglobin, bilirubin, albumin) and low concentrations of antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase, *glutathione-disulfide reductase) that may* limit the oxidative reactions mediated by ferryl iron, heme degradation products, and other free radicals formed during the redox cycling of HBOC solutions (29, 30). For example, Simoni *et al.* (24) have shown that the ascorbate–glutathione antioxidant system present in plasma is capable of preventing the formation of ferryl iron species and limiting membrane lipid peroxidation. This *in vitro* work is in agreement with our results, which demonstrates that the addition of plasma to HBOC and RBC hearts reduced the production of OxPC compounds and suggests an antioxidant role of plasma during EVHP. It is unknown if augmentation of the antioxidant capacity of the perfusate might further optimize donor heart preservation during EVHP.

We have attributed the diastolic dysfunction observed in HBOC hearts to the development of endothelial injury and subsequent myocardial edema. An important alternative consideration would be a reduction in oxygen delivery to HBOC hearts occurring as a result of the rising proportion of methemoglobin and increasing CVR observed over time. However, MRS spectra were equivalent among treatment groups, which argues against this possibility. Therefore, the decline in function observed was unlikely related to reduced oxygen delivery or availability of high-energy phosphate compounds. HBOC hearts were able to meet myocardial metabolic demands by increasing oxygen extraction.

Plasma propagated a proinflammatory cytokine milieu during EVHP. Despite this, plasma appeared to mediate a net protective effect in RBC+Plasma hearts as evidenced by lower troponin-I levels, less histologic evidence of myocardial injury, and a trend towards better preservation of myocardial function by T5 compared to RBC hearts. This benefit may be more pronounced in conditions of high oxidative stress, like in the resuscitation of donor hearts following circulatory death. Whether the addition of steroids to the perfusate would inhibit the generation of proinflammatory cytokines and further improve the preservation of myocardial function during EVHP requires investigation.

Previous animal and human studies have shown an association between HBOC solutions and increased troponin levels (31-33). McNeil *et al*. (32) observed significantly higher levels of CK-MB and a trend towards higher troponin-I levels when HBOC was used as a pump prime for cardiopulmonary bypass instead of whole blood. Neragi-Miandoab *et al*. (33) observed significantly higher troponin-I levels following cardioplegic arrest and cardiopulmonary bypass when HBOC was used as a pump prime instead of lactated ringers solution. Additionally, Jahr *et al*. (31) investigated the use of HBOC as an alternative to blood transfusion in patients undergoing elective orthopedic surgery and observed elevated troponin levels more commonly in HBOC treated patients. We also observed increased troponin-I levels and histologic myocardial injury scores in HBOC hearts; however, the etiology of this myocardial injury is unclear. The addition of plasma to HBOC hearts did not limit troponin-I release as it did in RBC hearts, suggesting a unique mechanism of myocardial injury associated with the use of HBOC solutions.

Our study has several important limitations. The impact of methemoglobin on endothelial cells and the development of myocardial edema was inferred based on previously published literature and wasn't directly quantified in this study. A leukocyte-depleting filter was incorporated into the *ex vivo* perfusion circuit to minimize the impact of circulating immune cells on the propagation of ischemia-reperfusion injury; however, the efficacy of the filter in reducing

circulating leukocytes was not confirmed by direct testing and cannot be confirmed with certainly. While we have provided evidence regarding the antioxidant capacity of donor plasma, other components may have contributed to the preservation of myocardial function observed in RBC+Plasma and HBOC+Plasma hearts (i.e. substrates for myocardial energy metabolism). Our results were obtained using plasma from a donor that had not suffered brain death and as a result did not contain the harmful hormonal and biochemical milieu present in the brain-dead donor. Therefore, application of these results to clinical transplantation would require the addition of banked plasma to washed donor RBC.

We conclude that a whole blood-based perfusate (RBC+Plasma) minimizes injury and provides superior preservation of myocardial function during EVHP. The beneficial effect of plasma on the preservation of myocardial function requires further investigation.

Chapter 5 Summary

In Chapter 5: *A whole blood-based perfusate provides superior preservation of myocardial function during ex vivo heart perfusion*, it was demonstrated that an oxygen carrier is required to maintain myocardial energy stores during EVHP in a physiologic working mode; however, a hemoglobin concentration of only 40 g/L is sufficient to meet metabolic demands. Additionally, myocardial function was best preserved when a whole blood-based perfusate was used. This observation may be related to the antioxidant properties or metabolic substrates present in donor plasma. Unfortunately, commercial HBOC solutions appear to cause significant myocardial edema that limits their applicability in clinical donor heart preservation. The data presented in this chapter also demonstrates that myocardial function declines in a time-dependent fashion during EVHP, the extent of which is out of proportion to the severity of injury sustained by the organ. This highlights a need for further investigation regarding the optimal means of supporting the heart from a metabolic point of view during prolonged EVHP.

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Supplemental Information

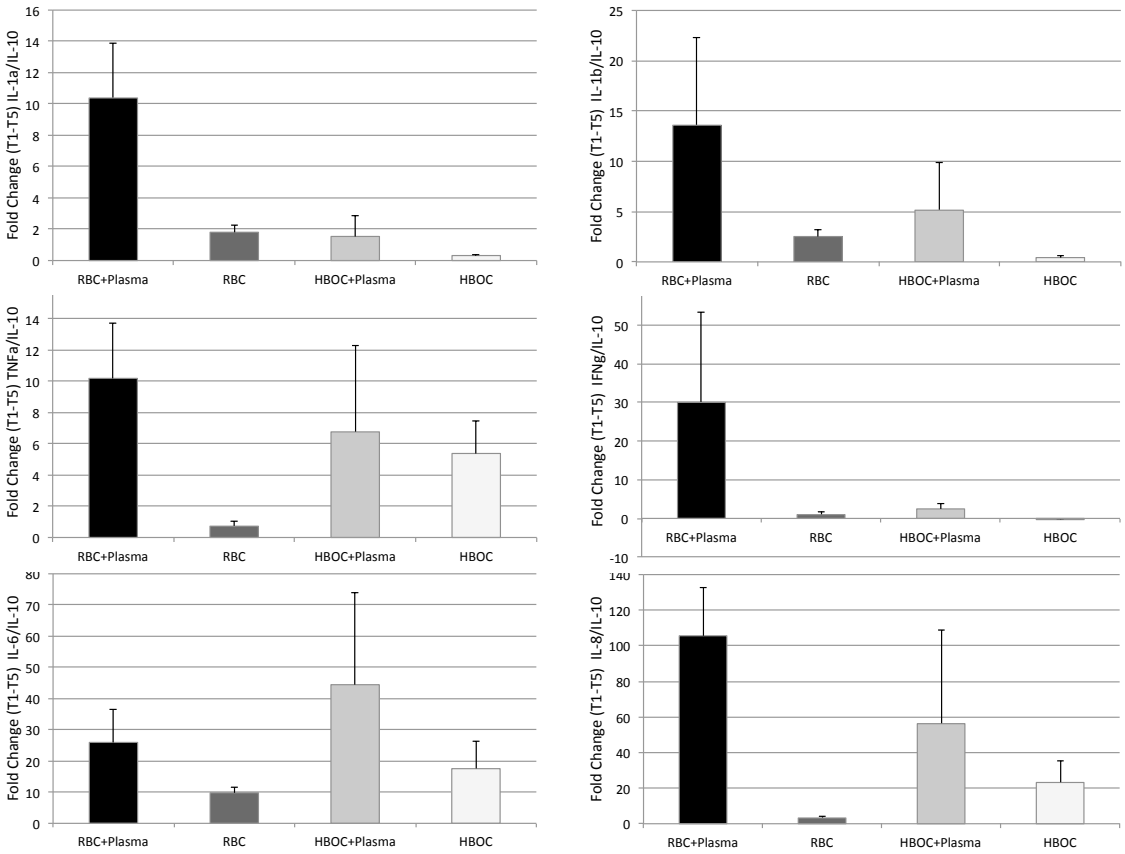
Supplement Table 5.1. *Ex vivo* heart perfusion parameters

	RBC+Plasma	RBC	HBOC+Plasma	HBOC	*p value*
Defibrillation following initial reperfusion (joules), median [IQR]	20 [0-48]	25 [18-160]	50 [20-210]	70 [38-288]	0.194
T1 working mode (minutes), mean (SE)	45 (7)	41 (7)	36 (2)	33 (4)	0.353
T3 working mode (minutes), mean (SE)	44 (9)	33 (4)	43 (2)	39 (5)	0.502
T5 working mode (minutes), mean (SE)	44 (6)	36 (5)	34 (3)	38 (4)	0.471
Total working mode (minutes), mean (SE)	138 (21)	110 (5)	113 (3)	110 (6)	0.164
Total EVHP time (minutes), mean (SE)	360 (0)	360 (0)	360 (0)	360 (0)	NA

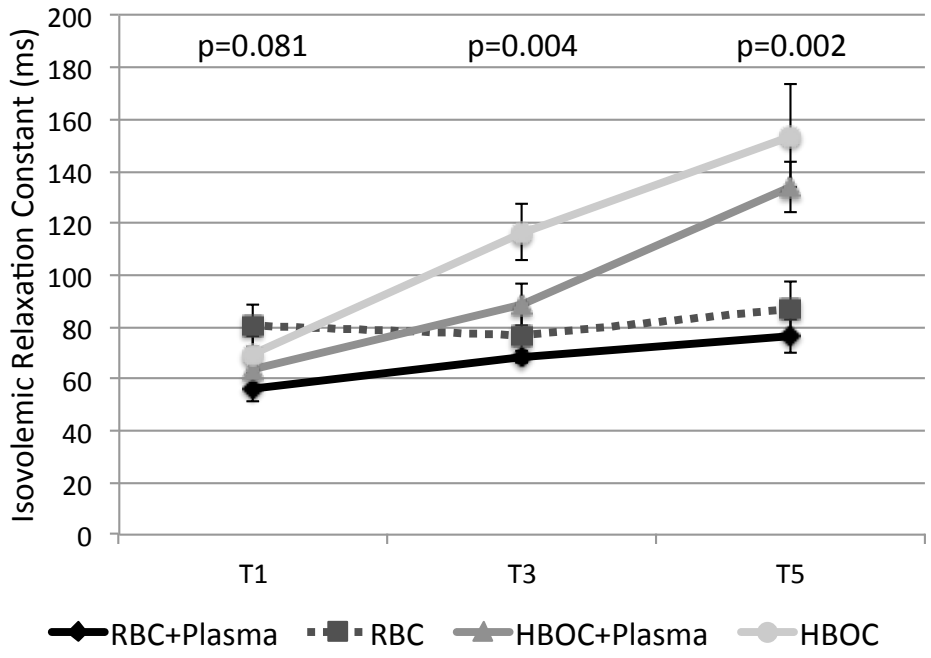
EVHP, ex vivo heart perfusion; *T1*, 1-hour following initiation of *ex vivo* heart perfusion; *T3*, 3-hours following initiation of *ex vivo* heart perfusion; *T5*, 5-hours following initiation of *ex vivo* heart perfusion

Supplement Table 5.2. Perfusate characteristics

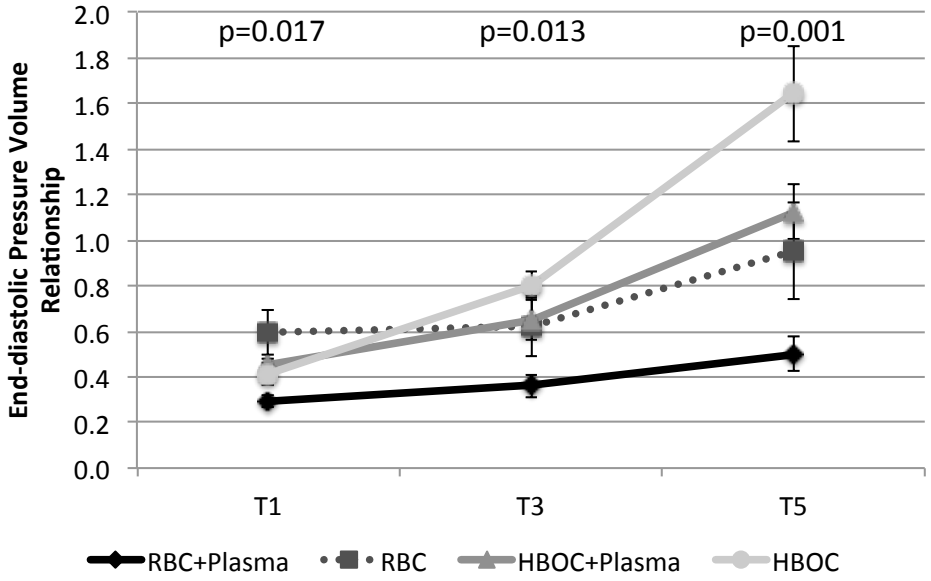
	RBC+Plasma	RBC	HBOC+Plasma	HBOC	*p value*
Osmotic Pressure (mOsmol/kg), mean (SE)	308 (2)	291 (3)	295 (4)	307 (3)	0.007
Oncotic Pressure (mmHg), mean (SE)	33.8 (1.3)	36.1 (0.3)	32.2 (0.5)	37.4 (0.3)	<0.001
Na^+ (mmol/L), mean (SE)	145 (1)	146 (2)	145 (2)	148 (1)	0.506
Cl^- (mmol/L), mean (SE)	115 (1)	116 (2)	110 (2)	116 (1)	0.014
K^+ (mmol/L), mean (SE)	5.6 (0.5)	4.2 (0.1)	4.5 (0.1)	4.6 (0.1)	0.003
Ca^{2+} (mmol/L), mean (SE)	1.1 (0.0)	0.8 (0.0)	1.0 (0.0)	1.0 (0.0)	<0.001
HCO_3^- (mmol/L), mean (SE)	22 (1)	19 (1)	19 (0)	20 (1)	0.008
Glucose (mmol/L), mean (SE)	7.8 (0.7)	7.7 (0.2)	5.2 (0.2)	7.3 (0.9)	0.0310
Hemoglobin (g/L), mean (SE)	43 (2)	39 (1)	39 (0)	36 (0)	<0.001



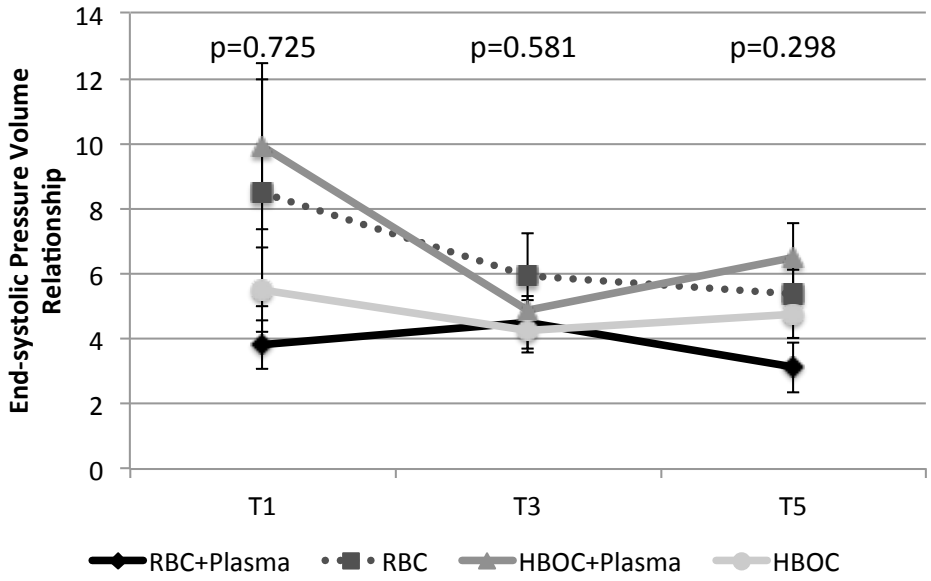
Supplement Figure 5.1. Fold change (T1-T5) in the ratio of IL-1a/IL-10, IL-1b/IL-10, TNF-α /IL-10, INF-γ/IL-10, IL-6/IL-10, IL-8/IL-10.



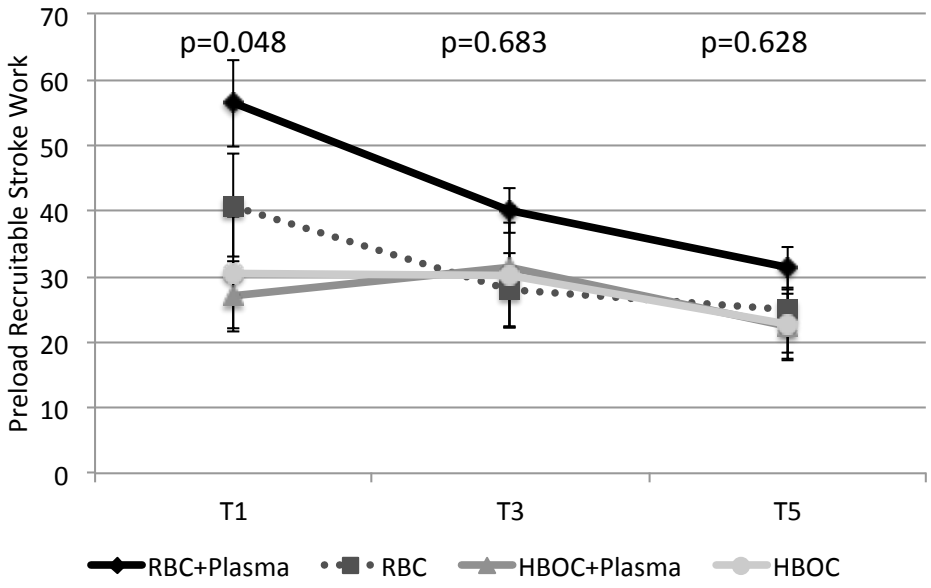
Supplement Figure 5.2. Diastolic function as assessed by the isovolumic relaxation constant at 1 (T1), 3 (T3), and 5 (T5) hours of *ex vivo* heart perfusion.



Supplement Figure 5.3. Diastolic function as assessed by the end-diastolic pressure volume relationship at 1 (T1), 3 (T3), and 5 (T5) hours of *ex vivo* heart perfusion.



Supplement Figure 5.4. Systolic function as assessed by the end-systolic pressure volume relationship at 1 (T1), 3 (T3), and 5 (T5) hours of *ex vivo* heart perfusion.



Supplement Figure 5.5. Systolic function as assessed by the preload recruitable stroke work at 1 (T1), 3 (T3), and 5 (T5) hours of *ex vivo* heart perfusion.

Chapter 6

Assessment of donor heart viability during *ex vivo* heart perfusion

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Chapter 6 Preface

Optimizing donor heart evaluation is a crucial component of a successful DCD transplant protocol (Figure 1.1). Hearts from DCD donors have sustained significant ischemic injury prior to organ procurement, placing these organs at risk of primary graft dysfunction following transplantation. Therefore, it is necessary to assess organ function to ensure only viable organs are transplanted. An evidence based approach to DCD heart evaluation ensures that donor organ utilization is maximized while recipient risk is minimized. In Chapter 6: *Assessment of donor heart viability during ex vivo heart perfusion*, the reliability of various functional and metabolic parameters to identify organs at risk of primary graft dysfunction is investigated. The data presented in this chapter suggests that functional parameters provide the best assessment of donor heart viability during EVHP, while value of metabolic parameters is limited.

Abstract

Ex vivo heart perfusion (EVHP) may facilitate resuscitation of discarded donor hearts and expand the donor pool; however, a reliable means of demonstrating organ viability prior to transplantation is required. Therefore, we sought to identify metabolic and functional parameters that predicted myocardial performance during EVHP. To generate a broad spectrum of organ function, the hearts from 9 normal and 37 donation after circulatory death pigs were perfused *ex vivo*. Functional parameters obtained from a left ventricular conductance catheter, oxygen consumption, coronary vascular resistance, and lactate concentration were measured, and linear regression analyses were performed to identify which parameters best correlated with myocardial performance (cardiac index: mL/minute/gram). Functional parameters exhibited excellent correlation with myocardial performance and demonstrated high sensitivity and specificity for identifying hearts at risk of poor post-transplant function (ejection fraction: R^2=0.80, sensitivity=1.00, specificity=0.85; stoke work: R^2=0.76, sensitivity=1.00, specificity=0.77; dP/dt minimum: R^2=0.74, sensitivity=1.00, specificity=0.54; tau: R^2=0.51, sensitivity=1.00, specificity=0.92), while metabolic parameters were limited in their ability to predict myocardial performance (oxygen consumption: R^2=0.28, coronary vascular resistance: R^2=0.20, lactate concentration: R^2=0.02). We conclude that evaluation of functional parameters provides the best assessment of myocardial performance during EVHP, highlighting the need for an EVHP device capable of assessing the donor heart in a physiologic working mode.

Introduction

Cardiac transplantation is the gold-standard treatment for eligible patients with advanced heart failure; however, it is limited by a critical shortage of suitable donor organs (1). A low utilization rate of hearts that are offered for transplantation contributes to this organ shortage. In North America, only 36-39% of the available donor hearts are actually transplanted and the majority are discarded (2, 3). Echocardiographic evidence of myocardial dysfunction is the most

common reason for donor heart non-utilization (2), which often results from exposure to increased levels of catecholamines in the brain-dead donor (1). However, the majority of myocardium in such hearts is histologically normal and the observed organ dysfunction may be reversible (4). *Ex vivo* heart perfusion (EVHP) has been proposed as a means of resuscitating such discarded donor hearts by providing an opportunity for recovery from catecholamine mediated myocardial injury in an *ex vivo* environment where the hormonal milieu can be controlled.

Donation after circulatory death (DCD) donors may represent an additional source of organs for transplantation. EVHP may facilitate resuscitation of DCD hearts through restoration of intracellular ion homeostasis, support of oxidative metabolism, and delivery of pharmaceutical agents that limit ischemia-reperfusion injury (5, 6). Application of such resuscitation strategies has suggested that DCD hearts can be successfully transplanted in large animal models (7-10).

Non-utilized hearts from brain-dead and DCD donors may represent alternative sources of organs for transplantation and expand the donor pool; however, a reliable method for evaluating donor heart viability prior to transplantation is required before such organs can be utilized clinically (9-11). The Organ Care System Heart (OCS Heart, TransMedics, Andover, USA) is an EVHP apparatus that preserves the donor heart in a normothermic beating state and is currently being evaluated as a means to improve donor heart preservation (ClinicalTrials.gov Identifier: NCT00855712). While OCS Heart preservation occurs in a non-working mode that prevents assessments of myocardial function to be undertaken, this device facilitates monitoring of arterial and venous oxygen saturation, lactate concentration, aortic pressure, and coronary blood flow that have been utilized to predict post-transplant viability (12). Yet, there may be a reluctance to transplant discarded hearts unless sufficient functional recovery can be demonstrated (13, 14).

A variety of *ex vivo* functional parameters have been shown to accurately predict the post-transplant viability of resuscitated hearts (15). Therefore, assessments of myocardial

function may become an important metric for the evaluation and selection of hearts for transplantation in the future (15, 16). In order to inform further device innovation and the development of protocols for the identification of marginal donor hearts suitable for transplantation, we sought to identify which metabolic and functional parameters best predicted myocardial performance during EVHP.

Materials and Methods

All animals received humane care in compliance with the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals*. Institutional Animal Care Committees approved the experimental protocol. To ensure a broad range of myocardial performance conditions over which the discriminatory ability of the various functional and metabolic parameters could be evaluated, the hearts of 9 normal and 37 DCD pigs were procured. Hearts were perfused *ex vivo* in a normothermic beating state and transitioned into a working mode for functional and metabolic assessments.

Donor heart procurement and preparation for EVHP

Female domestic pigs (44±1 kg) were induced with an intramuscular injection of tiletamine (2.4 mg/kg), zolazepam (2.4 mg/kg), and xylazine (0.9 mg/kg). Orotracheal intubation was established and general anesthesia was maintained using mechanical ventilation (10 mL/kg) with 1.5-3% isoflurane. A median sternotomy was performed and 1000 units/kg of heparin was delivered intravenously. 7F and 6F catheters were placed into the internal jugular vein and common carotid artery for monitoring of central venous and aortic pressures, respectively. Normovolemic hemodilution with 1000 mL of warmed Lactated Ringer's solution was undertaken to facilitate the harvest of 500 mL of whole donor blood for priming of the EVHP circuit.

Normal hearts (N=9):

Donor animals were exsanguinated into a Brat2 cell saver (Sorin Group Canada Inc., Burnaby, Canada) and a simultaneous rapid cardiectomy was performed. The heart was emptied of blood and weighed. The aortic cannula was inserted and the heart was immediately connected to the EVHP circuit and perfused via the aortic root at 40 mmHg.

DCD hearts (N=37):

The fraction of inspired oxygen was reduced to 25% and then mechanical ventilation was discontinued. When pulsatility on the arterial pressure tracing was no longer evident, circulatory death was declared and a 15-minute warm ischemic standoff period was observed. Donor animals were then exsanguinated into a Brat2 cell saver (Sorin Group Canada Inc.) and a simultaneous rapid cardiectomy was performed. The heart was emptied of blood, weighed, and reperfused with an oxygenated, normokalemic, adenosine-lidocaine crystalloid cardioplegia for 3 minutes using the MPS2 (Quest Medical Inc., Allen, USA), and then connected to the EVHP circuit and perfused via the aortic root at 40 mmHg.

Ex vivo perfusate solution

The *ex vivo* perfusate solution consisted of 1000 mL of STEEN solution™ (XVIVO Perfusion, Goteborg, Sweden), 500 mL of whole donor blood, and 3.375 grams of piperacillin-tazobactam. STEEN solution is a buffered extracellular-type salt solution with a colloid osmotic pressure optimized for *ex vivo* organ perfusion (17). Additional blood collected during the donor exsanguination was centrifuged and washed with 1000 mL of 0.9% saline solution. The red blood cell concentrate obtained was added to the perfusate solution to achieve a hemoglobin concentration of 45 g/L. Perfusate pH, PO_2, PCO_2, and electrolyte, hemoglobin, and lactate concentrations were measured using the ABL800 Flex Analyzer (Radiometer Medical ApS, Brønshøj, Denmark).

Ex vivo heart perfusion

A Medtronic Affinity NT oxygenator, venous reservoir, 2 modified BioMedicus 540 centrifugal pumps (Medtronic, Minneapolis, Minnesota, USA), and a leukocyte filter (LeukoGuard LG, PALL Medical, Port Washington, USA) were used to construct the EVHP circuit (Figure 6.1). Computer control of the centrifugal pumps with automated feedback loops was used to maintain precise control of aortic diastolic and left atrial pressures.

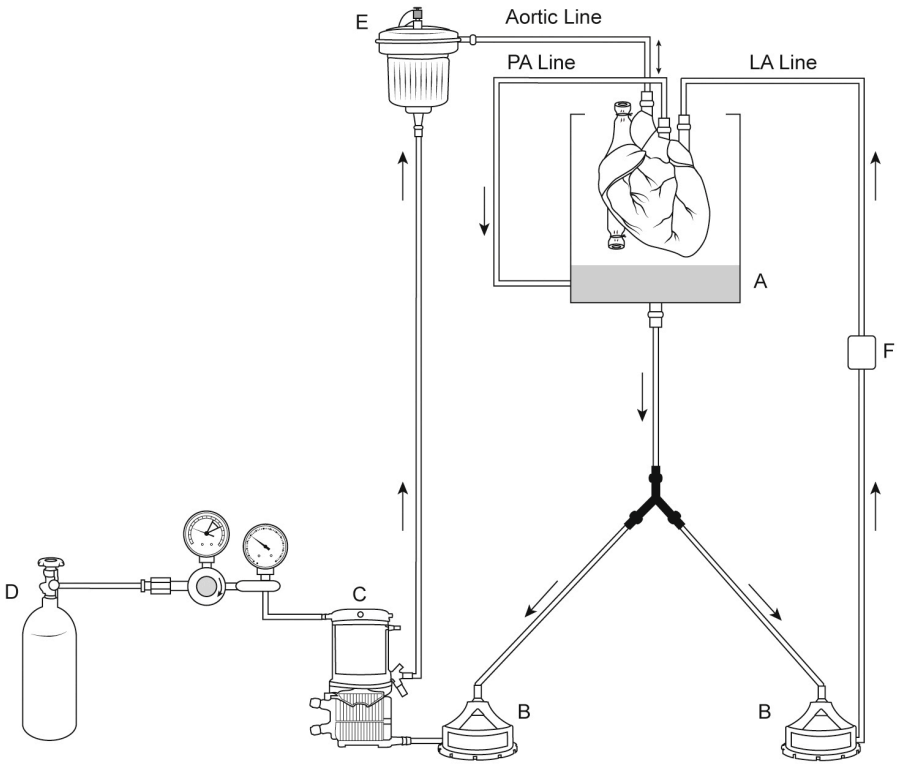

Figure 6.1. Simplified diagram of the *ex vivo* heart perfusion circuit.

The transition from Langendorff perfusion mode (left atrial pressure = 0 mmHg) to working heart mode (left atrial pressure = 8 mmHg) is accomplished by increasing the revolutions/minute on the LA line centrifugal pump, while aortic diastolic pressure (40 mmHg) is maintained by controlling the revolutions/minute on the aortic line centrifugal pump. Myocardial performance is

determined by measuring the flow through the LA line (mL/minute) in working heart mode under precisely controlled preload, afterload, chronotropic, and inotropic conditions.
A, venous reservoir; *B,* centrifugal pumps; *C,* oxygenator/heat exchanger; *D,* oxygen/medical air source; *E,* leukocyte filter; *F,* flow probe; *LA*, left atrium; *PA*, pulmonary artery.

Hearts were initially reperfused at 22°C, an aortic diastolic pressure of 40 mmHg, and a left atrial pressure of -1 mmHg. Hearts were then gradually warmed to 37°C over a 30-minute period, during which time a cannula was placed in the pulmonary artery, a cone shaped left atrial cannula (XVIVO Perfusion) was sewn to the common orifice of the pulmonary veins, and the superior and inferior vena cava were oversewn. Oxygen and gas flow through the membrane oxygenator were titrated to maintain a pH of 7.25-7.35 and a PaO_2 of 100-200 mmHg. Infusions of insulin (2.25 units/hour) and dobutamine (4 mcg/minute) were maintained over the duration of EVHP. At 1 hour following initial reperfusion the left atrial pressure was increased to 8 mmHg and the atria were paced at 110 beats/minute. The transition from an empty, non-working state (left atrial pressure = -1 mmHg) into a physiologic working mode (left atrial pressure = 8 mmHg) was accomplished by increasing the revolutions per minute on the left atrial centrifugal pump (Figure 6.1). Once a steady state was reached, perfusate samples were obtained from the aortic root and coronary sinus, and assessments of myocardial function, metabolism, and coronary blood flow were carried out.

Myocardial performance

Myocardial performance was determined by measuring the flow on the left atrial line, (Bio-Probe Transducer Model TX-40, Medtronic, Minneapolis, Minnesota, USA) indexed for heart weight (mL/minute/gram), that was achieved at a left atrial pressure of 8 mmHg, aortic diastolic pressure of 40 mmHg, and a heart rate 110 beats/minute.

Functional parameters

Nineteen left ventricular functional parameters were obtained using a 5F conductance catheter (Ventri-Cath 507, Millar Inc., Houston, USA) and were analyzed using LabChart 7 Pro Version 7.2.5 (ADInstruments Inc., Colorado Springs, USA). Catheter pressure calibration was performed using a hand-held manometer and volume calibration was carried out following measurement of the perfusate solution conductivity and resistivity. Functional parameters were obtained in a steady state (Figure 6.2) and during a computer-controlled linear reduction in left atrial pressure (Figure 6.3).

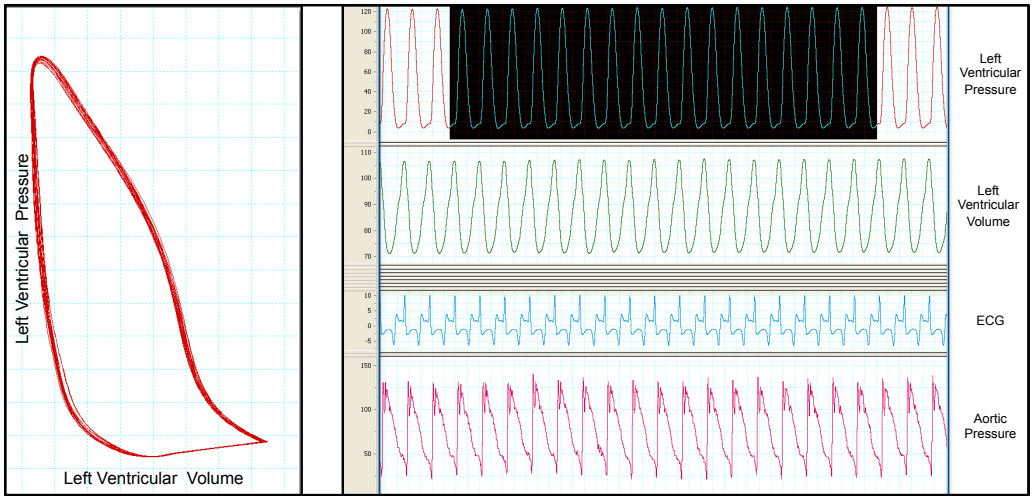

Figure 6.2. Pressure volume loops obtained under steady-state hemodynamic conditions.

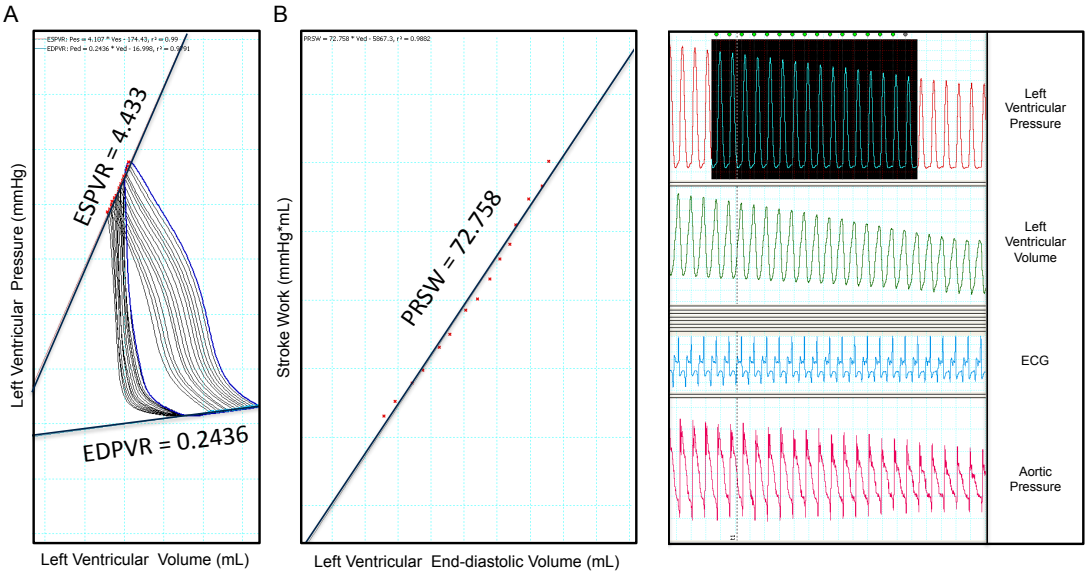

Figure 6.3. Pressure volume loops obtained during a computer controlled linear reduction in revolutions/minute on the LA line centrifugal pump, used to calculate load-independent assessments of myocardial function including A) end-systolic pressure volume relationship, end-diastolic pressure volume relationship, and B) preload recruitable stroke work.

Metabolic parameters

Coronary blood flow (CBF) was determined by averaging 2 timed collections of pulmonary arterial blood flow (18). Coronary vascular resistance (CVR) was then calculated as follows:

$$\text{CVR}\left(\frac{\text{mmHg} * \frac{\text{min}}{\text{mL}}}{100\ \text{grams}}\right) = \frac{\frac{\text{aortic diastolic pressure (mmHg)}}{\text{CBF}\ \left(\frac{\text{mL}}{\text{min}}\right)}}{100\ \text{grams heart weight}}$$

Oxygen consumption (MVO_2) was determined by multiplying CBF with the arterial-venous oxygen content difference as follows:

$$\mathrm{MVO2}\left(\frac{\frac{\mathrm{mLO2}}{\mathrm{minute}}}{100\ \mathrm{grams}}\right)=\frac{(\mathrm{CaO2}-\mathrm{CvO2})*\mathrm{CBF}}{100\ \mathrm{grams\ heart\ weight}}$$

Arterial (CaO_2) and venous (CvO_2) oxygen content were calculated as follows:

$$\mathrm{CaO2}\left(\frac{\mathrm{mLO2}}{100\mathrm{mL}}\right)=\left(1.34\left(\frac{\mathrm{mLO2}}{\mathrm{gram\ hemoglobin}}\right)*\mathrm{hemoglobin\ concentration}\left(\frac{\mathrm{grams}}{100\ \mathrm{mL}}\right)*\right.$$

$$\left.\mathrm{oxygen\ saturation}\ (\%)\right)+\left(0.00289\left(\frac{\frac{\mathrm{mLO2}}{\mathrm{mmHg}}}{100\ \mathrm{mL}}\right)*\mathrm{PaO2}\ (\mathrm{mmHg})\right),$$

and

$$\mathrm{CvO2}\left(\frac{\mathrm{mLO2}}{100\mathrm{mL}}\right)=\left(1.34\left(\frac{\mathrm{mLO2}}{\mathrm{gram\ hemoglobin}}\right)*\mathrm{hemoglobin\ concentration}\left(\frac{\mathrm{grams}}{100\ \mathrm{mL}}\right)*\right.$$

$$\left.\mathrm{oxygen\ saturation}\ (\%)\right)+\left(0.00289\left(\frac{\frac{\mathrm{mLO2}}{\mathrm{mmHg}}}{100\ \mathrm{mL}}\right)*\mathrm{PvO2}\ (\mathrm{mmHg})\right),$$

where PaO_2 and PvO_2 represent the partial pressure of oxygen in blood samples from the aortic root and coronary sinus, respectively.

Oxygen extraction was calculated as follows:

$$\mathrm{O2\ extraction}\ (\%)=\ 1\ -\ \left(\frac{\mathrm{CvO2}}{\mathrm{CaO2}}\right)*\ 100.$$

Myocardial lactate metabolism was determined by measuring the difference in lactate concentration between coronary sinus and aortic root blood samples as follows:

$$\mathrm{VA\ lactate\ difference}\left(\frac{\mathrm{mmol}}{\mathrm{L}}\right)=\ \mathrm{Coronary\ sinus\ lactate}\left(\frac{\mathrm{mmol}}{\mathrm{L}}\right)-\ \mathrm{Arterial\ lactate}\left(\frac{\mathrm{mmol}}{\mathrm{L}}\right).$$

Statistical Analysis

Normally distributed continuous variables were reported as mean ± standard error (SE) and non-normally distributed continuous variables were reported as median [range]. To determine the proportion of the total variation in myocardial performance that was explained by each functional and metabolic parameter examined, a linear regression for each variable was performed and the correlation coefficient (R^2) was calculated. The sensitivity and specificity of each metabolic and functional parameter to identify hearts with a cardiac index < 11 mL/minute/gram was also determined. A cutoff value was defined for each parameter that optimized the sensitivity of the test. Successful transplantation of hearts achieving an equivalent cardiac index during EVHP has been demonstrated previously (19), and therefore represents a conservative cutoff for identifying hearts at risk of developing post-transplant graft failure. However, these cutoff values should not be extrapolated beyond the scope of this study and are meant to be hypothesis generating only. A *p*-value <0.05 was considered statistically significant. Analyses were performed using GraphPad Prism V6.0c (GraphPad Software Inc., La Jolla, USA).

Results

The electrolyte composition, osmotic, and oncotic pressures of the perfusate solution are displayed in Supplementary Table 6.1. Prior to the initiation of EVHP, normal and DCD hearts (225±3 grams) sustained 4.6±0.2 and 27.6±0.3 minutes of warm-ischemia, respectively. This resulted in a broad range of myocardial performance conditions (cardiac index range: 1.2 – 19.4 mL/minute/gram, cardiac output range: 250 – 4050 mL/minute) over which the discriminatory ability of the various functional and metabolic parameters could be evaluated. To improve the reliability of each assessment, the preload (left atrial pressure = 8±0 mmHg), afterload (aortic diastolic pressure = 40±0 mmHg), chronotropic (heart = 108±2 beats/minute), and inotropic (dobutamine infusion = 4 mcg/minute) conditions were precisely controlled in each heart.

Functional assessments

The correlation coefficients for each functional parameter examined are displayed in Table 6.1. There was significant variation in the ability of the measured functional parameters to predict myocardial performance (R^2 range: 0.002 – 0.795). The best functional predictors (6.4) included assessments of systolic function (ejection fraction, R^2 = 0.795, p<0.001), myocardial work (stroke work, R^2 = 0.759, p<0.001), and diastolic function (dP/dt minimum, R^2 = 0.738, p<0.001).

Metabolic assessments

The correlation coefficients for each metabolic parameter examined are displayed in Table 6.1. There was significant variation in the ability of the measured metabolic parameters to predict myocardial performance (R^2 range: 0.001 – 0.283). MVO_2 (R^2 = 0.283, p<0.001) and coronary vascular resistance (R^2 = 0.200, p=0.002) demonstrated statistically significant correlation with myocardial performance; however, arterial (R^2 = 0.019, p=0.360) and venous (R^2 = 0.001, p=0.833) lactate concentration, venoarterial lactate difference (R^2 = 0.006, p=0.610), and oxygen extraction (R^2 = 0.029, p=0.261) failed to demonstrate a significant correlation (Figure 6.5).

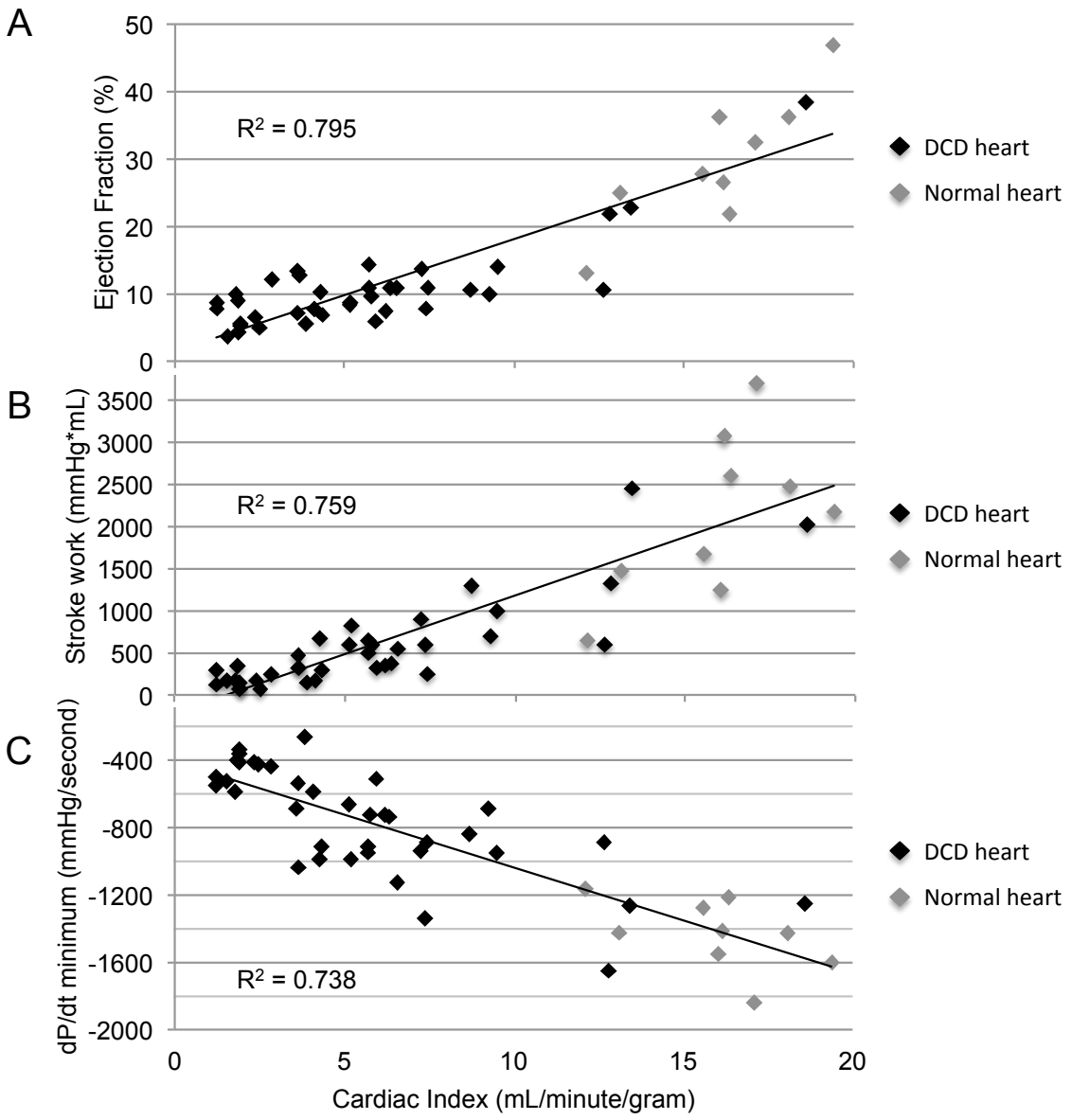

Figure 6.4. Linear regression models for A) ejection fraction (%), B) stroke work (mmHg*mL), and C) dP/dt minimum (mmHg/second).

Table 6.1. Correlation coefficients for functional and metabolic parameters examined

Functional parameter	Correlation coefficient (R^2)	*p* value
End-systolic volume (mL)	0.081	0.055
End-diastolic volume (mL)	0.004	0.658
Ejection fraction (%)	0.795	<0.001
End-systolic pressure (mmHg)	0.512	<0.001
End-diastolic pressure (mmHg)	0.202	0.002
Developed pressure (mmHg)	0.569	<0.001
Stroke work (mmHg*mL)	0.759	<0.001
Arterial elastance (mmHg/mL)	0.404	<0.001
Maximum power (mmHg*mL/second)	0.202	0.002
dP/dt maximum (mmHg/second)	0.537	<0.001
dP/dt minimum (mmHg/second)	0.738	<0.001
Pressure at dP/dt maximum (mmHg)	0.047	0.146
dV/dt maximum (mL/second)	0.616	<0.001
dV/dt minimum (mL/second)	0.321	<0.001
Pressure at dV/dt maximum (mmHg)	0.216	0.001
Tau (millisecond)	0.508	<0.001
End-systolic pressure volume relationship	0.012	0.466
End-diastolic pressure volume relationship	0.143	0.010
Preload recruitable stroke work	0.201	0.002
Metabolic parameter	**Correlation coefficient (R^2)**	***p* value**
MVO_2 (mL O_2 / minute / 100 grams)	0.283	<0.001
CVR (mmHg*min / mL / 100 grams)	0.198	0.002
Oxygen extraction (%)	0.029	0.261
Arterial lactate concentration (mmol/L)	0.019	0.360
Venous lactate concentration (mmol/L)	0.001	0.833
Venoarterial lactate difference (mmol/L)	0.006	0.610

CVR, coronary vascular resistance; *MVO_2*, myocardial oxygen consumption; *Tau*, isovolumic relaxation time constant.

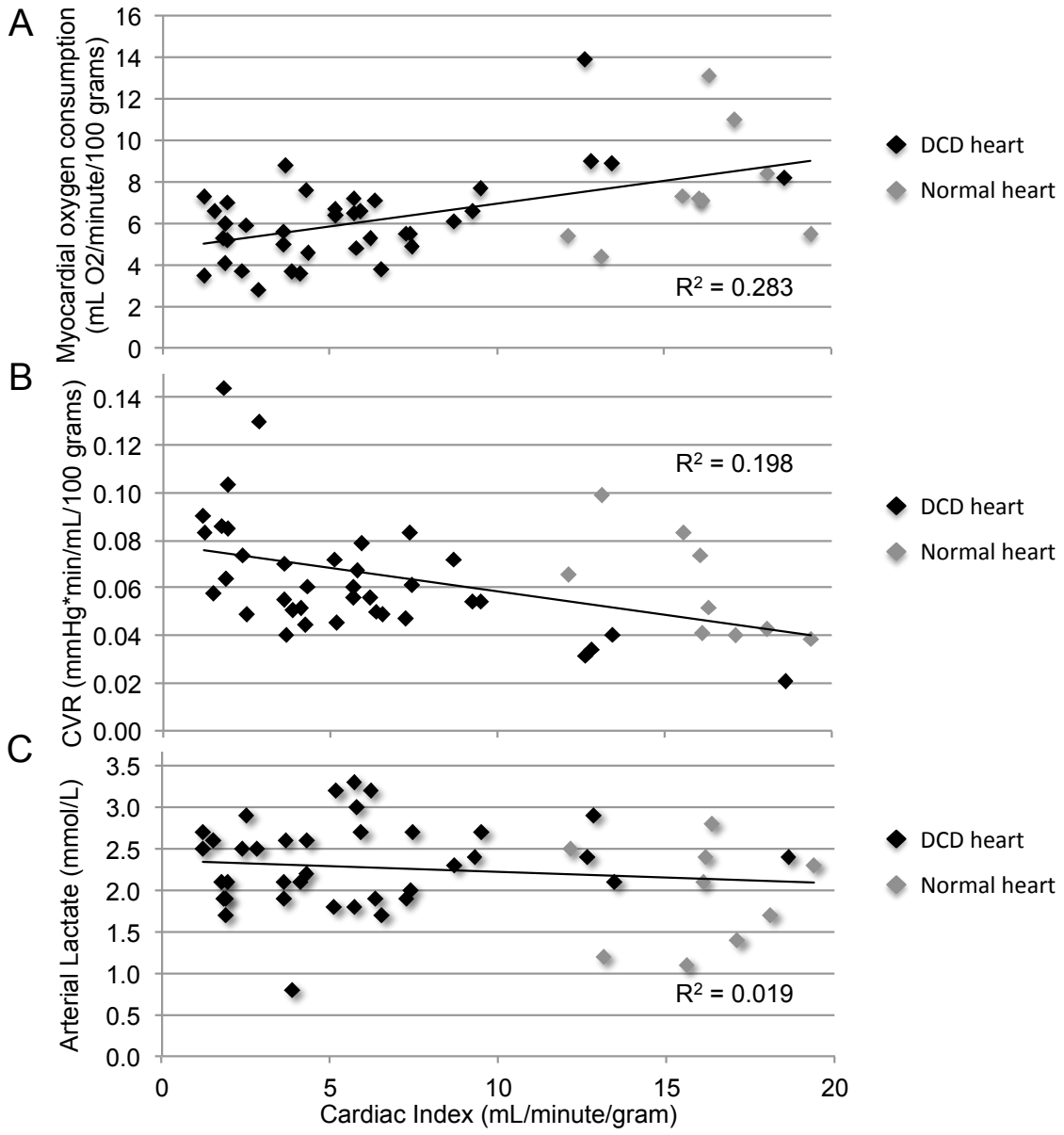

Figure 6.5. Linear regression models for A) myocardial oxygen consumption (mL O_2/minute/100grams), B) coronary vascular resistance (mmHg*min/mL/100 grams), C) arterial lactate concentration (mmol/L).

Sensitivity and specificity analysis

The sensitivity and specificity of metabolic and functional parameters to identify hearts at risk of developing post-transplant graft failure (cardiac index < 11 mL/minute/gram) are

displayed in Table 6.2. Cutoff values were identified that optimized the sensitivity of each parameter (a negative test would exclude the possibility of poor performance). At these cutoff values, functional parameters (specificity: tau = 0.92, ejection fraction = 0.85, stroke work = 0.77, dP/dt minimum = 0.54) demonstrated a greater ability than metabolic assessments (specificity: MVO_2 = 0.39, coronary vascular resistance = 0.38, oxygen extraction = 0.14, arterial lactate concentration = 0.23) to correctly identify hearts with poor function.

Table 6.2. Sensitivity and specificity of functional and metabolic parameters to identify hearts with a cardiac index < 11mL/minute/gram.

Functional parameter	Cutoff Value	Sensitivity (95% CI)	Specificity (95% CI)	Likelihood Ratio
Tau (milliseconds)	> 44	1.00 (0.89-1.00)	0.92 (0.64-1.00)	13.00
Ejection fraction (%)	< 18	1.00 (0.89-1.00)	0.85 (0.55-0.98)	6.50
Stroke work (mmHg*mL)	< 1309	1.00 (0.89-1.00)	0.77 (0.46-0.95)	4.33
dP/dt minimum (mmHg/seconds)	> -1377	1.00 (0.89-1.00)	0.54 (0.25-0.81)	2.17
dV/dt maximum (mL/seconds)	< 455	1.00 (0.89-1.00)	0.38 (0.14-0.68)	1.63
dP/dt maximum (mmHg/seconds)	< 1562	1.00 (0.89-1.00)	0.31 (0.09-0.61)	1.44
Developed pressure (mmHg)	< 153	1.00 (0.89-1.00)	0.15 (0.02-0.45)	1.18
Metabolic parameter	**Cutoff Value**	**Sensitivity (95% CI)**	**Specificity (95% CI)**	**Likelihood Ratio**
MVO_2 (mL O_2/minute/100 grams)	< 8.8	1.00 (0.89-1.00)	0.39 (0.14-0.69)	1.63
CVR (mmHg*min/mL/100 grams)	> 0.040	1.00 (0.89-1.00)	0.38 (0.09-0.61)	1.44
Oxygen extraction (%)	> 17	0.97 (0.84-1.00)	0.14 (0.02-0.43)	0.97
Venoarterial lactate difference (mmol/L)	< 5.75	0.97 (0.84-1.00)	0.00 (0.00-0.25)	0.97
Arterial lactate concentration (mmol/L)	> 1.55	0.97 (0.84-1.00)	0.23 (0.05-0.54)	1.26
Venous lactate concentration (mmol/L)	< 4.95	0.97 (0.84-1.00)	0.15 (0.02-0.45)	1.15

CVR, coronary vascular resistance; *dP/dt,* rate of pressure change; *dP/dt,* rate of volume change; MVO_2, myocardial oxygen consumption; *Tau*, isovolumic relaxation time constant.

Discussion

EVHP may facilitate resuscitation and preservation of donor hearts deemed unsuitable for transplantation and expand the donor pool; however, to minimize the risk of primary graft

dysfunction it is necessary to demonstrate organ viability prior to transplantation (6). Our results suggest that assessments of systolic function (ejection fraction), diastolic function (dP/dt minimum), and myocardial work (stroke work) provide the most reliable estimates of myocardial performance during EVHP. This highlights the need for an EVHP device capable of performing assessments of myocardial function in a physiologic working mode, and suggests that metabolic assessments, such as lactate metabolism, may not provide a reliable evaluation of organ viability.

Several methods have been utilized to predict organ viability during EVHP. Two-dimensional echocardiography has been used to determine the fractional area reduction as a marker of myocardial contraction (20). Magnetic resonance imaging has been used to measure the fractional anisotropy of arrested heats during EVHP to characterize cellular integrity and differentiate reversible from lethal injury (11). Biomarkers such as troponin-I (10), caspase-3 (11), and endothelin-1 (11) have been quantified to determine the severity of injury sustained by the donor heart. Oxygen consumption has been measured as an assessment of myocardial metabolic function (20). Measurement of perfusate lactate concentration during EVHP has also been proposed as a means of predicting post-transplant graft failure (12). Lactic acid is a byproduct of anaerobic glucose metabolism and may be used as a surrogate marker for insufficient oxygen delivery or compromised oxidative metabolism. Hamed *et al* (12) found serum lactate to be the most powerful predictor of post-transplant graft failure (lactate > 4.96 mmol/L, sensitivity = 0.63, specificity = 0.98); however, this evaluation did not include any assessments of myocardial function. While a high perfusate lactate concentration correctly identified hearts that developed primary graft failure (high specificity), its ability to rule out the possibility of graft dysfunction in hearts with a low lactate was much less (low sensitivity). This is exemplified by a recent case report describing the preservation of an ideal donor heart on the OCS Heart over an 8.4-hour period (21). Despite the maintenance of normal perfusion parameters and lactate levels during the preservation interval, the heart became edematous and

primary graft dysfunction occurred following transplantation that required support with extracorporeal membrane oxygenation. It is imperative that *ex vivo* parameters utilized to evaluate the viability of discarded donor hearts have a high sensitivity in order to reduce the risk of transplanting a non-viable organ.

We determined cutoff values for functional and metabolic parameters that optimized the sensitivity for predicting a cardiac index < 11 mL/minute/gram, in order to simulate the ability of these parameters to identify hearts at risk of post-transplant graft failure. At these cutoff values, functional parameters maintained high specificity for identifying non-viable organs; however, MVO_2, coronary vascular resistance, and lactate concentration were limited in their ability to identify organs with poor function. These results emphasize the importance of myocardial functional parameters in discriminating between viable and non-viable organs for transplantation.

Reproducible, reliable, and easily acquired parameters are required to assess myocardial function prior to transplant. Pressure-volume loop catheters have been utilized extensively and provide a broad range of myocardial functional metrics. Those most commonly reported include dP/dt maximum and minimum (10, 11, 15, 16, 20, 22 - 24), developed pressure (11, 22, 23), stroke work (16, 24), preload recruitable stroke work (10, 16), end systolic pressure volume relationship (9, 10, 15, 16), and the end diastolic pressure volume relationship (10, 16). However, which parameters most reliably predict myocardial performance is not well established. Our results suggest that some commonly reported functional parameters exhibit poor correlation with myocardial performance during EVHP and should not be used. Conversely, ejection fraction, stroke work, dP/dt minimum, and Tau demonstrate excellent correlation with myocardial performance during EVHP, exhibit a high sensitivity and specificity for identifying non-viable organs, and represent robust functional parameters. However, it is important that these parameters be acquired under equivalent preload, afterload, chronotropic, and inotropic conditions to ensure that serial assessments and those reported in different studies can be

reliably compared. This requires an EVHP device capable of precisely controlling such parameters.

Left ventricular ejection fraction is a well-established echocardiographic parameter utilized for the assessment of systolic function. An *in vivo* ejection fraction >40% is employed clinically as a cutoff for identifying donor hearts suitable for transplantation (25). The results presented here suggest that conductance catheter assessment of ejection fraction provides a reliable assessment of donor heart function in the *ex vivo* environment. Recent reports have also demonstrated that the ejection fraction of a heart in a physiologic working mode can be measured during EVHP using 2D transesophageal echocardiography (26). Therefore, *ex vivo* assessments of ejection fraction can be acquired using technology that is readily available in the clinical setting and may provide reliable evaluations of donor heart viability prior to transplantation. For this to be realized, standardization of the echocardiographic approach to the *ex vivo* perfused heart will be required.

Stroke work represents the mechanical work performed during systole and represents a comprehensive assessment of myocardial function, being most accurately determined by measuring the area confined within the pressure-volume loop (27). Stroke work can also be estimated from the product of stroke volume and developed pressure (27), which could be determined through non-invasive assessments of stroke volume (2D echocardiography) and developed pressure (aortic pressure minus left atrial pressure). Such an approach could eliminate the need for conductance catheter assessment and facilitate translation of such a functional parameter to clinical practice.

dP/dt minimum (maximum rate of ventricular pressure decline) and tau (relaxation time constant) characterize the isovolumic relaxation phase of diastole (28). In contrast to the process of calcium release during systole, relaxation during diastole is an energy-dependent process that requires high-energy phosphates for the reuptake of intracellular calcium into the sarcoplasmic reticulum and deactivation the troponin-tropomyosin complex (29). Under normal conditions the

rate of calcium reuptake is rapid, energy stored in compressible interstitial elements during systolic contraction is released during diastole, and relaxation occurs quickly (28). However, intracellular calcium overload, alterations to calcium handling, myofibril contracture, and interstitial edema that occur following ischemia and reperfusion impair the rate of calcium reuptake, reduce elastic recoil properties of the myocardium, and result in diastolic dysfunction (28, 29). This might explain why impaired diastolic function has been observed despite relatively preserved systolic function following transplantation (15), and why dP/dt minimum and Tau exhibited good correlation with myocardial performance and reliably identified poorly functioning hearts in our study.

MVO_2 represents the amount of oxygen consumed by the myocardium while performing work, and therefore reflects both the presence of functioning myocardium capable of consuming oxygen and intact coronary endothelial cells capable of increasing coronary flow to meet metabolic demands of the heart. MVO_2 can be measured non-invasively and was the best metabolic correlate of myocardial performance; however, it had a lower coefficient of determination compared to many of the functional parameters examined, and the likelihood ratio for identifying non-viable organs was limited. Similarly, lactate concentration did not demonstrate a significant correlation with myocardial performance and may have a limited role in pre-transplant assessments of donor heart viability.

This study has some limitations. We investigated metabolic and functional parameters that predicted myocardial performance during EVHP; however, transplant studies are required to determine cutoff values of these parameters that predict post-transplant graft failure. The cutoff values presented here are theoretical and should not be extrapolated beyond the scope of this study. Functional and metabolic parameters were only measured at a single point during EVHP; however, repeated assessments during the *ex vivo* preservation interval may be a more clinically relevant approach. We only evaluated predictors of left ventricular performance in this study and a comprehensive evaluation of donor heart viability during EVHP should also include right

ventricular functional assessments.

We conclude that functional parameters evaluating systolic function (ejection fraction), diastolic function (dP/dt minimum) and myocardial work (stroke work) provide the most reliable assessment of myocardial performance during EVHP, while metabolic assessments may be limited in their ability to identify organs at high risk of post-transplant graft failure. Further confirmatory transplant studies are required to establish cutoff values and/or trends in a broad range of *ex vivo* functional, biochemical, and metabolic parameters that predict post-transplant graft failure. This highlights the need for an EVHP device capable of assessing the donor heart in a physiologic working mode.

Chapter 6 Summary

In Chapter 6: *Assessment of donor heart viability during ex vivo heart perfusion*, it was demonstrated that assessments of myocardial function are the most reliable indicators of donor heart viability during EVHP, while metabolic parameters are of limited value. These results highlight the need for a clinical EVHP device capable of assessing donor heart function in a physiologic working mode. The results of this chapter must be confirmed in transplant studies in order to identify cutoff values of these parameters that predict successful transplantation.

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Supplemental Information

Supplementary Table 6.1. *Ex vivo* perfusate characteristics

Variable	Mean (SE)
pH	7.30 (0.01)
PaO_2 (mmHg)	181 (10)
$PaCO_2$ (mmHg)	31 (1)
Hemoglobin (g/L)	45 (1)
Potassium (mmol/L)	3.7 (0.0)
Sodium (mmol/L)	146 (1)
Calcium (mmol/L)	0.91 (0.01)
Chloride (mmol/L)	120 (1)
Glucose (mmol/L)	7.2 (0.1)
Osmotic pressure (mOsmol/kg)	299 (1)
Oncotic pressure, (mmHg)	33 (1)

$PaCO_2$, arterial partial pressure of carbon dioxide; *PaO_2,* arterial partial pressure of oxygen.

Chapter 7

Avoidance of profound hypothermia during initial reperfusion improves the functional recovery of hearts donated after circulatory death

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Chapter 7 Preface

Optimizing donor heart resuscitation is a crucial component of a successful DCD transplant protocol (Figure 1.1). Initial reperfusion of ischemic myocardium with a cardioplegic solution provides an opportunity to restore ionic homeostasis in the myocyte, and has been shown to minimize ischemia–reperfusion injury and improve functional recovery. Previous authors have suggested that the reparative processes that are necessary to resuscitate the DCD heart may be inhibited if the cardioplegic solution is delivered under profoundly hypothermic conditions; however, initial reperfusion temperature has not been systemically examined. In Chapter 7: *Avoidance of profound hypothermia during initial reperfusion improves the functional recovery of hearts donated after circulatory death*, the impact of initial reperfusion temperature on myocardial injury and DCD heart resuscitation is explored. The data presented in this chapter suggests that the avoidance of profound hypothermia during the initial reperfusion of DCD hearts minimizes injury and optimizes DCD heart resuscitation.

Abstract

The resuscitation of hearts donated after circulatory death (DCD) is gaining widespread interest; however, the method of initial reperfusion (IR) that optimizes functional recovery has not been elucidated. We sought to determine the impact of IR temperature on myocardial functional recovery during *ex vivo* heart perfusion (EVHP). Eighteen pigs were anesthetized, mechanical ventilation was discontinued, and cardiac arrest ensued. A 15-minute standoff period was observed and then hearts were reperfused for 3 minutes at 3 different temperatures (5°C; N=6, 25°C; N=5, and 35°C; N=7) with a normokalemic adenosine-lidocaine crystalloid cardioplegia. Hearts then underwent normothermic EVHP for 6 hours during which time myocardial function was assessed in working mode. We found that IR coronary blood flow differed among treatment groups (5°C=483±53, 25°C=722±60, 35°C=906±36 mL/minute, $p<0.01$). During subsequent EVHP, less myocardial injury (troponin-I: 5°C=91±6, 25°C=64±16, 35°C=57±7 pg/mL/gram, $p=0.04$) and greater preservation of endothelial cell integrity (electron microscopy injury score: 5°C=3.2±0.5, 25°C=1.8±0.2, 35°C=1.7±0.3, $p=0.01$) were evident in hearts initially reperfused at warmer temperatures. IR under profoundly hypothermic conditions impaired the recovery of myocardial function (cardiac index: 5°C=3.9±0.8, 25°C=6.2±0.4, 35°C=6.5±0.6 mL/minute/gram, $p=0.03$) during EVHP. We conclude that the avoidance of profound hypothermia during IR minimizes injury and improves the functional recovery of DCD hearts.

Introduction

Cardiac transplantation remains the gold-standard treatment for eligible patients with advanced heart failure. A critical shortage of suitable organs from traditional brain dead donors has prompted a renewed interest in donation after circulatory death (DCD) to expand the donor pool; however, following donor extubation the DCD heart is forced to function in an increasingly hypoxemic environment while attempting to maintain systemic oxygen delivery. Consequently, the DCD heart experiences significant global ischemia during the hypoxemic cardiac arrest and warm-ischemic standoff period that ethically define death (1). Subsequent reperfusion causes intracellular calcium overload and the propagation of myocyte death through the development of hypercontracture, activation of calcium-dependent proteases, generation of reactive oxygen species, activation of the mitochondrial permeability transition pore, and initiation of apoptotic pathways (2-4). Concern regarding the degree of ischemic injury suffered following donor extubation, and the severity of reperfusion injury sustained at the time of organ procurement has limited the clinical transplantation of DCD hearts.

An extensive body of literature supports the notion that therapeutic interventions delivered at the onset of reperfusion provide a significant opportunity to resuscitate the ischemic heart; however, the therapeutic window is narrow and optimal success is realized in the first minutes after reperfusion (5, 6). Therefore in the DCD context, the pathogenesis of ischemia-reperfusion injury can be mitigated by optimizing the composition of the initial reperfusion solution that is delivered at the time of organ procurement. Administration of reactive oxygen species scavengers, sodium-hydrogen exchange inhibitors, and activators of ischemic postconditioning pathways (I.E. erythropoietin, glyceryl trinitrate, adenosine, insulin) have been shown to minimize reperfusion injury and improve the functional recovery of DCD hearts (7-10). Clinical application of this tailored approach has now resulted in the successful transplantation of human hearts donated after circulatory death (11).

Optimizing the conditions of the initial reperfusion may further improve DCD heart resuscitation. Initial reperfusion with a cardioplegic solution inhibits the myocardial contractile unit at the onset of reoxygenation, facilitates the repletion of myocardial energy stores and restoration of intracellular ion homeostasis prior to myocardial contraction, and prevents the development of myocardial hypercontracture (12, 13). However, these reparative processes may be inhibited if the cardioplegia is delivered under profoundly hypothermic conditions. Hypothermia markedly lowers the activity of the ion pumps that are required to restore intracellular ion homeostasis in the DCD heart and may exacerbate ischemia-reperfusion injury (14-17). Previous studies have suggested that the avoidance of profound hypothermia during initial reperfusion may support aerobic metabolism and improve functional recovery (12, 18), yet the optimal temperature for the initial reperfusion of DCD hearts has not been previously investigated. Therefore, we sought to determine if the avoidance of profound hypothermia during initial reperfusion would minimize myocardial injury and improve the functional recovery of DCD hearts during *ex vivo* heart perfusion (EVHP).

Materials and Methods

All animals received humane care in compliance with the National Institute of Health's *Guide for the Care of Laboratory Animals*. Institutional Animal Care Committees approved the experimental protocol. Eight-teen DCD pig hearts were allocated to 3 treatment groups according to the temperature of the initial reperfusion solution that was delivered at the time of organ procurement: 5°C (N=6), 25°C (N=5), 35°C (N=7). In addition, the hearts from 3 pigs that did not undergo the DCD withdrawal protocol were included as normal controls.

DCD heart procurement

Pigs (42±1 kg) were sedated with an intramuscular injection of tiletamine (2.4mg/kg), zolazepam (2.4 mg/kg), and xylazine (0.9 mg/kg). Orotracheal intubation was established and general anesthesia was maintained with 2-3% isoflurane. A median sternotomy was performed and 1000 units/kg of heparin was delivered intravenously. 7F and 6F catheters were placed into the internal jugular vein and common carotid artery for monitoring of central venous and aortic pressures, respectively. Normovolemic hemodilution with 1000 mL of warmed Lactated Ringer's solution was undertaken to facilitate the harvest of 500 mL of whole donor blood for priming of the EVHP circuit. The fraction of inspired oxygen was reduced to 25% and then mechanical ventilation was discontinued. When pulsatility on the arterial pressure tracing was no longer evident, circulatory death was declared and a 15-minute warm ischemic standoff period was observed (Figure 7.1). Donor animals were then exsanguinated into a Brat2 cell saver (Sorin Group Canada Inc., Markham, Canada) and a simultaneous rapid cardiectomy was performed. The heart was emptied of blood, weighed, and the aortic cannula was inserted.

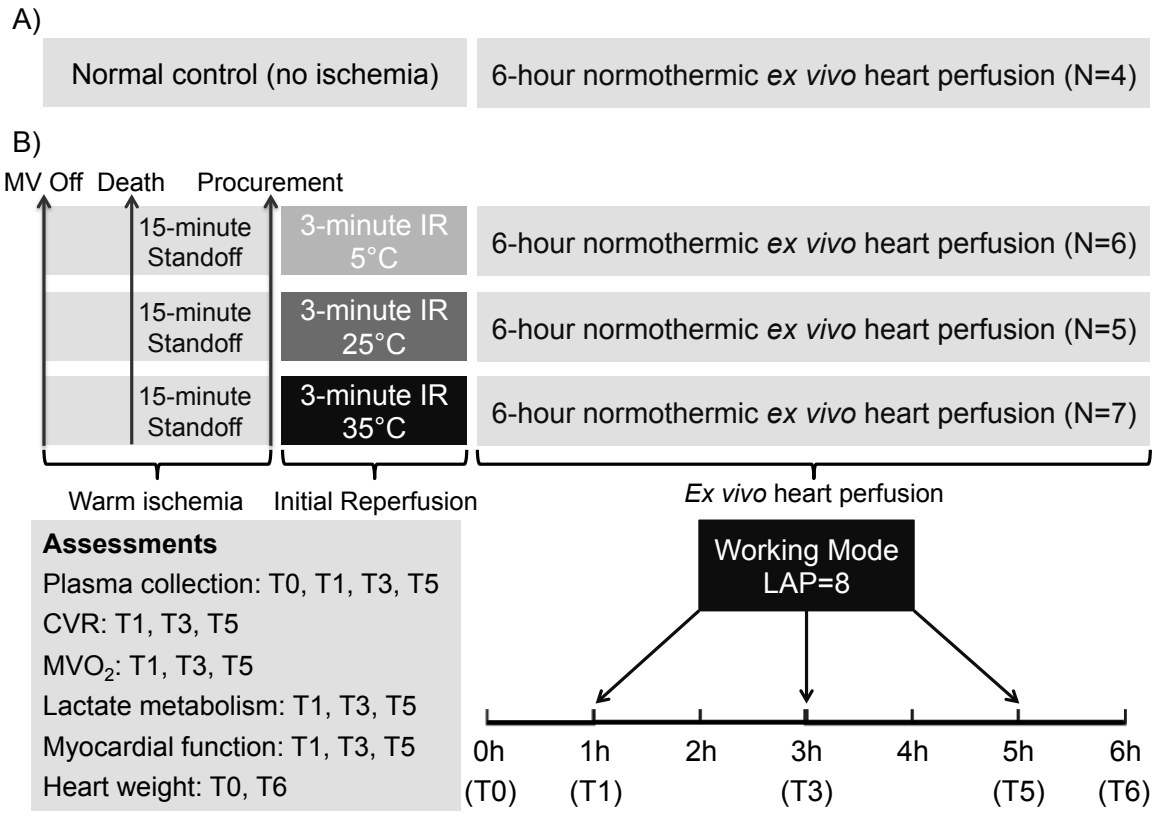

Figure 7.1. Experimental protocol for A) normal control hearts (N=4), and B) hearts donated after circulatory death that were reperfused at 5°C (N=6), 25°C (N=5), and 35°C (N=7).

CVR, coronary vascular resistance; *IR*, initial reperfusion with normokalemic adenosine-lidocaine crystalloid cardioplegia; *LAP*, left atrial pressure; *MV*, mechanical ventilation; MVO_2, myocardial oxygen consumption

DCD heart initial reperfusion

Hearts were assigned to 3 treatment groups according to the temperature of the initial reperfusion solution: 5°C (N=6), 25°C (N=5), and 35°C (N=7). Following procurement hearts were reperfused for 3 minutes at the target temperature and an aortic root pressure of 40 mmHg (Figure 7.1). The Myocardial Protection System (MPS) 2 (Quest Medical Inc., Allen, USA) was utilized to precisely control the pressure and temperature during the initial reperfusion.

Myocardial temperature was also monitored using a temperature probe inserted into the anterolateral wall of the left ventricle.

The initial reperfusion solution was a normokalemic adenosine-lidocaine cardioplegia prepared daily, and was comprised of NaCl (111.8 mmol/L), KCl (5.9 mmol/L), $CaCl_2$ (0.22 mmol/L), $MgCl_2$ (2.6 mmol/L), $NaHCO_3$ (32 mmol/L), NaH_2PO_4 (1.2 mmol/L), glucose (10 mmol/L), mannitol (120 mmol/L), pyruvate (1 mmol/L), reduced glutathione (3 mmol/L), adenosine (400 μmol/L), lidocaine (500 μmol/L), and insulin (10 IU/L). This solution was developed based on previous studies demonstrating improved myocardial protection compared with depolarizing hyperkalemic cardioplegic solutions (46). The solution was rendered hypocalcemic to minimize calcium influx via reverse sodium-calcium exchange during the initial reperfusion (46, 47). In addition, reduced glutathione was included as an antioxidant, pyruvate as a metabolic substrate, and adenosine and insulin as ischemic postconditioning agents (4).

Normal control heart procurement

Normal control animals (N=4) were anesthetized and prepared in the same fashion as the DCD donor animals. Following normovolemic hemodilution, the donor animals were exsanguinated and a simultaneous rapid cardiectomy was performed. The heart was emptied of blood, weighed, the aortic cannula was inserted, and the heart was immediately connected to the EVHP circuit.

Ex vivo heart perfusion

A Medtronic Affinity NT oxygenator, venous reservoir, 2 modified BioMedicus 540 centrifugal pumps (Medtronic, Minneapolis, Minnesota, USA), and a leukocyte filter (LeukoGuard LG, PALL Medical, Port Washington, USA) were used to construct the EVHP circuit as described previously (19). Computer control of the centrifugal pumps with automated feedback loops was used to maintain precise control of aortic diastolic and left atrial pressures. The

transition from a Langendorff perfusion mode (left atrial pressure = -1 mmHg) to a working heart mode (left atrial pressure = 8 mmHg) was accomplished by increasing the revolutions per minute on the left atrial centrifugal pump, while aortic diastolic pressure was maintained by controlling the revolutions per minute on the aortic centrifugal pump. Cardiac output was determined by measuring the flow through the left atrial line in working heart mode.

Immediately following the 3-minute initial reperfusion period (DCD hearts) or the rapid cardiectomy (normal control hearts), hearts were connected to the EVHP circuit and perfused via the aortic root at 40 mmHg, a left atrial pressure of -1 mmHg, and a temperature of 22°C. Hearts were then gradually rewarmed to 37°C over a 30-minute period, during which time a cannula was placed in the pulmonary artery, a cone shaped left atrial cannula (XVIVO Perfusion AB, Göteborg, Sweden) was sewn to the common orifice of the pulmonary veins, and the superior and inferior vena cava were oversewn. Hearts were perfused *ex vivo* in a normothermic beating state for a total duration of 6 hours (Figure 7.1). At 1 (T1), 3 (T3), and 5 (T5) hours the hearts were paced in an AAI mode at 105 beats/minute and were transitioned into a working mode by increasing the left atrial pressure to 8 mmHg. Once a steady state was reached, perfusate samples were obtained from the aortic root and coronary sinus, and assessments of myocardial function, metabolism, and coronary blood flow were carried out (Figure 7.1).

Ex vivo perfusate solution

The *ex vivo* perfusate solution consisted of 1000 mL of STEEN solution (XVIVO Perfusion AB) and 500 mL of whole donor blood. Additional blood collected during donor exsanguination was centrifuged and washed with 1000 mL of 0.9% saline solution. The red blood cell concentrate obtained was added to the perfusate solution to achieve a hemoglobin concentration of 45 g/L. Perfusate pH, partial pressure of oxygen, partial pressure of carbon dioxide, and electrolyte, hemoglobin, and lactate concentrations were measured using an ABL800 Flex Analyzer (Radiometer Medical ApS, Brønshøj, Denmark). Oxygen and gas flow

through the membrane oxygenator were titrated to maintain a pH of 7.25-7.35 and an arterial partial pressure of oxygen (P_aO_2) of 100-200 mmHg. Infusions of insulin (2.25 units/hour) and dobutamine (4 mcg/minute) were maintained over the duration of EVHP.

Myocardial injury

Myocardial edema

Hearts were emptied of blood and weighed at the beginning (T0) and end (T6) of EVHP (Figure 7.1). The total weight gained was expressed as a percent of the original heart weight as follows:

Weight gain (%) = [End-EVHP heart weight (grams) – Start-EVHP heart weight (grams)] / Start-EVHP heart weight (grams).

Troponin-I

Plasma samples were obtained from coronary sinus effluent and the amount of troponin-I that accumulated in the perfusate between T0 and T5 was determined using a Pig Cardiac Troponin-I ELISA Kit (Life Diagnostics, Pennsylvania, USA) and normalized to the heart weight (pg/mL/gram).

Electron microscopy

Myocardial specimens from a random subset of animals (Normal control, N=3; 5°C, N=3; 25°C, N=3; 35°C, N=3) were examined using electron microscopy. Three samples of the anterolateral wall of the left ventricle (subepicardium, middle, subendocardium) were obtained and fixed in a 0.1 M phosphate buffer with 3% glutaraldehyde and then 1% osmium tetroxide. Samples were then dehydrated and embedded in Epon 812. Three blocks from each sample (9 blocks per animal) were randomly selected, sectioned, stained with uranyl acetate and lead citrate, and photographed using a Philips CM 10 electron microscope (Philips Electronics,

Eindhoven, Netherlands). An anatomist (JT) evaluated samples in a blinded fashion using a coded grid, and assigned endothelial and myocyte injury scores (0=normal, 1=mild-moderate, 2=moderate, 3=moderate-severe, 4=severe injury). Myocytes were examined for alterations in cell integrity, including disruption of myofibrils and loss of organelles. Capillaries were examined for the loss of endothelial cell integrity, including the presence and severity of endothelial cell edema.

Coronary vascular resistance

Coronary blood flow (CBF) was determined by averaging 2 timed collections of pulmonary arterial blood flow. Coronary vascular resistance (CVR) was then calculated as follows:

*CVR (mmHg*minute/mL/100 grams) = aortic diastolic pressure (mmHg)/CBF (mL/minute)/100 grams heart weight.*

Myocardial metabolism

Oxygen consumption (MVO_2) was determined by multiplying CBF with the arterial-venous oxygen content difference:

$$MVO_2 \text{ (mL } O_2\text{/minute/100 grams)} = [(C_aO_2 - C_vO_2) * CBF]/100 \text{ grams heart weight.}$$

Arterial (C_aO_2) and venous (C_vO_2) oxygen content were calculated as follows:

$$C_aO_2 \text{ (mL}O_2\text{/100mL)} = [1.34 \text{ (mL}O_2\text{/gram hemoglobin)} * \text{hemoglobin concentration (grams/100 mL)} * \text{oxygen saturation (\%)}] + [0.00289 \text{ (mL}O_2\text{/mmHg/100 mL)} * P_aO_2 \text{ (mmHg)}],$$

and

$$C_vO_2\ (mLO_2/100mL) = [1.34\ (mLO_2/gram\ hemoglobin) * hemoglobin\ concentration\ (grams/100\ mL) * oxygen\ saturation\ (\%)] + [0.00289\ (mLO_2/mmHg/100\ mL) * P_vO_2\ (mmHg)],$$

where P_aO_2 and P_vO_2 represent the partial pressure of oxygen in blood samples from the aortic root and coronary sinus, respectively. Myocardial lactate metabolism was determined by measuring the difference in lactate concentration between coronary sinus and aortic root blood samples as follows:

$$Lactate\ metabolism\ (mmol/L) = Coronary\ sinus\ lactate\ (mmol/L) - Arterial\ lactate\ (mmol/L).$$

Myocardial function

The cardiac index was determined by measuring the blood flow on the left atrial line (Bio-Probe Transducer Model TX-40, Medtronic, Minneapolis, USA) indexed for heart weight (mL/minute/gram). Additionally, the maximum (dP/dt maximum) and minimum (dP/dt minimum) rate of pressure change, and the developed pressure were measured using a 5F conductance catheter (Ventri-Cath 507, Millar Inc., Houston, USA) placed in the left ventricle and analyzed using LabChart 7 Pro Version 7.2.5 (ADInstruments Inc., Colorado Springs, USA).

Statistical Analysis

Normally distributed continuous variables were reported as mean ± standard error (SE) and non-normally distributed continuous variables were reported as median [range]. The analysis of variance was used to evaluate differences among the 3 treatment groups. A *p*-value

<0.05 was considered statistically significant. Analyses were performed using GraphPad Prism V6.0c (GraphPad Software Inc., La Jolla, USA).

Results

Initial Reperfusion

DCD hearts sustained equivalent periods of warm ischemia (5°C=28±1, 25°C=29±1, 35°C=28±1 minutes, *p*=0.498) prior to initial reperfusion. There was no significant difference in the electrolyte composition, osmotic, or oncotic pressures of the initial reperfusion solution among treatment groups (Supplement Table 7.1). Average myocardial temperature during the 3-minute initial reperfusion period varied according to treatment group (5°C=10±1, 25°C=25±1, 35°C=35±0 °C, *p*<0.001, Table 7.1). Initial reperfusion under profoundly hypothermic conditions resulted in increased CVR, reduced CBF, and lower coronary sinus lactate concentration (Table 7.1).

Table 7.1. Impact of cardioplegia temperature on myocardial temperature, coronary vascular resistance, and coronary sinus lactate concentration during the 3-minute initial reperfusion period.

Parameter, mean (SE)	5°C	25°C	35°C	*p value*
Myocardial temperature (°C)	10 (1)	25 (1)	35 (0)	<0.001
CVR (mmHg*minute/mL/100 grams)	0.042 (0.005)	0.021 (0.002)	0.018 (0.001)	<0.001
Coronary blood flow (mL/minute)	483 (53)	772 (60)	906 (36)	<0.001
Coronary sinus lactate (mmol/L)	0.73 (0.06)	1.33 (0.03)	1.75 (0.15)	<0.001

CVR, coronary vascular resistance

Myocardial injury during ex vivo heart perfusion

Myocardial edema

There was no significant difference in the development of myocardial edema over the 6-hour EVHP interval among treatment groups (5°C=29±5, 25°C=27±6, 35°C=25±3 % weight gain, *p*=0.800). Normal control hearts gained 12±1 % of their original heart weight during EVHP.

Troponin-I

The accumulation of troponin-I in the EVHP perfusate solution during EVHP was significantly higher in hearts exposed to profound hypothermia during the 3-minute initial reperfusion period (Figure 7.2).

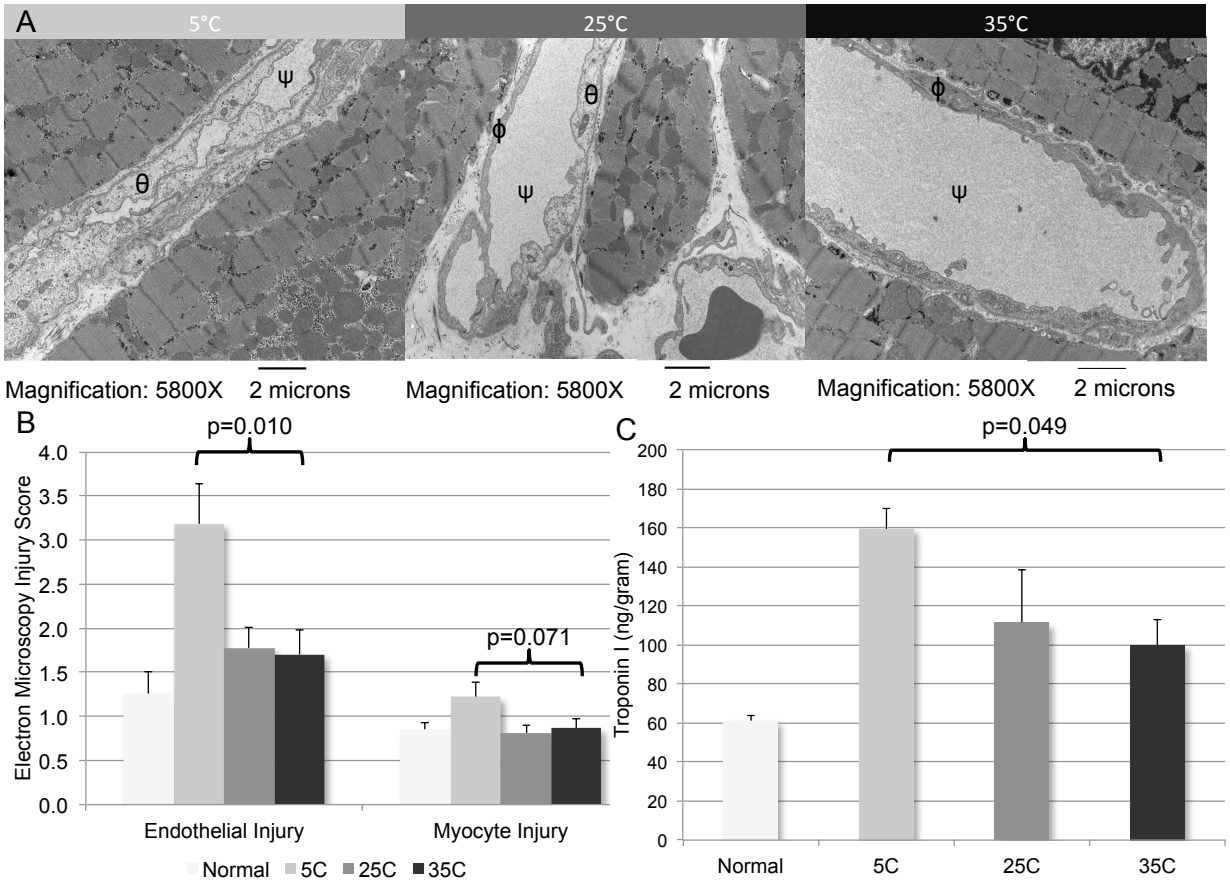

Figure 7.2. A) Electron microscopy of myocardial specimens from the anterolateral wall of the left ventricle, B) electron microscopy injury scores, and C) concentration of troponin-I in the perfusate solution according to initial reperfusion temperature. Electron microscopy injury scores and troponin-I concentrations from normal control hearts are included for reference.

Ψ, capillary lumen; θ, edematous capillary endothelium resulting in capillary occlusion; Φ, normal capillary endothelium

Electron microscopy

Examination of myocardial specimens obtained from the anterolateral wall of the left ventricle at T6 revealed greater capillary endothelial cell edema and a trend towards greater myocyte injury in hearts exposed to profound hypothermia during the 3-minute initial reperfusion period (Figure 7.2). Endothelial cell edema was of sufficient severity to cause capillary occlusion in many specimens obtained from 5°C hearts.

Coronary vascular resistance during ex vivo heart perfusion

There was no significant difference in CVR among treatment groups at T1; however, hearts initially reperfused at 35°C exhibited significantly lower CVR at T3 and a trend towards lower CVR at T5 (Figure 7.3).

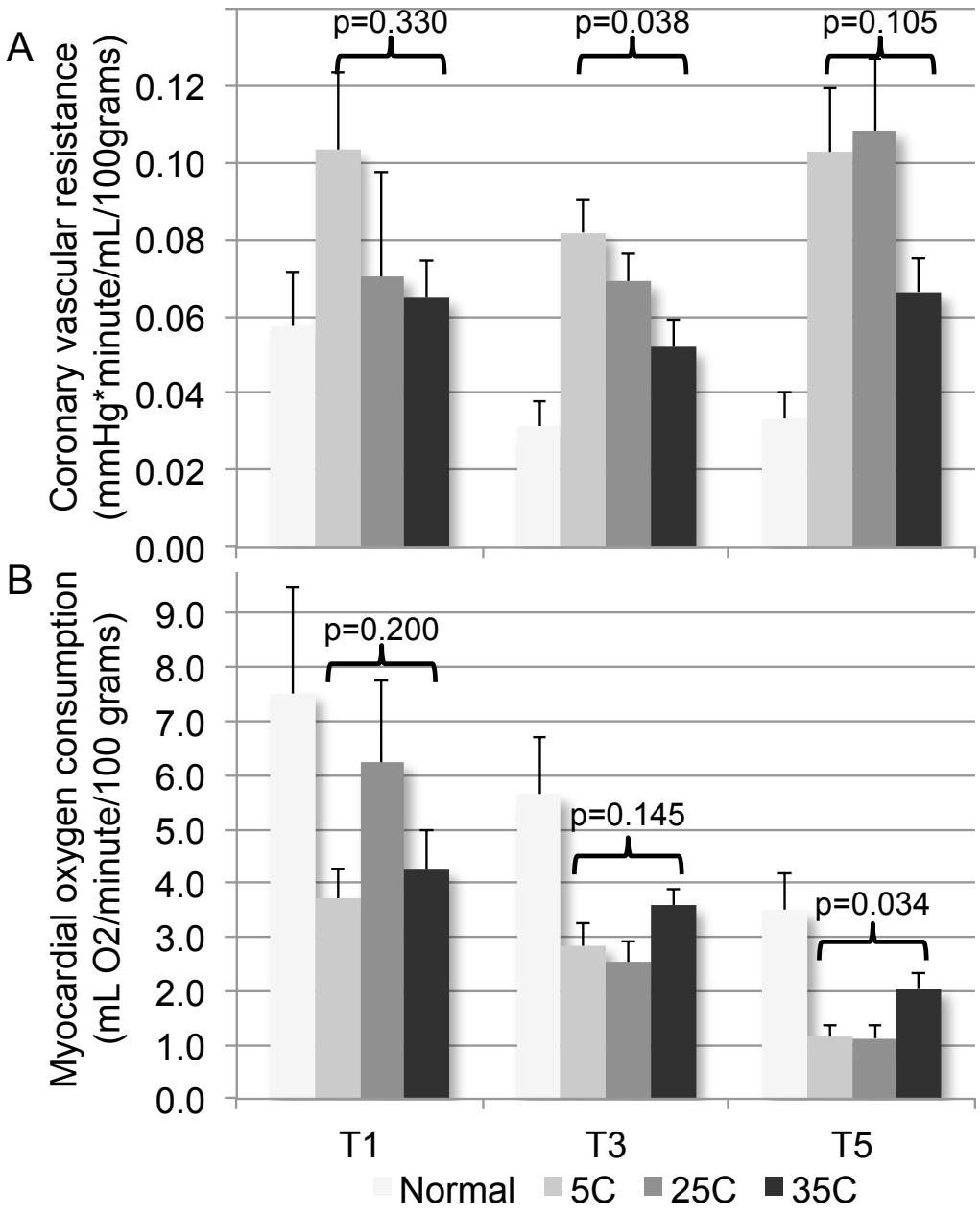

Figure 7.3. A) Coronary vascular resistance and B) myocardial oxygen consumption measured in working mode after 1 (T1), 3 (T3), and 5 (T5) hours of normothermic *ex vivo* heart perfusion. p values represent results of the ANOVA comparing the 3 treatment groups (5°C, 25°C, and 35°C)

Myocardial metabolism during ex vivo heart perfusion

There was no significant difference in MVO_2 among treatment groups at T1 and T3; however, hearts initially reperfused at 35°C exhibited greater MVO_2 at T5 compared to hearts initially reperfused at 25°C and 5°C (Figure 7.3). There was no significant difference in the lactate concentration among treatment groups at T1; however, at T3 and T5 the arterial and venous lactate concentrations were significantly lower in hearts initially reperfused at 35°C (Figure 7.4).

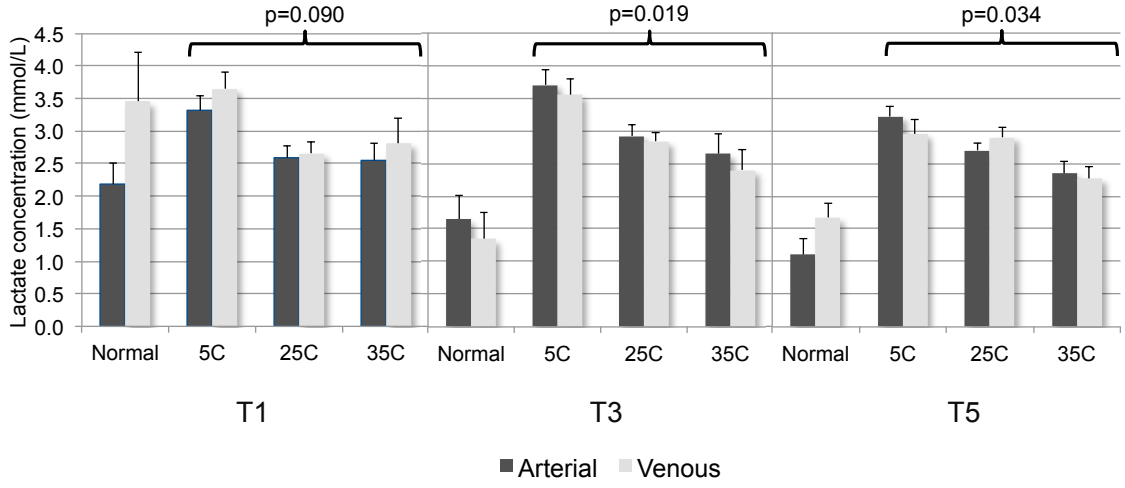

Figure 7.4. Arterial and venous lactate concentrations measured in working mode after 1 (T1), 3 (T3), and 5 (T5) hours of normothermic *ex vivo* heart perfusion.

p values represent results of the ANOVA comparing the 3 treatment groups (5°C, 25°C, and 35°C)

Myocardial function during ex vivo heart perfusion

Initial reperfusion temperature significantly impacted the recovery of myocardial function during EVHP. Systolic (dP/dt maximum) and diastolic (dP/dt minimum) function of donor hearts are displayed in Figure 7.5. In hearts initially reperfused at 35°C, the diastolic function was significantly better at all time points during EVHP and the systolic function was superior at T5.

The impact of initial reperfusion temperature on the cardiac index and developed pressure are displayed in Supplement Figures 7.1-7.2.

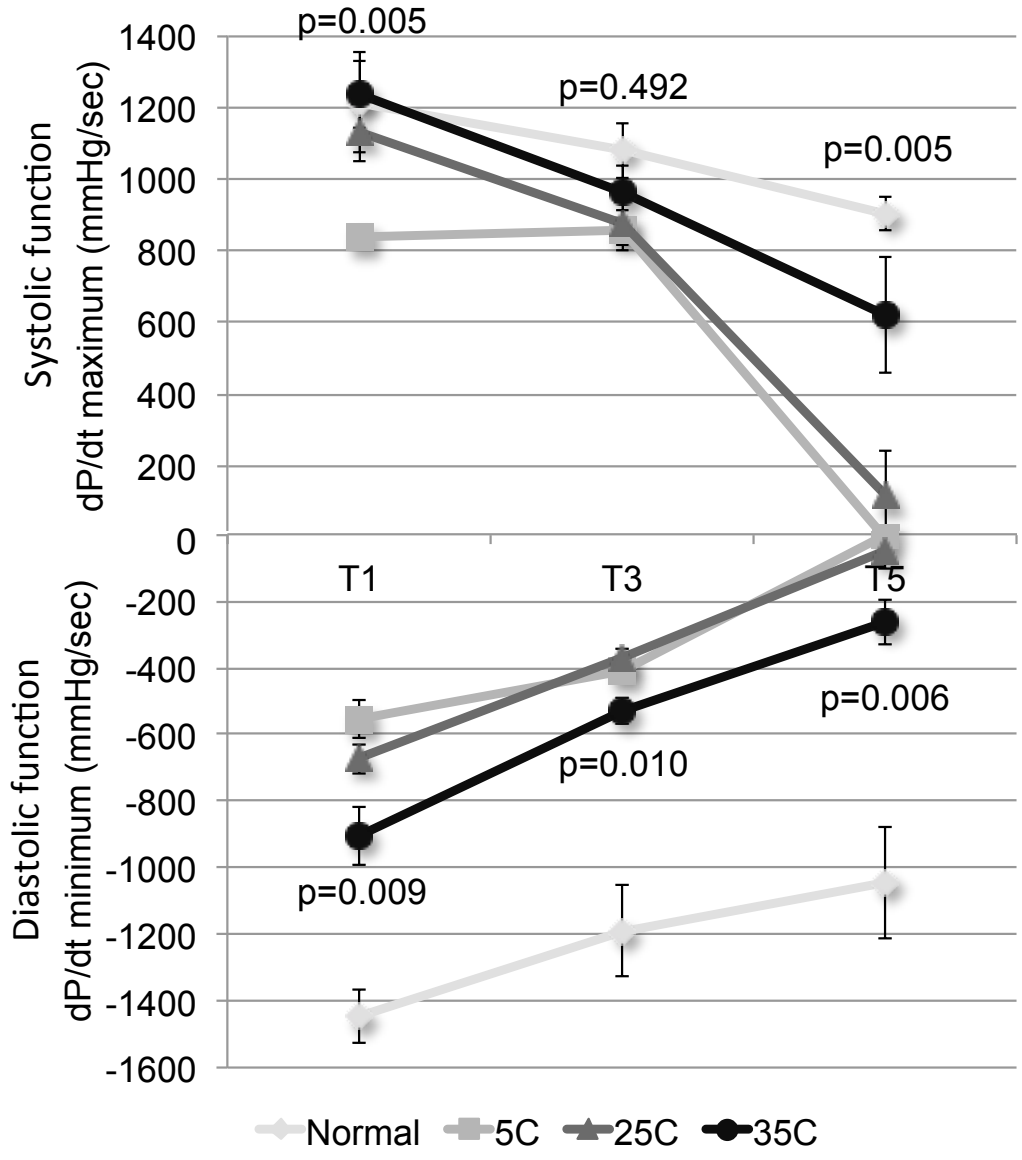

Figure 7.5. Systolic (dP/dt maximum) and diastolic (dP/dt minimum) of hearts measured in working mode after 1 (T1), 3 (T3), and 5 (T5) hours of normothermic *ex vivo* heart perfusion.
p values represent results of the ANOVA comparing the 3 treatment groups (5°C, 25°C, and 35°C)

Discussion

DCD hearts have been proposed as an additional donor source in an attempt to mitigate the current organ shortage that limits cardiac transplantation. Translational experiments have clearly demonstrated that optimizing the composition of the initial reperfusion solution and the conditions of its delivery at the time of organ procurement impacts DCD heart viability. In order to realize the potential of DCD heart transplantation, it is important to establish an evidence-based resuscitation strategy that optimizes functional recovery and organ viability (20). Many authors have speculated that the avoidance of profound hypothermia during initial reperfusion may optimize DCD heart resuscitation; however, this has not been systematically evaluated. We have now demonstrated in a large animal model that the avoidance of profound hypothermia during initial reperfusion minimizes myocyte and endothelial injury, and improves the functional recovery of DCD hearts.

The delivery of a profoundly hypothermic cardioplegic solution at the time of organ procurement is standard practice when procuring hearts from brain dead donors, to minimize metabolic demands during the subsequent period of cold static storage. In the context of DCD; however, organ preservation using normothermic ex vivo perfusion has been shown to provide superior outcomes in experimental models (21) and has been employed in clinical DCD transplantation (11). With this in mind, the potential benefits of cooling the DCD heart at the time of procurement only to rewarm it minutes later upon initiation of EVHP seem questionable. Simply unloading and arresting the donor heart reduces myocardial metabolic demands by 80-90% (22, 23). The benefit of further reducing metabolic demands, by inducing a profoundly hypothermic state in the short period of time between procurement and initiation of EVHP, may be outweighed by the negative impact of profound hypothermia on the reparative processes essential for resuscitation of the vulnerable DCD heart. This proposal; however, must be accompanied by the caveat that the time between organ procurement and initiation of EVHP is

short. We accomplished this by inserting only the aortic cannula before initiation of EVHP and completing the remaining donor heart dissection and cannulation with the heart beating.

Ischemia reperfusion injury in the DCD heart

Following donor extubation the DCD heart is forced to function in an increasingly hypoxemic environment while attempting to maintain systemic oxygen delivery. This hypoxemia results in a transition to anaerobic metabolism, the development of intracellular acidosis, and activation of the sodium-hydrogen exchanger (2). Reperfusion at the time of organ procurement rapidly normalizes the extracellular pH and creates a large hydrogen ion gradient across the plasma membrane that causes further sodium influx via the sodium-hydrogen exchanger and the sodium-bicarbonate co-transporter (4). This increase in intracellular sodium forces the sodium-calcium exchanger to function in reverse mode and import calcium ions across the sarcolemma. The resultant intracellular calcium overload that develops during the initial reperfusion of ischemic myocardium plays an important role in the pathogenesis of myocardial hypercontracture and ischemia-reperfusion injury (24).

Optimizing the composition of the solution delivered at the time of organ procurement may mitigate the detrimental effects of ischemia-reperfusion injury and enhance the resuscitation of DCD hearts. For example, the addition of a sodium-hydrogen exchange inhibitor (7, 9) or an antioxidant (8) to the initial reperfusion solution has been shown to minimize ischemia-reperfusion injury and optimize functional recovery. The delivery of pharmaceutical agents that activate ischemic postconditioning pathways (erythropoietin, glyceryl trinitrate, p38 mitogen-activated protein kinase inhibitors) have also been shown to optimize DCD heart resuscitation (7, 25). Taken together these studies demonstrate that interventions delivered at the onset of reperfusion that target pathways involved in the pathogenesis of ischemia-reperfusion injury can optimize the resuscitation of DCD hearts.

Controlling the conditions of the initial reperfusion may further minimize ischemia-reperfusion injury. The reactivation of ATP production in calcium overloaded cardiomyocytes after prolonged ischemia causes calcium oscillations that provoke uncontrolled myofibrillar activation and the development of hypercontracture (26, 27). Therefore, initial reperfusion of ischemic myocardium should maintain cardiac arrest to provide an opportunity for the restoration intracellular ion homeostasis prior to myocardial contraction (8). Previous studies have demonstrated that hearts subjected to a period of ischemia exhibit better functional recovery when reperfused with a cardioplegic solution compared to reperfusion with unmodified blood (28, 29). Further, Rosenkranz et al (30) have shown that following warm ischemia, hearts reperfused with blood recover 33% of baseline function, while those reperfused with cold or warm cardioplegia recover 63% and 85% of baseline function, respectively. These results not only suggest that initial reperfusion of ischemic myocardium with a cardioplegic solution is protective, but also that delivery of the solution at warmer temperatures provides additional benefit.

Rationale for avoiding profound hypothermia during initial reperfusion

The avoidance of profound hypothermia during the initial reperfusion of ischemic myocardium may produce an environment that facilitates the resumption of metabolic activity at a sufficient rate to allow restoration of myocardial energy stores and the recovery of ionic homeostasis (30-32). The cardiomyocyte is dependent on the sodium-potassium ATPase to restore the normal sodium gradient and repolarize the cell membrane. Once this has been achieved, the sodium-calcium exchanger can return to a forward mode of operation, extrude excess calcium from the cell, and restore ionic homeostasis (27). However, there is evidence to suggest that hypothermia inhibits sodium-potassium ATPase activity during the reperfusion of ischemic myocardium (17). Most enzymatic processes have a 1.5–2.5 fold decrease in enzyme activity rate for every 10°C decrease in temperature (33), and profound hypothermia has been

shown to decrease the efficiency of oxidative phosphorylation and limit the synthesis of ATP (34). Taken together these studies suggest that initial reperfusion with a profoundly hypothermic cardioplegia is likely to inhibit sodium-potassium ATPase activity and the generation of ATP required for the restoration of ionic homeostasis (17).

The protective effect of terminal warm blood cardioplegia in cardiac surgery exemplifies the potential benefits of avoiding hypothermia during the initial reperfusion of DCD hearts. Previous work in animal models have demonstrated that delivery of a warm cardioplegia following ischemic arrest reduces reperfusion arrhythmias, lowers troponin levels, and improves functional recovery (35-37). Luo et al (38) have demonstrated in a clinical randomized trial of patients undergoing elective valve replacement that terminal warm cardioplegia limited myocardial injury and improved function postoperatively. In this study the protective effect of terminal warm cardioplegia was equivalent to that achieved with ischemic preconditioning. Likewise, Toyoda et al (39) showed in a randomized trial of pediatric patients that terminal warm cardioplegia reduced reperfusion arrhythmias and lowered troponin levels. Since DCD hearts have sustained ischemic injury during the anoxic arrest and warm ischemic standoff period prior to organ procurement, application of the terminal warm cardioplegia concept to the initial reperfusion of DCD hearts may optimize their resuscitation (20).

Translational experiments in animal models have advocated for the avoidance of profound hypothermia during the initial reperfusion to facilitate DCD heart resuscitation. In these studies the initial reperfusion solution was delivered at temperatures ranging from 20-37°C (8, 18, 20, 40-42); however, the optimal temperature has not been examined systematically. Our results demonstrate that avoidance of profound hypothermia during the initial reperfusion reduced CVR, increased oxygen delivery, and improved the washout of lactate from the myocardium. These changes may have optimized the restoration ion homeostasis prior to myocardial contraction and minimized ischemia-reperfusion injury, as evidenced by the lower troponin levels during subsequent EVHP and preserved myocyte ultrastructure. The recovery of

myocardial function was best preserved in hearts reperfused at warmer temperatures. The beneficial impact of initial reperfusion at 35°C was most apparent in the preservation of diastolic function during EVHP. The negative effects of hypothermia on sarcoplasmic reticular calcium handling may account for this observation (43). Finally, initial reperfusion at 5°C was associated with endothelial cell injury in our study, which is in accordance with previous research demonstrating that endothelial cells exhibit significantly decreased cell adhesion and increased permeability at profoundly hypothermic temperatures (44). The endothelial cell protection afforded by initial reperfusion at warmer temperatures is important given the known association between endothelial injury, the development of cardiac allograft vasculopathy, and long term graft survival (45). The importance of optimal endothelial function is not limited to cardiac allografts, and our observations regarding the importance of initial reperfusion temperature may be applicable to other DCD organs as well.

Limitations

The results of this study suggest that initial reperfusion temperature impacts the recovery of myocardial function during EVHP; however, further studies are required to determine if post-transplant outcomes would be similarly affected. We included uninjured hearts as a normal control group; however, successful DCD heart transplantation may be possible without achieving normal heart function in the ex vivo setting. Future transplant studies are required to determine cutoff values for functional and metabolic parameters that predict successful transplantation. It may be beneficial to include hearts from brain-dead donor animals as a control group in future studies. The impact of initial reperfusion temperature on myocardial energy stores, and intracellular sodium and calcium concentrations were inferred based on previously published literature and were not directly quantified in this study.

Conclusions

How the DCD heart is treated in the first minutes of reperfusion significantly impacts the severity of ischemia-reperfusion injury. The avoidance of profound hypothermia during initial reperfusion with a normokalemic adenosine-lidocaine cardioplegia minimizes myocyte and endothelial injury, and improves the functional recovery of DCD hearts during ex vivo perfusion. Therefore, the conditions under which cardioplegia is delivered during organ procurement is an important variable impacting organ viability.

Chapter 7 Summary

In Chapter 7: *Avoidance of profound hypothermia during initial reperfusion improves the functional recovery of hearts donated after circulatory death*, it was demonstrated that initial reperfusion at 35°C minimizes injury and optimizes the functional recovery of DCD hearts during EVHP. It is clear that how the DCD heart is treated in the first minutes of reperfusion significantly impacts the severity of ischemia-reperfusion injury. Further, the condition under which the resuscitative cardioplegia is delivered at the time of organ procurement is an important variable impacting organ viability. Further research in this area may identify additional strategies that optimize DCD heart resuscitation and improve transplant outcomes.

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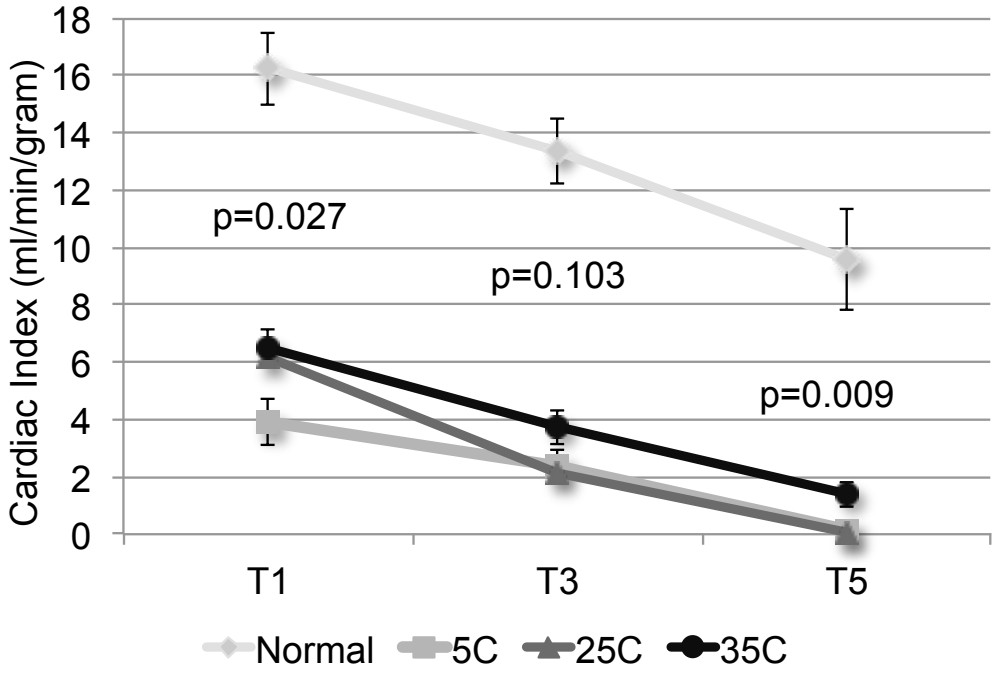
Supplemental Information

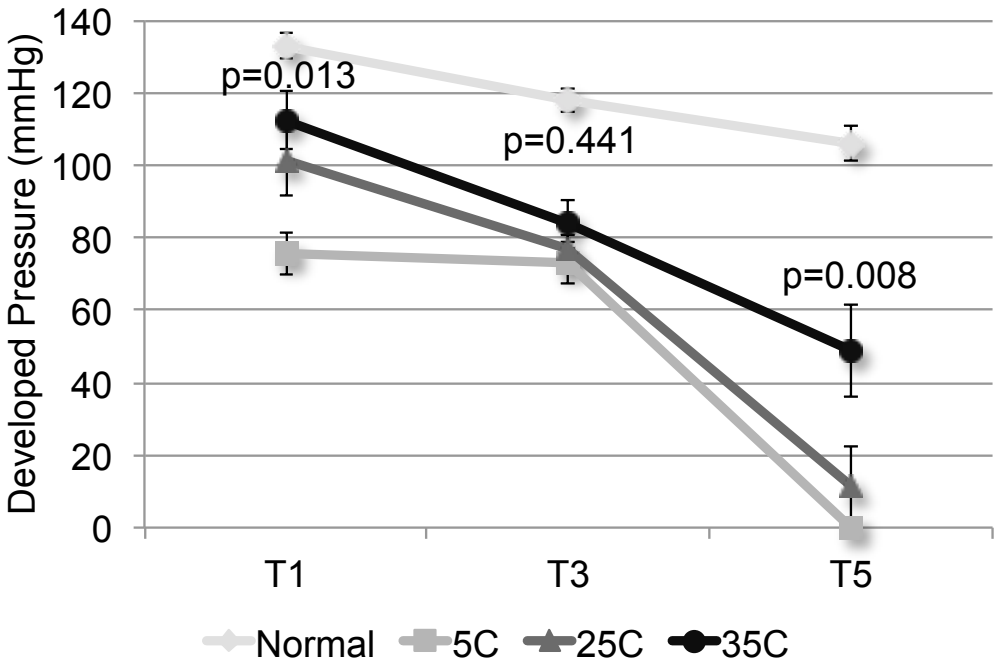
Supplement Table 7.1. Initial reperfusion solution properties

	5°C	25°C	35°C	*p value*
pH, mean (SE)	7.3 (0.1)	7.4 (0.1)	7.3 (0.1)	0.312
PO_2 (mmHg), mean (SE)	327 (95)	211 (57)	143 (4)	0.117
PCO_2 (mmHg), mean (SE)	48 (6)	36 (4)	44 (4)	0.208
Na^+ (mmol/L), mean (SE)	144 (1)	145 (1)	144 (1)	0.749
K^+ (mmol/L), mean (SE)	5.8 (0.0)	5.9 (0.1)	5.9 (0.0)	0.657
Cl^- (mmol/L), mean (SE)	124 (1)	124 (1)	123 (1)	0.803
Glucose (mmol/L), mean (SE)	8.3 (0.7)	9.1 (0.2)	9.4 (0.1)	0.266
Osmolality (mOsmol/Kg), mean (SE)	297 (2)	298 (1)	297 (1)	0.838
Oncotic pressure (mmHg), mean (SE)	34 (1)	34 (1)	36 (1)	0.395



Supplement Figure 7.1. Cardiac index of hearts measured in working mode after 1 (T1), 3 (T3), and 5 (T5) hours of normothermic *ex vivo* heart perfusion.

p values represent results of the ANOVA comparing the 3 treatment groups (5C, 25C, and 35C)



Supplement Figure 7.2. Developed pressure of hearts measured in working mode after 1 (T1), 3 (T3), and 5 (T5) hours of normothermic *ex vivo* heart perfusion.

p values represent results of the ANOVA comparing the 3 treatment groups (5C, 25C, and 35C)

Chapter 8

Impact of reperfusion calcium and pH on the resuscitation of hearts donated after circulatory death

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Chapter 8 Preface

Optimizing donor heart resuscitation is a crucial component of a successful DCD transplant protocol (Figure 1.1). In Chapter 7: *Avoidance of profound hypothermia during initial reperfusion improves the functional recovery of hearts donated after circulatory death*, it was demonstrated how the conditions under which the resuscitative cardioplegia is delivered at the time of organ procurement can significantly impact DCD heart resuscitation. In Chapter 8: *Impact of reperfusion calcium and pH on the resuscitation of hearts donated after circulatory death*, we will explore how the composition of the cardioplegic solution (pH and the calcium concentration) can impact the functional recovery of DCD hearts during EVHP. The data presented in this chapter suggests that initial reperfusion with an acidic and hypocalcemic cardioplegia optimizes DCD heart resuscitation.

Abstract

Background:

Hearts donated after circulatory death (DCD) may represent an additional donor source. The influx of sodium and calcium ions across the sarcolemma play a central role in the pathogenesis of ischemia reperfusion injury; however, this process may be inhibited if the initial reperfusion (IR) solution is rendered hypocalcemic and acidic. We sought to determine the calcium concentration and pH of the IR solution that yielded optimal functional recovery of DCD hearts during *ex vivo* heart perfusion (EVHP).

Methods:

Pigs were anesthetized, mechanical ventilation was discontinued, and a 15-minute standoff period was observed following circulatory arrest. Hearts were reperfused with a normothermic cardioplegia of varying calcium concentrations (Part 1: 50μmol/L; N=4, 220μmol/L; N=9, 500μmol/L; N=4, 1250μmol/L; N=5), and pH (Part 2: 7.9; N=5, 7.4; N=9, 6.9; N=8, 6.4; N=6). Myocardial function was then assessed in a physiologic working mode 1 hour following initiation of normothermic EVHP.

Results:

The calcium concentration and pH of the cardioplegic solution impacted the development of myocardial edema (Part 1: 50μmol/L=5.8±0.9, 220μmol/L=4.3±0.4, 500μmol/L=7.0±0.6, 1250μmol/L=6.6±0.8 % weight gain, p=0.015; Part 2: 7.9=3.6±0.4, 7.4=4.3±0.4, 6.9=3.7±0.6, 6.4=6.4±1.3 % weight gain, p=0.056) and the recovery of myocardial function (Cardiac Index Part 1: 50μmol/L=2.6±0.6, 220μmol/L=6.0±0.8, 500μmol/L=2.3±0.5, 1250μmol/L=1.9±0.6 mL/minute/gram, p<0.001; Part 2: 7.9=1.5±0.7, 7.4=6.0±0.8, 6.9=8.4±1.8, 6.4=3.1±0.8 mL/minute/gram, p=0.003) during EVHP.

Conclusions:

Initial reperfusion of DCD hearts with a hypocalcemic and moderately acidic cardioplegia minimizes edema and optimizes functional recovery during subsequent EVHP.

Introduction

Cardiac transplantation remains the gold-standard treatment for patients with advanced heart failure (1). Despite a growing number of eligible patients, a critical shortage of suitable organs from brain dead donors has resulted in a static transplant volume (1). Donation after circulatory death (DCD) may represent a novel avenue to expand the donor pool. The DCD heart; however, experiences global ischemia during the hypoxemic cardiac arrest and warm-ischemic standoff period that ethically define death (2). Reperfusion at the time of organ procurement creates a large hydrogen ion gradient across the sarcolemma that activates the sodium-hydrogen exchanger (NHE) and causes sodium influx into the myocyte (3). With loss of the sodium gradient, the sodium-calcium exchanger (NCX) functions in reverse mode and imports calcium ions into the cell (Figure 8.1B). This intracellular calcium overload plays a central role in the pathogenesis of ischemia-reperfusion injury (IRI) (3-5). Concern regarding the severity of IRI sustained following organ procurement has limited the clinical transplantation of DCD hearts.

Application of a tailored approach to the initial reperfusion of DCD hearts provides an opportunity to resuscitate these organs and facilitate successful clinical transplantation (6). The pathogenesis of IRI can be mitigated by optimizing the composition of the cardioplegic solution delivered at the time of organ procurement. Pharmacologic inhibition of the NHE and NCX have been shown to minimize IRI (7-11); however, such inhibitors are not clinically available. Alternatively, delivery of acidic and hypocalcemic solutions have been shown to limit sodium entry via NHE (12-14) and calcium entry via NCX, respectively (15). Taken together these results suggest that initial reperfusion of DCD hearts with a hypocalcemic and acidic cardioplegia may minimize IRI. Therefore, we sought to determine what initial reperfusion solution calcium concentration and pH would optimize the functional recovery of DCD hearts during *ex vivo* heart perfusion (EVHP).

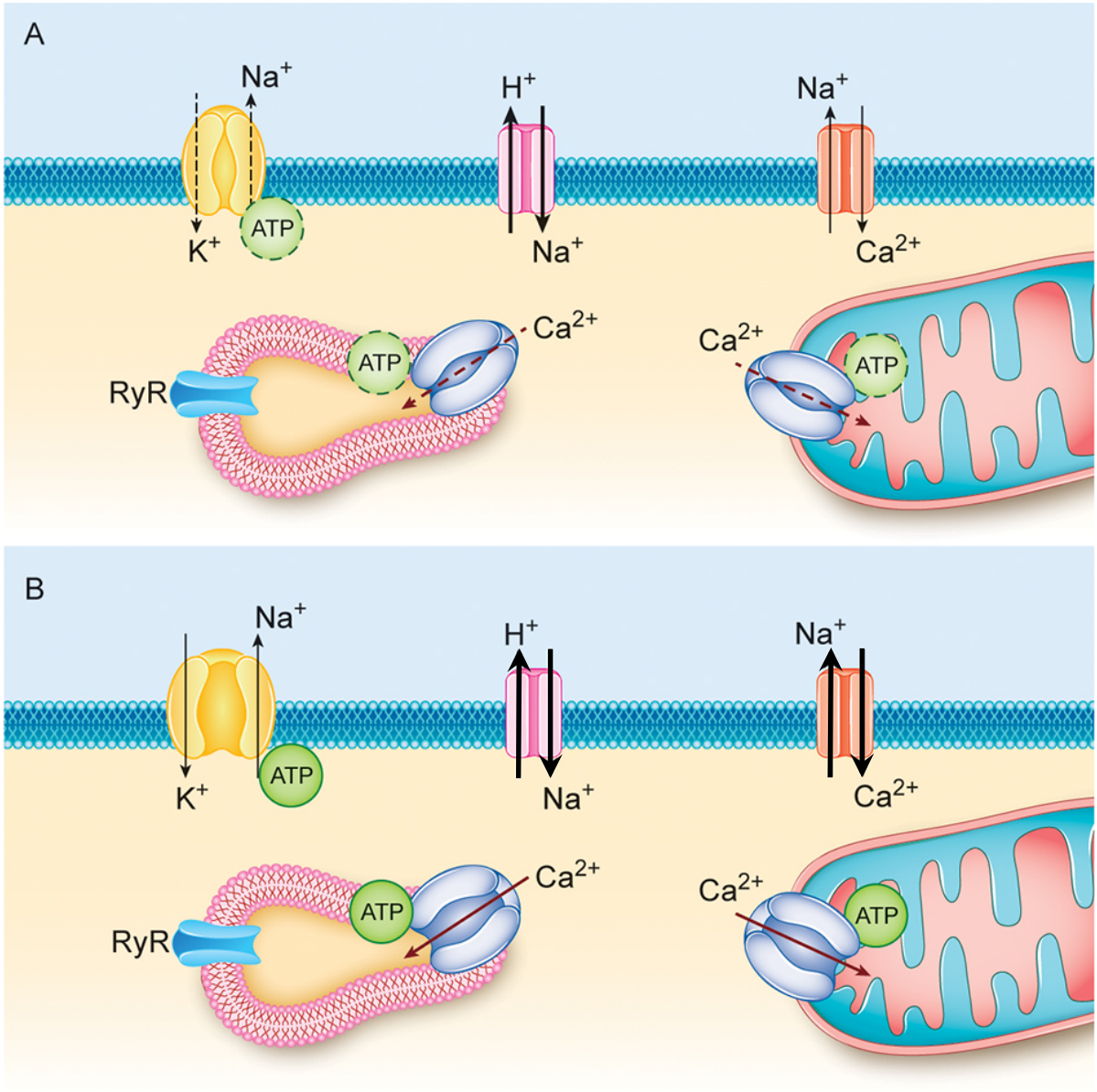

Figure 8.1. A) Ionic changes during ischemia. Anaerobic metabolism results in the production of hydrogen ions that activate the sodium-hydrogen exchanger and the accumulation of sodium ions inside the myocyte. The sodium-potassium ATPase is not able to extrude the excess sodium ions and maintain the normal membrane potential due to a lack of available ATP. Consequently, as ischemia progresses there is an accumulation of sodium and hydrogen ions inside the myocyte and depolarization of the membrane potential. B) Ionic changes during reperfusion. Reperfusion washes out the hydrogen ions that have accumulated in the interstitial

space and creates a large gradient for sodium-hydrogen exchange. The influx of sodium ions into the myocyte during early reperfusion forces the sodium-calcium exchanger to function in reverse mode and import calcium ions across the sarcolemma. Intracellular ionic homeostasis cannot be restored until the sodium-potassium ATPase is able to reestablish the resting membrane potential and normal intracellular sodium levels, which will allow the sodium-calcium exchanger to return to a forward mode of operation and extrude excess calcium from the cytoplasm.

ATP: adenosine triphosphate, RyR: ryanodine receptor

Materials and Methods

The University of Manitoba Animal Care Committee approved the experimental protocol. All animals received humane care in compliance with the National Institute of Health's *Guide for the Care of Laboratory Animals*. A total of 41 DCD pig hearts were allocated to 8 treatment groups according to the composition of the initial reperfusion solution that was delivered following organ procurement (Figure 8.2). Part 1 of the experimental protocol examined the impact of initial reperfusion calcium concentration on DCD heart resuscitation: 50μmol/L (N=4), 220μmol/L (N=9), 500μmol/L (N=4), and 1250μmol/L (N=5). Part 2 examined the impact of initial reperfusion pH on DCD heart resuscitation: 7.9 (N=5), 7.4 (N=9), 6.9 (N=8), and 6.4 (N=6). Hearts allocated to the 220μmol/L group in Part 1 were also used as the pH 7.4 group in Part 2 of the experimental protocol (Figure 8.2, Table 8.1, Supplement Tables 8.1-8.4).

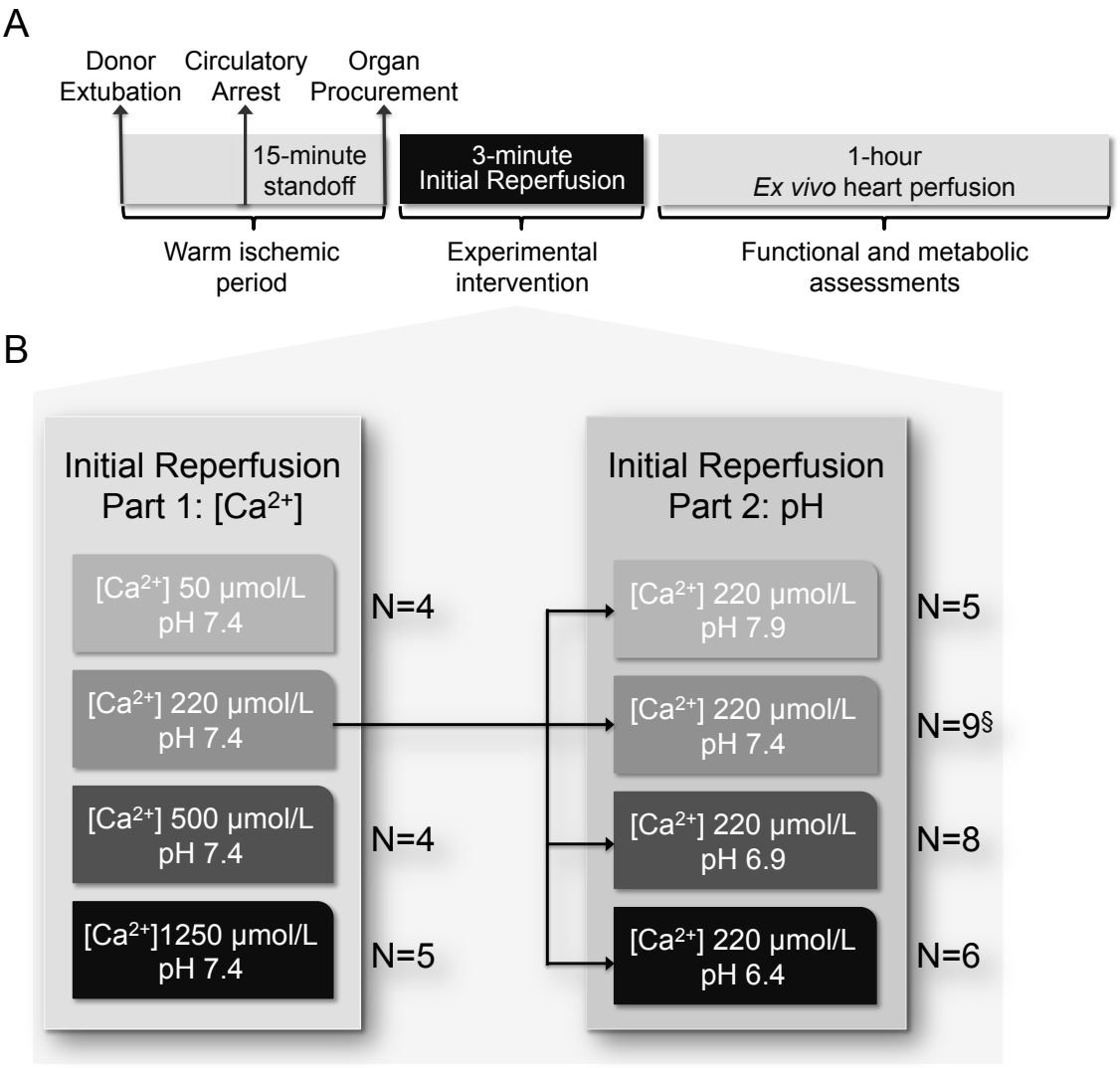

Figure 8.2. Experimental protocol examining the impact of initial reperfusion calcium concentration (Part 1) and pH (Part 2) on the resuscitation of hearts donated after circulatory death. A) Donor animals were extubated and a 15-minute standoff period was observed following declaration of circulatory arrest. Hearts were reperfused with a cardioplegic solution of varying [Ca^{2+}] and pH for 3 minutes. Hearts were then perfused ex vivo in a normothermic beating state for 1-hour before functional and metabolic assessments were carried out in a working mode. B) Experimental groups according to the [Ca^{2+}] and pH of the cardioplegic solution delivered during the 3-minute initial reperfusion period.

§ Hearts from the [Ca^{2+}] 220µmol/L group in Part 1 also represented the pH 7.4 group in Part 2

Table 8.1. Impact of initial reperfusion calcium concentration and pH on myocardial function during ex vivo heart perfusion.

Functional parameter, mean (SE)	$[Ca^{2+}]$=50μmol/L	$[Ca^{2+}]$=220μmol/L[§]	$[Ca^{2+}]$=500μmol/L	$[Ca^{2+}]$=1250μmol/L	*p* value
dP/dt maximum (mmHg/second)	962 (93)	1217 (102)	757 (44)	745 (81)	0.006
dP/dt minimum (mmHg/second)	-640 (123)	-904 (117)	-476 (74)	-391 (62)	0.013
Developed pressure (mmHg)	81 (13)	111 (9)	74 (8)	60 (10)	0.008
Stroke work (mmHg*mL)	496 (162)	1520 (231)	403 (110)	348 (123)	<0.001
	pH=7.9	pH=7.4[§]	pH=6.9	pH=6.4	*p* value
dP/dt maximum (mmHg/second)	506 (212)	1217 (102)	1310 (109)	816 (97)	<0.001
dP/dt minimum (mmHg/second)	-281 (122)	-904 (117)	-980 (79)	-490 (92)	<0.001
Developed pressure (mmHg)	47 (20)	111 (9)	114 (6)	73 (10)	<0.001
Stroke work (mmHg*mL)	312 (165)	1471 (253)	1957 (395)	696 (251)	0.005

[§] 220μmol/L group in part 1 of the experimental protocol is equivalent to the pH=7.4 group in part 2 of the experimental protocol

Donation after circulatory death and heart procurement

Hearts were procured from anesthetized donor pigs (43±1 kg) following cessation of mechanical ventilation as previously described (16). Briefly, following donor extubation, circulatory arrest was declared when pulsatility on the arterial pressure tracing was no longer evident. An additional 15-minute warm ischemic standoff period was observed, and then donor animals were exsanguinated into a cell saver device (Brat2, Sorin Group Canada Inc., Markham, Canada) and a simultaneous rapid cardiectomy was performed. The heart was emptied of blood, weighed, and the aortic cannula was inserted.

Initial Reperfusion

Hearts were assigned to 8 treatment groups according to the composition of the initial reperfusion solution (Figure 8.2, Supplement Tables 8.1-8.2). All hearts underwent a controlled reperfusion at an aortic root pressure of 40 mmHg for 3 minutes at 35°C (16). The MPS2 (Quest Medical Inc., Allen, USA) was utilized to precisely control the pressure and temperature during the initial reperfusion.

Ex vivo heart perfusion

An Affinity NT oxygenator, venous reservoir, 2 modified BioMedicus 540 centrifugal pumps (Medtronic, Minneapolis, Minnesota, USA), and a leukocyte filter (LeukoGuard LG, PALL Medical, Port Washington, USA) were used to construct the EVHP circuit as previously described (17). Immediately following completion of the 3-minute initial reperfusion period, hearts were connected to the EVHP circuit, perfused via the aortic root at 40 mmHg, and gradually rewarmed to 37°C over a 30-minute period (16). During this time a cannula was placed in the pulmonary artery and a cone shaped left atrial cannula (XVIVO Perfusion, Englewood, Colorado, USA) was sewn to the common orifice of the pulmonary veins. One hour following initiation of EVHP, hearts were paced in an AAI mode at 105 beats/minute and were transitioned into a working mode by increasing the left atrial pressure to 8 mmHg. Once a steady state was reached, perfusate samples were obtained from the aortic root and coronary sinus, and assessments of myocardial function and metabolism were carried out.

Ex vivo perfusate solution

The *ex vivo* perfusate solution consisted of 1000 mL of STEEN solution (XVIVO Perfusion) and 500 mL of whole donor blood. Additional blood collected during the donor exsanguination was centrifuged and washed with 1000 mL of 0.9% saline solution. The red blood cell concentrate obtained was added to the perfusate solution to achieve a hemoglobin concentration of 45 g/L. Perfusate pH, partial pressure of oxygen, partial pressure of carbon dioxide, and electrolyte, hemoglobin, and lactate concentrations were measured using an ABL800 Flex Analyzer (Radiometer Medical ApS, Brønshøj, Denmark). Oxygen and gas flow through the membrane oxygenator were titrated to maintain a pH of 7.25-7.35 and an arterial partial pressure of oxygen (P_aO_2) of 100-200 mmHg. Infusions of insulin (2.25 units/hour) and dobutamine (4 mcg/minute) were maintained over the duration of EVHP.

Myocardial injury

Myocardial edema

Hearts were emptied of blood and weighed at the beginning and end of EVHP. The total amount of weight gained was normalized to the initial heart weight.

Troponin-I

The amount of troponin-I accumulation in the perfusate was determined using a Pig Cardiac Troponin-I ELISA Kit (Life Diagnostics, Pennsylvania, USA).

Metabolic assessments

Coronary vascular resistance (CVR), myocardial oxygen consumption (MVO_2), and lactate metabolism were calculated as described in the Supplemental Equations.

Myocardial function

Cardiac output was determined by measuring the blood flow on the left atrial line (Bio-Probe Transducer TX-40, Medtronic, Minneapolis, USA) indexed for heart weight (mL/minute/gram). Additionally, the maximum (dP/dt_{max}) and minimum (dP/dt_{min}) rate of pressure change, and developed pressure were measured using a 5F conductance catheter (Ventri-Cath 507, Millar Inc., Houston, USA) and analyzed using LabChart 7 Pro Version 7.2.5 (ADInstruments Inc., Colorado Springs, USA). Stroke work was also calculated as described in the Supplemental Equations.

Statistical Analysis

Normally distributed continuous variables were reported as mean ± standard error. The one-way analysis of variance was used to evaluate differences among the treatment groups. A *p*-value <0.05 was considered statistically significant. Analyses were performed using GraphPad Prism V6.0c (GraphPad Software Inc., La Jolla, USA).

Results

Part 1: Calcium concentration

Initial Reperfusion

The composition of the initial reperfusion solution for each treatment group in Part 1 is displayed in Supplement Table 3. Hearts sustained equivalent periods of warm ischemia (50μmol/L=27.8±0.5, 220μmol/L=27.7±0.4, 500μmol/L=28.3±0.5, 1250μmol/L=28.4±0.4 minutes, *p*=0.551) prior to initial reperfusion. CVR and CBF during the initial reperfusion were comparable among treatment groups (data not shown).

Myocardial injury

Hearts reperfused with a calcium concentration of 220μmol/L developed less myocardial edema during EVHP (Figure 8.3); however, the accumulation of troponin-I in the perfusate during EVHP was not significantly different among treatment groups (50μmol/L=28±6, 220μmol/L=22±5, 500μmol/L=21±5, 1250μmol/L=41±9 pg/mL, *p*=0.855).

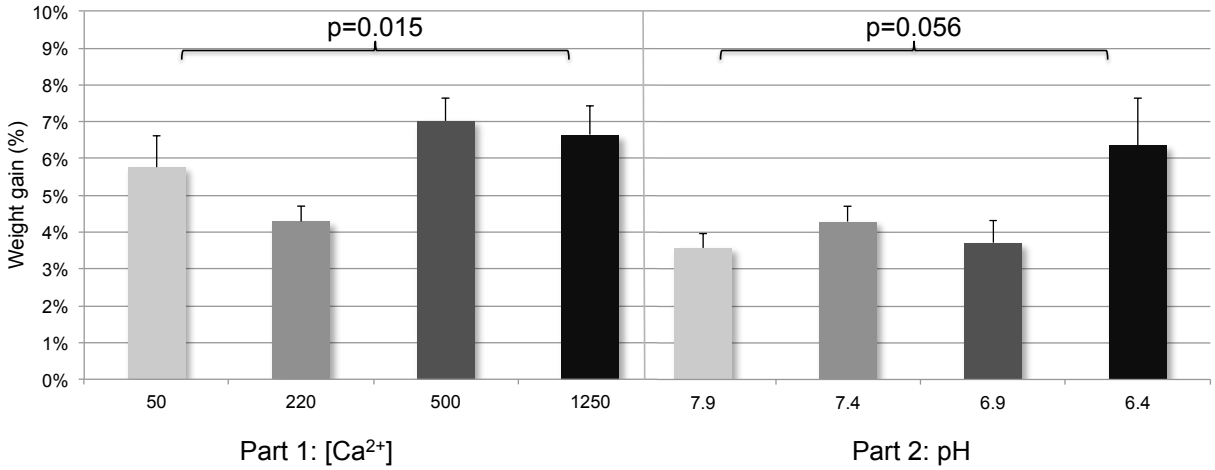

Figure 8.3. Myocardial edema during ex vivo heart perfusion

Myocardial metabolism

There was no significant difference in CVR, myocardial oxygen consumption, arterial lactate, venous lactate, or the veno-arterial lactate difference among treatment groups during EVHP (Figures 8.4-8.6).

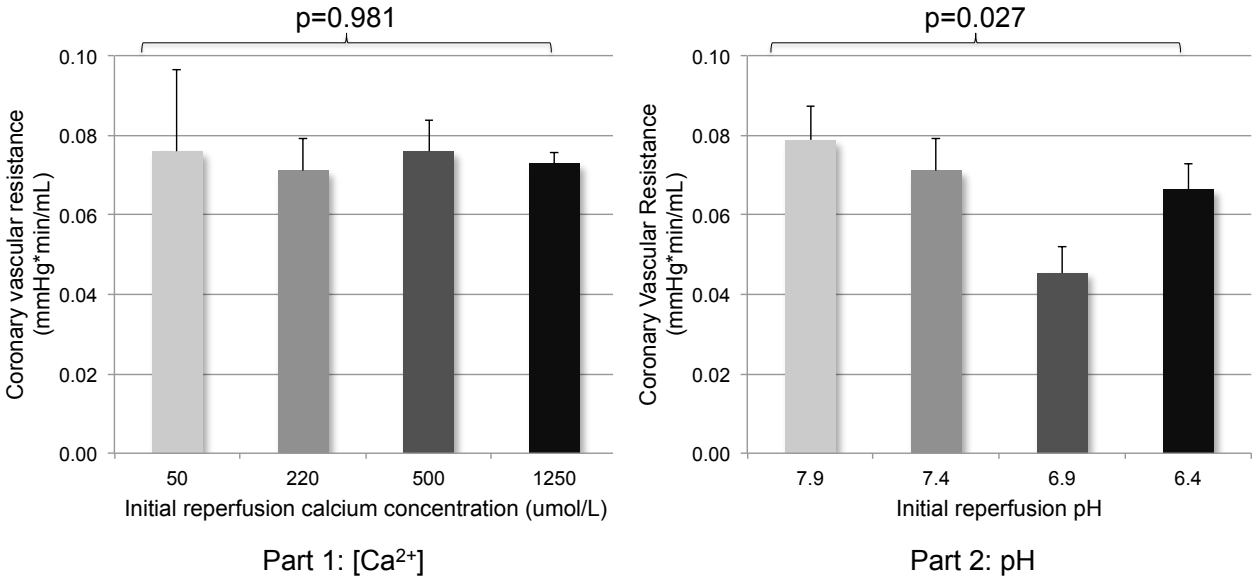

Figure 8.4. Coronary vascular resistance during ex vivo heart perfusion

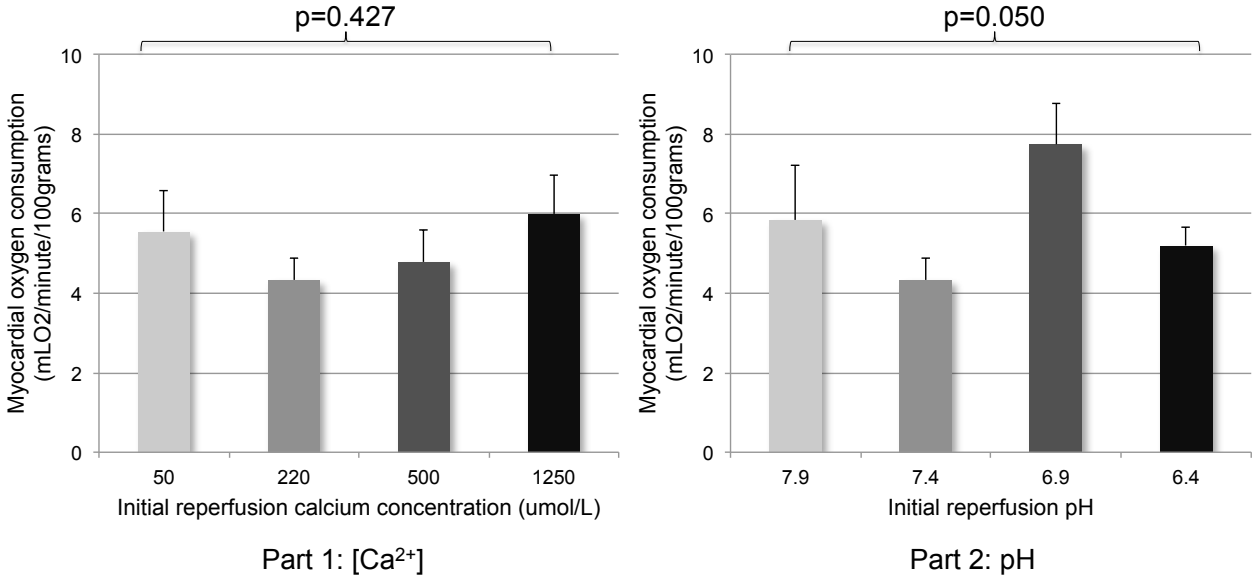

Figure 8.5. Myocardial oxygen consumption during ex vivo heart perfusion

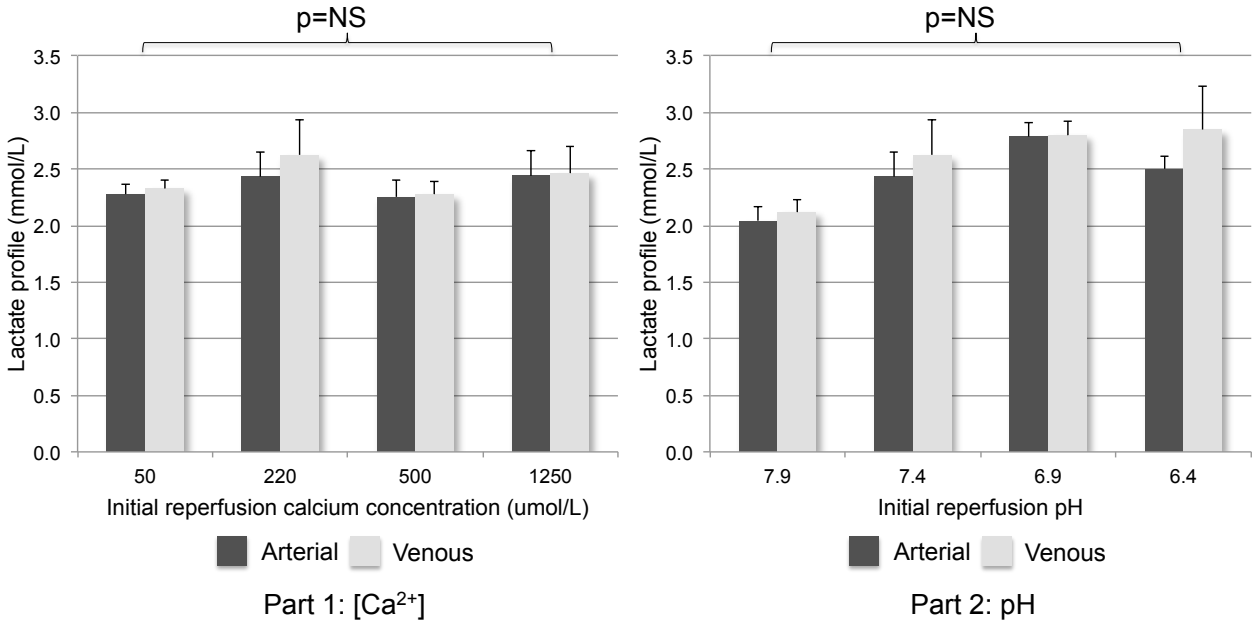

Figure 8.6. Lactate profile during ex vivo heart perfusion

p values represent results of the ANOVA for arterial lactate, venous lactate, and the veno-arterial lactate difference among the 4 treatment groups in Part 1 and Part 2.

Myocardial function

Initial reperfusion calcium concentration significantly impacted the recovery of myocardial function. Hearts initially reperfused with a calcium concentration of 220μmol/L exhibited the highest cardiac index during subsequent EVHP (Figure 8.7). The dP/dt_{max}, dP/dt_{min}, developed pressure, and stroke work improved progressively as the calcium concentration in the initial reperfusion solution was reduced from 1250 to 220μmol/L; however, a further reduction to 50μmol/L was detrimental (Table 8.1).

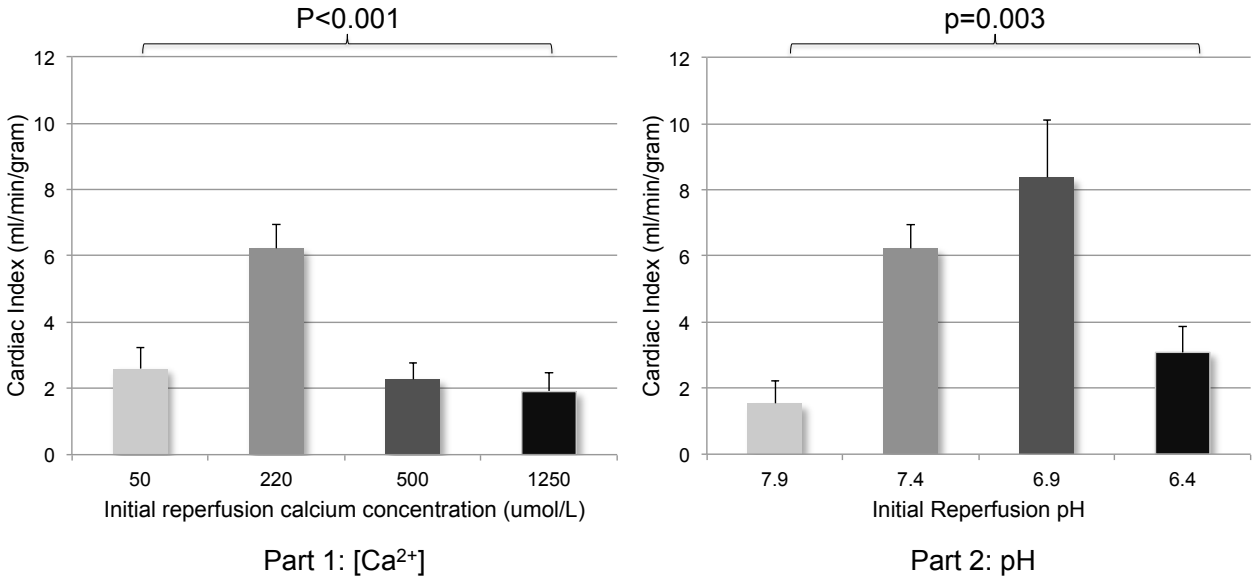

Figure 8.7. Cardiac index (mL/minute/gram) of hearts in a working mode

Part 2: pH

Initial Reperfusion

The composition of the initial reperfusion solution for each treatment group in Part 2 is displayed in Supplement Table 8.4. Hearts sustained equivalent periods of warm ischemia (7.9=28.8±1.2, 7.4=27.7±0.4, 6.9=27.5±0.3, 6.4=27.0±0.7 minutes, *p*=0.279) prior to initial reperfusion. CVR and CBF during the initial reperfusion were comparable among treatment groups (data not shown).

Myocardial injury

Hearts initially reperfused with a pH of 6.4 displayed a trend towards worse myocardial edema during subsequent EVHP (Figure 8.3); however, the accumulation of troponin-I in the perfusate during EVHP was not significantly different among treatment groups (7.9=18±5, 7.4=22±5, 6.9=19±5, 6.4=53±12 pg/mL, *p*=0.593).

Myocardial metabolism

Hearts initially reperfused with a pH of 6.9 demonstrated lower CVR during subsequent EVHP (Figure 8.4). Myocardial oxygen consumption, arterial lactate, venous lactate, and the veno-arterial lactate difference were comparable among treatment groups (Figure 8.5 and 8.6).

Myocardial function

Initial reperfusion pH significantly impacted the recovery of myocardial function. Hearts that were initially reperfused with a moderately acidic (pH 6.9) solution exhibited the highest cardiac index during subsequent EVHP, while alkalotic (pH 7.9) and profoundly acidic (pH 6.4) solutions negatively impacted the recovery of myocardial function (Figure 8.7). Similar results were also observed for the dP/dt_{max}, dP/dt_{min}, developed pressure, and stroke work (Table 8.1).

Discussion

DCD hearts have been proposed as an additional donor source in an attempt to mitigate the current organ shortage that limits cardiac transplantation; however, the development of an evidence-based resuscitation strategy that optimizes the recovery of organ function is essential to realize the potential of DCD transplantation. The severity of myocardial IRI may be significantly impacted by the calcium concentration and pH of the initial reperfusion solution; however, these variables have not been systematically evaluated in the context of DCD. In this study it was demonstrated that initial reperfusion with a hypocalcemic and moderately acidic cardioplegia minimizes myocardial edema and optimizes the functional recovery of DCD hearts during EVHP.

Ischemia reperfusion injury

Following donor extubation the DCD heart functions in an increasingly hypoxemic environment while attempting to maintain systemic oxygen delivery (2). During this time

adenosine triphosphate (ATP) stores are depleted and anaerobic metabolism predominates, which cause intracellular acidosis, activation of the NHE, and sodium influx into the myocyte (4). The sodium-potassium ATPase normally functions to extrude sodium ions entering the myocyte via the NHE (Figure 8.1A). In the DCD context however, intracellular acidosis develops concurrently with the depletion of ATP stores. The combined effect of increased NHE activity and inhibition of the sodium-potassium ATPase produces a pathological accumulation of intracellular sodium (Figure 8.1A) (11). Subsequent reperfusion at the time of organ procurement rapidly normalizes the extracellular pH and creates a large hydrogen ion gradient across the plasma membrane that causes further sodium influx via the NHE (3). This increase in intracellular sodium forces the NCX to function in reverse mode and import calcium ions across the sarcolemma (Figure 8.1B). The resultant intracellular calcium overload propagates myocyte death through the development of hypercontracture, activation of calcium-dependent proteases, generation of reactive oxygen species, activation of mitochondrial permeability transition pores, uncoupling of mitochondrial ATP production, and initiation of apoptotic pathways (3, 4).

DCD heart resuscitation

At the time of organ procurement the DCD heart is energy deplete and vulnerable to the influx of sodium and calcium upon reperfusion. The reactivation of ATP production in this pathologic scenario can propagate calcium oscillations and the development of hypercontracture (18). Okamoto *et al* have shown that irreversible hypercontracture develops in hearts reperfused with blood; however, reperfusion with a cardioplegic solution mitigates this pathologic process (19). Therefore, hypercontracture can be prevented if initial reperfusion of the DCD heart maintains cardiac arrest and provides an opportunity for the restoration of intracellular ion homeostasis prior to myocardial contraction (20, 21). The resuscitation of DCD hearts may be further enhanced by optimizing the composition of the cardioplegic solution to target pathways involved in the pathogenesis of IRI.

Initial reperfusion calcium concentration

Reverse NCX activity causes a profound increase in cytosolic calcium during the reperfusion of ischemic myocardium (Figure 8.1B); however, inhibiting this pathologic process can minimize IRI (10). NCX can move calcium ions in either direction depending on the resting membrane potential and the transmembrane gradients of sodium and calcium (21). The aforementioned membrane depolarization and sodium influx that occur during ischemia and upon reperfusion, force the NCX to function in reverse mode and import calcium ions across the sarcolemma (3). This process can be limited if the initial reperfusion solution is rendered hypocalcemic; thereby, minimizing the calcium gradient that favors reverse NCX activity during early reperfusion (15). The sodium-potassium ATPase can then simultaneously restore the normal sodium gradient and repolarize the cell membrane, allowing NCX to return to a forward mode of operation and restore calcium homeostasis (18).

The majority of experimental evidence to date suggests that hypocalcemic reperfusion of ischemic myocardium minimizes calcium overload, facilitates the restoration of energy stores (phosphocreatine and ATP), limits myocyte injury, and improves functional recovery (15, 22-26). Variability in the optimal calcium concentration (50-750 μmol/L) described in these studies may be explained by the potassium and magnesium concentrations of the solutions (15, 22-27). In this study, it was demonstrated that initial reperfusion of DCD hearts with an adenosine-lidocaine cardioplegia optimized functional recovery when the calcium concentration is 220 μmol/L. This was a normokalemic solution with a magnesium concentration of 2.6 mmol/L, variables that must be taken into account when interpreting our results in the context of previously published literature.

It was also observed that initial hypocalcemic reperfusion minimized myocardial edema during EVHP; however, the troponin concentration in the perfusate was not significantly different among treatment groups. These results suggest that the primary benefit of hypocalcemic

reperfusion in our study was to minimize myocardial stunning, a pathologic impairment in function that persists following ischemia despite the complete reperfusion of viable myocardium (28). This functional impairment is thought to result from calcium overload and the generation of reactive oxygen species during reperfusion, which cause decreased myofilament responsiveness to calcium during contraction (28, 29). Kusuoka *et al* have demonstrated that these pathologic changes can be mitigated by reperfusion with a hypocalcemic solution (29).

It this study, it was observed that reducing the calcium concentration of the reperfusion solution from 1250 to 220 μmol/L significantly improved DCD heart resuscitation; however, the protective effect of hypocalcaemia was lost when the calcium concentration was reduced further to 50 μmol/L. Such a bell-shaped dose response curve to initial reperfusion calcium concentration has been demonstrated previously and may be explained by the calcium paradox (23). Reperfusion with a calcium free solution followed by the restoration of a normal perfusate calcium concentration precipitates myocardial hypercontracture and profound creatine kinase release (30). This calcium paradox is thought to result from sodium influx into the myocyte during calcium free perfusion, precipitating profound calcium entry via NCX following the restoration of a normal perfusate calcium concentration (5, 31). Other authors have also demonstrated that a calcium concentration of 50 μmol/L is not sufficient to protect the myocardium from the calcium paradox (32, 33).

Initial reperfusion pH

The reperfusion of ischemic myocardium rapidly normalizes the extracellular pH and creates a large hydrogen ion gradient across the plasma membrane that causes sodium influx via the NHE. This propagates intracellular calcium overload via reverse NCX activity (Figure 8.1B). The importance of NHE activity in IRI is highlighted by a host of studies demonstrating that NHE inhibitors limit IRI in animal models (7-9, 11). At present there are no clinically available NHE inhibitors, which significantly limits their application in the context of clinical DCD

transplantation. However, transient acidic reperfusion can inhibit NHE activity (12), activation of calcium-dependent proteases, and opening of the mitochondrial permeability transition pore (13, 14).

Previous studies seeking to optimize DCD heart resuscitation have rendered the initial reperfusion solution acidic (34); however, the impact of initial reperfusion pH has not been studied systematically. In Part 2 of this study we sought to determine if initial acidic reperfusion could provide an additive benefit over that achieved with a solution of optimized calcium concentration (i.e. does inhibition of NHE activity during initial reperfusion provide an additive benefit over and above that achieved with inhibition of reverse NCX activity). It was found that reperfusion with a moderately acidic (pH 6.9) solution improved the recovery of myocardial function during EVHP. This group also demonstrated lower coronary vascular resistance, which may have resulted from a higher oxygen demand coupled with better preservation of coronary endothelial cell integrity; however, further research would be required to confirm this. Gao *et al* have also shown that reperfusion with a hypocalcemic (calcium concentration 100-300 μmol/L) and moderately acidic (pH 6.8) solution reduces myocardial stunning and limits myofilament proteolysis (35). While these results suggest that a moderate acidity is protective, the magnitude of the benefit in this study compared to that achieved with a solution of normal pH was small.

The importance of NHE activity during the resuscitation of DCD hearts is underscored by the results of this study. We have shown that the protective effect of a hypocalcemic solution was completely abrogated when the cardioplegic solution was delivered under alkalotic (pH 7.9) or profoundly acidic (pH 6.4) conditions. An alkalotic solution would be expected to promote NHE activity and enhance calcium loading via reverse NCX during the reperfusion of ischemic myocardium, and has been previously shown to adversely impact myocardial recovery following ischemia (36). Interestingly, delivery of a profoundly acidic reperfusion solution was associated with the development myocardial edema during EVHP and very poor functional recovery. These results may be explained by the inhibitory effect of profound acidosis on oxidative

phosphorylation and sodium-potassium ATPase activity (37, 38), enzymatic processes that are required for the restoration of ionic homeostasis prior to myocardial contraction as described previously.

Limitations

The results of this study suggest that a hypocalcemic and moderately acidic cardioplegia optimizes myocardial functional recovery; however, further studies are required to determine if post-transplant outcomes would be similarly affected. We chose to optimize initial reperfusion calcium and pH in a sequential fashion, and the impact of other potential calcium-pH combinations on DCD heart resuscitation is not known. The impact of initial reperfusion calcium concentration and pH on intracellular sodium and calcium concentrations were inferred based on previously published literature and were not directly quantified in this study.

Conclusions

The composition of the cardioplegic solution delivered in the first minutes of reperfusion significantly impacts the recovery of myocardial function during *ex vivo* perfusion. Initial reperfusion of DCD hearts with a hypocalcemic and moderately acidic cardioplegia minimizes edema and optimizes functional recovery. Decades of research demonstrating the protective effect NHE and NCX inhibitors on IRI corroborate these results; however, since none of these drugs are currently available for clinical use, we suggest that the desired clinical endpoint may be achieved by optimizing the pH and calcium concentration of the cardioplegic solution.

Chapter 8 Summary

In Chapter 8: *Impact of reperfusion calcium and pH on the resuscitation of hearts donated after circulatory death*, it was demonstrated that initial reperfusion with an acidic and hypocalcemic cardioplegia optimizes the functional recovery of DCD hearts during EVHP. It is clear that how the DCD heart is treated in the first minutes of reperfusion significantly impacts DCD heart resuscitation. Further, the composition of the resuscitative cardioplegia delivered at the time of organ procurement is an important variable impacting organ viability. Further research in this area may identify additional strategies that optimize DCD heart resuscitation and improve transplant outcomes.

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Supplemental Information

Supplement Table 8.1. Initial reperfusion solution composition for Part 1 of the study

Part 1: [Ca2+], mean (SE)	50µmol/L	220µmol/L[§]	500µmol/L	1250µmol/L
NaCl (mmol/L)	111.8	111.8	111.8	111.8
KCl (mmol/L)	5.9	5.9	5.9	5.9
$CaCl_2$ (µmol/L)	50	220	500	1250
$MgCl_2$ (mmol/L)	2.6	2.6	2.6	2.6
$NaHCO_3$ (mmol/L)	32	32	32	32
NaH_2PO_4 (mmol/L)	1.2	1.2	1.2	1.2
D-Glucose (mmol/L)	10	10	10	10
D-Mannitol (mmol/L)	120	120	120	120
Pyruvate (mmol/L)	1	1	1	1
Glutathione (reduced) (mmol/L)	3	3	3	3
Lidocaine (µmol/L)	500	500	500	500
Adenosine (µmol/L)	400	400	400	400
Insulin (IU/L)	10	10	10	10

[§] equivalent to the 7.4 group in part 2 of the experimental protocol

Supplement Table 8.2. Initial reperfusion solution composition for Part 2 of the study

Part 2: pH, mean (SE)	7.9	7.4[§]	6.9	6.4
NaCl (mmol/L)	43.8	111.8	131.8	137.8
KCl (mmol/L)	5.9	5.9	5.9	5.9
$CaCl_2$ (µmol/L)	220	220	220	220
$MgCl_2$ (mmol/L)	2.6	2.6	2.6	2.6
$NaHCO_3$ (mmol/L)	100	32	12	6
NaH_2PO_4 (mmol/L)	1.2	1.2	1.2	1.2
D-Glucose (mmol/L)	10	10	10	10
D-Mannitol (mmol/L)	120	120	120	120
Pyruvate (mmol/L)	1	1	1	1
Glutathione (reduced) (mmol/L)	3	3	3	3
Lidocaine (µmol/L)	500	500	500	500
Adenosine (µmol/L)	400	400	400	400
Insulin (IU/L)	10	10	10	10

[§] equivalent to the 220µmol/L group in part 1 of the experimental protocol

Supplement Table 8.3. Initial reperfusion solution properties for Part 1 of the study

Part 1: [Ca2+]	50µmol/L	220µmol/L[§]	500µmol/L	1250µmol/L
pH, mean (SE)	7.33 (0.02)	7.34 (0.05)	7.35 (0.01)	7.37 (0.02)
PO_2 (mmHg), mean (SE)	134 (1)	140 (4)	133 (3)	131 (3)
PCO_2 (mmHg), mean (SE)	48 (1)	44 (3)	49 (1)	47 (2)
Na^+ (mmol/L), mean (SE)	143 (1)	143 (1)	142 (1)	143 (1)
K^+ (mmol/L), mean (SE)	5.7 (0.0)	5.8 (0.1)	5.7 (0.0)	5.7 (0.0)
Cl^- (mmol/L), mean (SE)	119 (1)	121 (2)	116 (1)	119 (0)
Ca^{2+} (µmol/L), mean (SE)	50[#]	220[#]	548 (13)	1070 (3)
Glucose (mmol/L), mean (SE)	9.5 (0.1)	9.3 (0.1)	9.5 (0.1)	9.4 (0.1)
Osmolality (mOsmol/Kg), mean (SE)	296 (2)	296 (1)	295 (1)	294 (2)

[#] value not measured (below reliable detection limit of blood gas analyzer)

[§] equivalent to the 7.4 group in part 2 of the experimental protocol

Supplement Table 8.4. Initial reperfusion solution properties for Part 2 of the study

Part 2: pH	7.9	7.4[§]	6.9	6.4
pH, mean (SE)	7.89 (0.02)	7.34 (0.05)	6.86 (0.03)	6.47 (0.05)
PO_2 (mmHg), mean (SE)	116 (2)	140 (4)	146 (1)	148 (2)
PCO_2 (mmHg), mean (SE)	38 (3)	44 (3)	35 (1)	26 (3)
Na^+ (mmol/L), mean (SE)	139 (1)	143 (1)	147 (1)	150 (5)
K^+ (mmol/L), mean (SE)	5.4 (0.0)	5.8 (0.1)	5.9 (0.1)	5.9 (0.1)
Cl^- (mmol/L), mean (SE)	76 (7)	121 (2)	138 (1)	143 (5)
Ca^{2+} (µmol/L), mean (SE)	220[#]	220[#]	220[#]	220[#]
Glucose (mmol/L), mean (SE)	9.4 (0.0)	9.3 (0.1)	9.3 (0.1)	9.1 (0.1)
Osmolality (mOsmol/Kg), mean (SE)	287 (1)	296 (1)	303 (2)	310 (11)

[#] value not measured (below reliable detection limit of blood gas analyzer)

[§] equivalent to the 220µmol/L group in part 1 of the experimental protocol

Chapter 9:

Thesis Summary

Thesis Summary

The clinical impact of cardiac transplantation on the treatment of advanced heart failure is limited by a critical shortage of suitable organs from DBD donors. Optimizing an approach to donor heart resuscitation, preservation, and evaluation that is tailored to the DCD context can facilitate successful transplantation and represents an avenue to expand the donor pool for transplantation.

This thesis explored the physiologic response to donor extubation and identified aspects with particular relevance to the context of DCD heart transplantation. The data presented demonstrates that the DCD heart is forced to function in an increasingly hypoxemic environment while attempting to maintain systemic oxygen delivery. During this time the heart is exposed to a profound catecholamine surge and the right ventricle experiences significant distension. This data highlights the importance of donor heart resuscitation to minimize ischemia-reperfusion injury at the time of organ procurement. Further, the evaluation of donor heart function is essential to select viable organs for transplantation, given the significant ischemic insult and right ventricular distention experienced following donor extubation. While this data provides insight into the physiologic response to donor extubation, the clinical relevance of DCD animal models could be significantly improved if an antecedent neurologic injury was induced prior to donor extubation. Finally, clinical studies have demonstrated that some donors do not immediately become apneic following extubation. Further studies are required to determine the impact of agonal respiratory efforts following donor extubation on the DCD heart.

This thesis established that DCD heart transplantation should utilize an approach to donor heart resuscitation, preservation, and evaluation that is tailored specifically to the DCD context. It is clear that the first minutes of reperfusion is a critical determinant of DCD heart viability. The data presented suggests that DCD heart resuscitation can be optimized if the

cardioplegic solution delivered at the time of organ procurement is tailored to minimize ischemia-reperfusion injury. The delivery of an acidic and hypocalcemic cardioplegic solution at near normothermic temperatures optimizes DCD heart resuscitation; however, these results must be confirmed in large animal transplantation studies. The addition of pharmacologic postconditioning agents to this resuscitative cardioplegia may further improve DCD heart resuscitation.

This thesis also demonstrated that an oxygen carrier is required to maintain myocardial energy stores during EVHP in a physiologic working mode; however, a hemoglobin concentration of only 40 g/L was sufficient to meet metabolic demands. Additionally, myocardial function was best preserved when a whole blood-based perfusate was used. This observation may be related to the antioxidant properties or metabolic substrates present in donor plasma. Further research is required to determine the optimal hemoglobin concentration, oncotic pressure, perfusion pressure, and additive strategies that optimize organ preservation. The data presented also demonstrates that myocardial function declines in a time-dependent fashion during EVHP, regardless of the severity of injury sustained by the organ. This highlights a need for further investigation regarding the optimal means of supporting the heart from a metabolic and hormonal point of view during prolonged EVHP. Advances in this area are likely to dramatically improve donor heart preservation.

Finally, this thesis demonstrated that assessments of myocardial function are the most reliable indicators of donor heart viability during EVHP, while metabolic parameters are of limited value. These results highlight the need for a clinical EVHP device capable of assessing donor heart function in a physiologic working mode. Metrics of organ function must be developed that enable simple, reliable, reproducible, and comprehensive assessments to be undertaken without the need for expensive and cumbersome equipment. This would significantly advance the field of cardiac transplantation, by providing surgeons with objective and definitive evidence of organ viability rather than relying on subjective metabolic surrogates.

Advances in our understanding of ischemia–reperfusion injury and pharmacologic postconditioning have facilitated the development of controlled reperfusion strategies that can successfully resuscitate the DCD heart. The application of other postconditioning strategies (mitochondrial permeability pore inhibitors, reactive oxygen species scavengers, etc.) during initial reperfusion may be beneficial. Further, investigation into the application of ischemic preconditioning protocols to the DCD donor before withdrawal of life sustaining therapy may provide a novel means of increasing the probability of successful organ resuscitation, provided they are ethically acceptable.

Donor heart preservation in a beating state provides an opportunity to deliver cardioprotective agents directly to the DCD heart, limit exposure to incremental cold ischemia prior to transplantation, and optimize post-transplant outcomes. However, prolonged EVHP is associated with a consistent decline in myocardial function that is out of proportion to the severity of myocardial injury. This decline may have a metabolic origin, stemming from a depletion of metabolic substrate, the inhibition of substrate utilization, or insufficient hormonal support (insulin, thyroid hormone, etc.). Further, Advances in our understanding regarding the optimal means of supporting the heart from a metabolic perspective will have a dramatic impact on the potential for EVHP to expand the donor pool.

Advances in donor heart evaluation during EVHP will continue to improve our ability to identify viable organs prior to transplantation. This will allow donor organ utilization to be maximized and recipient risk of primary graft dysfunction to be minimized. Device innovation is essential to facilitate the clinical application of EVHP in a physiologic working mode and the incorporation of functional metrics into viability protocols.

Future research investigating ways to optimize the resuscitation, preservation, evaluation, and transplantation of DCD hearts is vital to ensure a broader application of DCD heart transplantation in the future.