Regulation of Coronary Artery Flow during Normothermic *Ex Situ* Heart Perfusion

by

Xiao Qi

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Department of Surgery
University of Alberta

Abstract

The overall goal of this study is to improve the protocols for *Ex Situ* heart perfusion (ESHP) to better preserve the function and viability of donated hearts. ESHP has emerged as a new method of donor heart preservation. Unlike the standard cold storage technique, in ESHP, the heart is perfused with oxygenated, nutrient-rich solutions with warm blood during preservation, hence preventing ischemia. As a preservation method, there are solid theoretical advantages to storing donor heart in a physiological, functioning state: the organ can be resuscitated from ischemia or other acute injury. Unlike the cold storage method to decrease the metabolism, ESHP preserves the heart at normal temperature to maintain the metabolism in the myocardium. In addition, donor heart function can be used to predict viability and preservation time could be potentially prolonged. Ideal methods for maximally preserving cardiac function and graft quality using ESHP are sought after. However, the optimal approach for graft evaluation is yet to be established. Before successfully clinical application of this strategy, the establishment of effective techniques for resuscitation of the cardiac allograft and reliable methods for evaluating its transplantability is necessary.

During heart preservation, the coronary artery function is an important part. Myocardial perfusion is regulated by a variety of factors that influence arteriolar vasomotor tone. Many researchers indicate that there was endothelial damage during ESHP. Our preliminary results showed that there was a loss of coronary artery autoregulation. The mechanisms responsible for the loss of coronary regulation during ESHP are not understood but may be related to the metabolism alteration in the myocardium or oxidative stress state of endothelium appearing during ESHP. Such

change will exert its effect on the endothelium and affect the vasoactive function of the coronary artery. We aim to mitigate such functional loss and protect the coronary artery during ESHP. Also, we plan to use some additives such as oxygen-derived free radical scavengers to protect coronary artery function.

Our preliminary data suggest that regulation of coronary artery function is disturbed during ESHP, leading to apparent excessive coronary blood flow over time. This observation contrasts with a gradual reduction in myocardial function over time, suggesting a loss of coronary regulation during ESHP. The mechanisms responsible for the loss of coronary regulation during ESHP may be related to the metabolism alteration in the myocardium or oxidative stress state of endothelium appearing during ESHP. Several other factors may also be involved in the loss of coronary regulation during ESHP, such as endothelial factors, oxygen carriers, and vasoactive mediators. We hypothesize that the change of multiple factors during ESHP would affect the endothelium and affect the vasoactive function of the coronary artery. The change of multiple elements in the regulation of coronary blood flow will be studied during the isolated working heart perfusion model.

Understanding the physiological and pathophysiological factors that modulate coronary blood flow provides the basis for the judicious use of ESHP in providing ideal cardiac graft quality. We aim to investigate the mechanism underlying the loss of coronary autoregulation and find ways to protect coronary artery function.

Preface

This thesis is original work by Xiao Qi.

This work receive research ethics approval from the University of Alberta Animal Care Committee, protocol #AUP00000943.

Chapter 2 and 4 are currently in preparation for submission to peer-reviewed journal. Chapter 3 is currently in revision for *The Journal of Heart and Lung Transplantation*.

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Abbreviations

AA – Arachidonic acid

ATP – Adenosine triphosphate

CABG – Coronary artery bypass grafting

CAV – Cardiac allograft vasculopathy

cAMP – cyclin adenosine monophosphate

CBF – Coronary blood flow

CD138 – Syndecan-1

cGMP – cyclic guanosine monophosphate

CK-MB – Creatine kinase myocardial band

CI – Cardiac index

CS – Cold static storage

cTnI – Cardiac troponin I

CVR – Coronary vascular resistance

CYP – Cytochrome P450

DAMP – Damage associate molecular pattern

DCD – Donation after circulatory determined death

dP/dt_{max} – Maximum rates of left ventricle pressure change

dP/dt_{min} – Minimum rates of left ventricle pressure change

EDHF – Endothelium-dependent hyperpolarization

EET – Epoxyeicosatrienoic acid

ELISA – Enzyme-linked immunosorbent assay

ERK1/2 – Extracellular signal-related kinases 1/2

p-ERK1/2 – Phosphate extracellular signal-related kinases1/2

ESHP – *Ex situ* heart perfusion

GPx – Glutathione peroxidase

GPx1 – Glutathione Peroxidase 1

GSH – Reduced glutathione

HTx – Heart transplantation

5-HT – 5-hydroxytryptamine (serotonin)

IL-6 – Interleukin-6

IL-1β – Interleukin-1β

IL-8 – Interleukin-8

IL-10 – Interleukin-10

I/R – Ischemia and reperfusion

IVC – Inferior vena cava

K_{Ca} – Calcium-avtivated potassium channel

K_V – Voltage-dependent potassium channels

LA – Left atrium

LDH – Lactic dehydrogenase

LVSW – Left ventricle stroke work

MAPK – Mitogen-activated protein kinases

MDA – Malondialdehyde

MEO_2 – Myocardial oxygen extraction

MMP – Matrix metalloproteinases

MMP-9 – Matrix metallopeptidase 9

MPO – Myeloperoxidase

MPT – Mitochondria permeability transition

MVO_2 – Myocardial oxygen consumption

NADPH – Nicotinamide adenine dinucleotide phosphate

NO – Nitric oxide

NOS – Nitric oxide synthases

NOX – NADPH oxidase

OCS – Oran care system

oxLDL – Oxidized low-density lipoprotein

PEG – Polyethylene glycol

PF4 – Platelet factor 4

PGE_2 – Prostaglandin E_2

PGI_2 – Prostacyclin

PO_2 – Partial pressure of oxygen

PTP – Permeability transition pore

RNS – Reactive nitrogen species

ROS – Reactive oxygen species

RIRR – ROS-induced ROS release

SaO_2 – Oxygen saturation

sCD-40L – Soluble CD-40 ligand

SDH – succinate dehydrogenase

sTM – Soluble thrombomodulin

SVC – Super vena cava

TCA – Tricarboxylic acid cycle

β-TG – β-Thromboglobulin

TNF-α – Tumor necrosis factor-α

VCAM-1 – Vascular cell adhesion molecule 1

vWF – Von Willebrand factor

XO – Xanthine oxidase

Chapter One: Background

Introduction

Heart transplantation (HTx) is a recognized therapy for terminal heart failure patients. The clinical application of heart transplantation has expanded over time, and outcomes have improved substantially.[1] Meanwhile, waiting lists for donor heart continue to rise worldwide due to the discrepancy between the demand and supply of suitable organs.[2] A low rate of utilization of hearts that are offered for transplantation contributes to this organ shortage. In North America, only 30 % of the available donor hearts are transplanted and the majority are discarded.[3-5] To expand the heart donor pool, normothermic *Ex-Situ* heart perfusion (ESHP) has emerged as a platform for reconditioning marginal donor hearts and consequently increasing the number and quality of donor organs available for transplant. The EXPAND clinical trial has recently indicated that resuscitating extended criteria donor hearts using Organ Care System ™ (OCS) leads to excellent post-transplant outcomes and low primary graft dysfunction rates.[6] ESHP also allows continuous functional and metabolic assessment of donor hearts, thereby ensuring that only viable hearts are selected for transplantation. For example, the *ex-situ* metabolic assessment afforded by the OCS is a new capability that enables some biomarker data to be assessed by the surgeon up to the point of transplantation. Such an evaluation is not available in the cold storage method. In addition to determining graft viability, OCS technology can conceivably allow longer preservation time, potentially improving organ sharing and matching.[7]

Despite recent progress on ESHP, many questions remain on the optimal method for organ perfusion and preservation. These questions exist in different stages of heart transplantation. For example, pre-ischemic treatments, warm ischemic conditions,

procurement reperfusion, cardioprotective approaches applied during initial reperfusion, graft storage, and transplantation reperfusion.[8] Ideal methods for maximally preserving cardiac function and graft quality are sought after. Furthermore, the ideal approach for graft evaluation is yet to be established. Thus, preclinical research is required to develop the most effective and robust clinical protocols for ensuring adequate post-transplant graft function and recipient outcomes. The combined use of these modifications could contribute to better functional recovery.

During heart preservation, normal cardiac function is highly dependent on myocardial perfusion. So coronary arteries' function should be well evaluated and preserved as well. Myocardial perfusion is regulated by a variety of factors that influence arteriolar vasomotor tone. The ability of coronary resistance vessels to dilate in response to increment in myocardial oxygen demand, as illustrated by the tight correlation between myocardial oxygen consumption and coronary blood flow, is critical for maintaining an adequate supply of oxygen to the myocardium.[9] Our preliminary data suggest that regulation of coronary artery function is disturbed during ESHP, leading to apparent excessive coronary blood flow over time. This observation contrasts with a gradual reduction in myocardial function over time, suggesting a loss of coronary regulation during ESHP. The mechanisms responsible for the loss of coronary regulation during ESHP are not understood. Still, they may be related to the metabolism alteration in the myocardium or oxidative stress state of endothelium appearing during ESHP. Such change will exert its effect on the endothelium and affect the vasoactive function of the coronary artery. We aim to mitigate such functional loss and protect the coronary artery during ESHP. Also,

we plan to use some additives such as oxygen-derived free radical scavengers to protect coronary artery function.

In this project, we will study the underlying mechanisms for the loss of coronary regulation that occurs during ESHP and the methods to mitigate them. The overall goal of this study is to improve the protocols of ESHP for the aim of better preserving the function and viability of donated hearts. Also, we would like to find effective ways to evaluate coronary artery function during ESHP and protect the coronary artery function of the donated heart.

1.1 Heart preservation for Transplantation: Principles and strategies

It has been over half a century since the first successful clinical heart transplantation was done with a heart donated after circulatory death in 1967 by Christiaan Barnard and the South African Groote Schuur Hospital team.[10] With more than 6,000 heart transplants performed annually worldwide, according to the International Society for Heart and Lung Transplantation Registry data.[11] HTx now has become the standard treatment for end-stage heart failure. Significant developments have occurred in the field of HT over the past few years, especially the successful transplantation of donor hearts after circulatory determination of death (DCD).

Cold static storage (CS) is the current standard and most commonly used method of organ preservation. Despite being simple and inexpensive, the ischemic time that cold preservation can allow is short for the heart compared to other organs. Even though many studies have demonstrated prolonged storage time using cold preservation, the generally accepted ischemic time for cold preservation is limited to 6 hours. In clinical practice, it

has been accepted that cold ischemic time over 6.25 hours is associated with poor long-term cardiac graft survival, decreased patient survival, and higher 30-day and overall mortality.[12] Moreover, ischemic durations longer than 200 minutes have been associated with primary dysfunction and acute right heart failure, both of which substantially increased postoperative morbidity and mortality of transplant recipients.[13-15] Moreover, due to the static, non-physiologic condition of the heart in CS, assessing cardiac function or performing therapeutic interventions is impossible. This limitation is particularly important for extended criteria/suboptimal donor hearts in which viability should be evaluated carefully before transplantation.

1.1.1 Harmful effects of cold ischemic storage

Although cold preservation is commonly used in heart transplantation, there are disadvantages to this preservation method. First, cold preservation does not resuscitate the organs but only minimizes function deterioration and does not improve function. Secondly, cold ischemic storage can even lead to more deterioration through endothelial damage. Increased stress exerted on organs through endothelial damage induces antigen presentation to the recipient. It can lead to acute and long-term effects, such as rejection, coronary arteriopathy, and even mortality.[16-18] There are other harmful effects of cold ischemic storage, which will be described below:

1.1.1.1 Cellular swelling

Normally, cells are bathed in an extracellular solution with high Na^+ and low K^+, which is maintained by the Na^+-K^+ ATPase pump. Anaerobic and hypothermic preservation suppresses the activity of the Na^+ pump and decreases plasma membrane potential.[19] A

high concentration of Na^+ that cannot be transported outside the cardiomyocytes will lead to elevated crystal osmotic pressure. The water that follows leads to cell swelling. In the cold storage preservation method, colloids are added to the preservation solution to generate the same osmotic pressure in the intracellular compartment and counterbalance cell swelling. Impermeants are also used for this purpose, like raffinose, glucose, and mannitol.

1.1.1.2 Intracellular acidosis

It is well known that acidosis is harmful to normal cell function. During cold storage, cardiomyocyte acidosis is mainly attributed to the following two reasons: (1) anaerobic glycolysis is stimulated and lactate is gradually accumulated during ischemia; [20] Pyruvate is metabolized to lactic acid by lactic dehydrogenase (LDH).[21] When the concentration of lactic acid reaches 16 to 20 μmol,[13] it will injure cell organelles and activate macrophages, leading to cytokine production and the initiation of the inflammatory response. Efforts at limiting cellular acidosis include adding various hydrogen ion buffers to the preservation solutions, like sodium bicarbonate, histidine, and magnesium sulfate.[22]

1.1.1.3 Extracellular edema

During the process of organ procurement and storage, interstitial fluid accumulation results in extracellular edema.[13] This may be attributed to elevated hydrostatic pressure caused by the flushing of the organ vasculature with the preservation solution. This extracellular edema, in turn, can lead to the collapse of organ microvasculature, which can impede the flow of flush solution. Also, this may result in an uneven distribution of the preservation solution in the heart. To address this problem, impermeants like hydroxyethyl starch and other nontoxic colloids are added to the preservation solution to

exert colloid oncotic pressure in the intravascular space, preventing fluid accumulation in the interstitial space.

1.1.1.4 Reperfusion injury

Ischemia-reperfusion injury happens when blood circulation through the organ is restored, and oxygen supply is reestablished. Accumulated anaerobic metabolites in tissue during an ischemic situation react with oxygen to produce various harmful oxygen-free radicals. These superoxide anions, like hydrogen peroxide, hydroxyl radical, etc., can contribute to cell injury by involving lipid peroxidation and oxidation of protein sulfhydryl groups. Free radicals can also cross-link membrane proteins, cleave peptide bonds, alter the function of glycosaminoglycans, and promote DNA disruption.[23] On the other hand, since protective antioxidants will be depleted in ischemia, giving exogenous antioxidants became necessary. For example, glutathione can combine with reactive oxygen species (ROS) and free radicals to minimize tissue oxidative injury. It has long been known to play an active role in protecting tissue from reperfusion injury and be included as a specific additive to reduce oxygen-free radicals. It was known that free radicals derived from leukocytes are responsible for the major reperfusion injury.[24] It has also been reported that eliminating leukocytes from reaching the reperfused myocardium could prevent this aspect of reperfusion injury.[25] This suggests that leukocytes filtration before reperfusion the donor heart could protect the organ from reperfusion injury.

1.1.1.5 Calcium overload

Intracellular Ca^{2+} overload may play an essential role in reperfusion injury. Ca^{2+} balance is accomplished by some sarcolemmal transport enzymes, exchangers, and ion

pumps. For example, it would be harmful to the cell if Na^+- Ca^{2+} exchanger was in reverse mode (Ca^{2+} influx and Na^+ efflux) because a high level of Ca^{2+} in the cell can lead to activation of Ca^{2+}-dependent phospholipases and protease. Another intracellular influx of Ca^{2+} is through sarcolemmal Ca^{2+} channels. When intracellular ATP level is low, the sarcoplasmic reticulum Ca^{2+} pump cannot transfer the excess of Ca^{2+} out, and diastolic Ca^{2+} levels increase markedly, resulting in Ca^{2+} overload and cellular injury.[26] This Ca^{2+} paradox results in irreversible cell damage and the clinical phenomenon known as “stone heart.”[27] Several methods have been proposed to address Ca^{2+} overload and cellular injury, including the addition of Ca^{2+} channel blockers and giving exogenous ATP.

1.1.1.6 Oxidative stress injury

Oxidative stress is an imbalance between ROS production and degradation, such as superoxide anion, hydrogen peroxide, lipid peroxides, and peroxynitrite.[28] Several studies[29-31] identified that ROS is involved in the ischemic and reperfusion (I/R) process and in the absence of reperfusion. The addition of molecular oxygen during reperfusion causes the generation of oxygen-free radicals, and these cytotoxins induce peroxidation of the lipidic cell membrane.[32] Although NADPH oxidase (NOX) and xanthine oxidase can stimulate ROS formation during cold ischemia, the mitochondria pathway is the primary source of these ROS.[33] Mitochondria can also respond to elevated ROS concentrations by increasing their ROS production, a phenomenon termed ROS-induced ROS release (RIRR).[34] Ca^{2+} increase is a constant feature of pathological states associated with oxidative stress. ROS can cause calcium overload in cardiomyocytes by impairing Ca^{2+} transport systems.[35] On the other hand, hypothermia inhibits Na^+-K^+-ATPase, which will cause efflux of excess intracellular Na^+ through Na^+-Ca^{2+} exchanger.

Consequently, it will lead to mitochondrial calcium overload.[36] For both I/R injury and cold storage-induced injury, ROS seems to play an important pathogenic role, which is explained at least partly by the induction of lipid peroxidation. Attenuation of cold preservation-induced cell injury may prevent delayed graft function and consequently improve the long-term outcome of heart transplantation.

1.1.1.7 Cardiomyocytes apoptosis and necrosis

Prolonged cold ischemia is a major reason for delayed graft function. During isolated heart cold ischemia preservation, the increase of ROS will stimulate its downstream molecules, which will lead to cardiomyocyte apoptosis.[37] ROS is very active and can react with several cell substances such as membrane phospholipid, proteins, and nucleic acid. Consequently, it will cause cellular metabolism dysfunction and breakage of structure, which in the end, will lead to cardiomyocyte apoptosis and necrosis. Apoptosis is a kind of programming cell death and is also an ATP-depletion process.[38] As a consequence, the level of cellular adenosine decides the ways of cell death. In mitochondria permeability transition (MPT)-dependent I/R injury, the presence or absence of glycolytic ATP serves as a switch between the two forms of cell death.[39] For example, when reperfusion opens the permeability transition pore (PTP), and cellular ATP becomes depleted, apoptotic signaling is abrogated at the level of the apoptosome, and the fate of cell death is directed to ATP depletion-dependent necrosis. However, when glycolytic ATP is available, necrotic cell death is prevented, and apoptotic signaling occurs instead.[40] Halestrap[41] pointed out the evidence for the mitochondria permeability transition (MPT) being a major factor in the transition from reversible to irreversible injury during reperfusion of a heart following a period of ischemia.

In conclusion, simple hypothermic immersion is an effective and clinically employed method for donor heart storage. Although simple and efficient, this method might be reaching its limit concerning the duration, preservation, and quality of organs it can be preserved. It is an imperfect method associated with low-level but persistent anaerobic metabolism that induces discrete changes in myocardial gene expression.[42, 43] Currently, primary graft dysfunction affects approximately 3% of clinical heart transplants performed worldwide and accounts for 34% of death in the first 30 days after transplant.[15] Improvement in preservation solutions allows routine transplantation of livers and kidneys after 24 hours or more of cold ischemia. However, the time in hearts and lungs preservation is limited to 4-6 hours.

1.1.2 Normothermic *ex-situ* heart perfusion (ESHP)

ESHP is a novel method of donor heart preservation. In ESHP, the heart is perfused with oxygenated, nutrient-rich solutions with warm blood during preservation, preventing ischemia. ESHP preserves the donated heart in a beating, semi-physiological condition.[7] As a result of dynamic preservation of the heart during ESHP, it provides the opportunity to assess cardiac function, viability, and metabolism.[44] The transmedics OCS™ is the first commercially available device to transport donor hearts in a normothermic perfusate state.[45] The use of this system might allow for distant procurement of donor hearts, which could balance sharing of donor hearts among regions and possibly enable the resuscitation of marginal donor hearts, thus expanding the donor pool.[7]

Several clinical trials have been reported: PROTECT and PROCEED I both have shown excellent patient survival with preservation times within 5 hours;[46] PROCEED II[7]

showed that the clinical outcomes of donor hearts preserved with OCS™ platform were non-inferior to the outcomes of those preserved with static cold storage. The EXPAND clinical trial has recently indicated that resuscitating extended criteria donor hearts leads to excellent post-transplant outcomes and low primary graft dysfunction rates.[6] Despite recent progress on ESHP, many questions still await resolution before ESHP can become the preferred method of organ preservation. These questions exist in different stages of heart transplantation. For example, pre-ischemic treatments, warm ischemic conditions, procurement reperfusion, cardioprotective approaches applied during initial reperfusion, graft storage and transplantation reperfusion, etc.[8] Optimal, evidence-based strategies for graft reperfusion, storage and evaluation remain to be identified for ensuring adequate post-transplant graft function and recipient outcomes.[47] Thus, preclinical research is required to develop the most effective and robust clinical protocols for ensuring adequate post-transplant graft function and recipient outcomes.

1.1.2.1 Perfusate

The perfusate may have a significant impact on heart inflammatory and immune activation after transplantation.[48] The ideal perfusate for continuous perfusion of an organ has not yet been defined. However, the perfusate should deliver oxygen to the organ by using an oxygen carrier for the full benefit of perfusion to be realized.[49, 50] Simple oxygenated buffer solutions require higher flows for adequate oxygen delivery and create degenerative changes in the perfused tissues that are not seen when red blood cells are used as the oxygen carrier.[51-53] Perfusion preservation with oxygen-carrying solutions has the advantage of preventing ischemia, anaerobic metabolism, and reperfusion injury.[32] In addition to an oxygen carrier, any of the known free-radical scavengers, vasoactive

substances, inflammatory mediators, calcium-channel blockers, insulin, antibiotics, metabolic precursors and nutrition can be added to the perfusate to provide a continuous cellular supply, an advantage not offered by cold static storage.[54]

1.1.2.2 Heart viability assessment

A reliable method for assessing donor heart viability before transplantation is a must before brain dead and DCD donors can be utilized clinically.[55, 56] an essential limitation of preserving organs by cold storage is the lack of a method for assessing whether the organ will function properly after transplantation. Perfusion offers the attractive feature of assessing organ viability before transplantation; this can be done by studying the organ's perfusion characteristics (hemodynamics) and by analyzing the perfusate. Although this might not be necessary for organs harvested under ideal circumstances—these are almost certain to function—it becomes critically important for marginal organs that are more likely to fail. At present, organs are rejected if the chance of functioning is judged to be low. However, because there is no definitive measure of function, some viable organs are inevitably rejected. *Ex-situ* perfusion will allow assessing the viability of hearts before transplantation in a beating state, which would expand the donor pool and simultaneously keep risk to the recipient to a minimum. Additionally, the *ex-situ* metabolic assessment provided by the perfusion platform is a new capability that enables some biomarker data to be assessed by surgeons up to the point of transplantation.[7] Such evaluation cannot be achieved by cold storage.

1.1.3 Endothelial damage during myocardial preservation and storage

Optimal heart preservation for transplantation should include the protection not only of the myocardium but also the coronary artery. A key role of the endothelial dysfunction in the pathogenesis of early low coronary flow of heart transplants and late cardiac allograft vasculopathy (CAV) demonstrates the importance of optimal coronary endothelial preservation during heart storage.[57] Evidence indicates that endothelium plays a critical role in maintaining normal cardiac function after cardiac surgery, mainly by controlling coronary perfusion.[58, 59] Damage of endothelial integrity during preservation could lead to increased capillary permeability and organ edema after reperfusion.[60] Also, vasospasm by the release of vasoactive compounds could cause microvascular hypoperfusion and early organ dysfunction.[61] The following aspects of endothelial injury from cold static storage and machine perfusion are discussed below.

1.1.3.1 Endothelial injury from hypothermia

The static cold storage method primarily involves the slowing of myocardial metabolism with hypothermia. Hypothermia theoretically can exert protective effects by limiting the increase of permeability induced by hypoxia in cultured endothelial cells,[62] and by attenuating endothelial cell activation.[63, 64] However, there are several harmful effects associated with hypothermia. First, even at 4 °C, the metabolism does not completely stop, as some metabolic activity is still detectable. Hypothermia reduces oxygen consumption and high energy phosphate depletion, which is essential for cell viability.[65] It delays the occurrence of endothelial cell structures disruption, but the reparative process is also impaired. Additionally, the extent of adenosine triphosphate-generating reactions is slowing down, and the activity of ionic pumps is attenuated, which

facilitates the loss of transmembrane ionic gradient. This consequent event promotes endothelial swelling.[66]

Secondly, prolonged hypothermic storage causes an increased fusion of microvilli with blebbing of the plasma membrane, chromatin condensation and ruffling of the nuclear membrane, and DNA fragmentation, ultimately leading to loss of cell-cell connection.[67, 68] Also, the presence of blebbing as a prominent ultrastructural feature on plasma membranes and of nuclear fragmentation suggests apoptosis as the potential mechanism responsible for hypothermic endothelial injury and cell death.

Unlike the cold storage method to decrease the metabolism, ESHP preserves the heart at normal temperature to maintain the metabolism in the myocardium. Potentially it may get rid of the harmful effects associated with hypothermia.

1.1.3.2 Endothelial injury from preservation solutions

The use of preservation solutions, either intracellular or extracellular, cannot completely prevent endothelial damage.[69] It was reported by some studies[58, 59] that the hyperkalemic composition of the intracellular solution is harmful to the functional and structural integrity of endothelial cells of the coronary artery. A high level of potassium can induce swelling of the endothelium and disturb the initiation of microcirculation reperfusion.[70]

Normally, the endothelium synthesizes compounds that cause vascular smooth muscle relaxation, including nitric oxide (NO), endothelium-dependent hyperpolarization factor (EDHF), and prostacyclin. NO is a vasodilator and acts via the cyclic guanosine monophosphate (cGMP) pathway and is capable of affecting vascular tone and possesses properties that may be beneficial for the preservation of allograft function.[71]

Hyperkalemic damage to endothelial cells may reduce NO release and inhibit the formation of EDHF, which is likely a cytochrome P450 monooxygenase metabolite of arachidonic acid and normally causes vascular edema smooth muscles to relax via Ca^{2+} activated K^+ channels and prolonged membrane depolarization.[13] Finally, Hyperkalemia can result in the release of tissue plasminogen activator, fibronectin, interleukin-1, and endothelin, which may be involved in the endothelial injury.[72]

1.1.3.3 Endothelial injury from perfusate in machine perfusion

The perfusate used during normothermic machine perfusion may also cause endothelial damage. Different perfusion solutions have been used for machine perfusion, including leukocyte-depleted blood,[73-75] whole blood,[54, 76-79] modified Krebs solutions,[80, 81,] and other asanguineous crystalloid perfusate.[82-84] Preservation using a whole blood-based perfusate may improve donor heart preservation compared to a solution in which the plasma component has been removed.[78] This improvement may partially attribute to the protection of endothelial function. The addition of blood improves the preservation of both endothelium-dependent and endothelium-independent relaxation than crystalloid perfusate following circulatory death (DCD).[79]

The protective effects of blood perfusate on vascular endothelium may be derived from the counterbalance of ischemia-reperfusion injury. Blood is a potent inhibitor of oxygen-derived free radicals that may be released on initiation of reperfusion.[69] In addition, soluble O_2 from oxygenated blood may provide additional oxygen to the endothelial cells, thus reducing the ischemic damage. Thirdly, blood has favorable rheologic features with respect to crystalloid solution.[85] The protection from ischemia-reperfusion damage may

explain the beneficial effects on improved endothelial function with the addition of free-radical scavengers to crystalloid solutions.[86]

1.1.3.4 Endothelial injury from mechanical forces

The endothelium lining of the vasculature forms the physical barrier between the blood and underlying the myocardium. It is directly exposed to an environment composed of various biomechanical stimuli resulting from the pulsatile flow. These stimuli include shear stress and hydrostatic pressure. The endothelium can sense these stimuli and release cytokines, growth factors, nitric oxide, or hormones. They increase vascular permeability, elevate the expression of leukocyte adhesion molecules, induce monocyte recruitment and modulate endothelial cells proliferation and repair.[87, 88] The mechanical stress is transduced by flow-sensitive receptors in the endothelium into the nucleus through a distinct signaling pathway, inducing gene transcription. Additionally, shear stress-induced changes in cell shape modify endothelial barrier function. Disassembly of endothelial cell-cell junctions increases vascular permeability and facilitate leukocyte transmigration.[89]

In addition to shear stress, the endothelium of coronary arteries is exposed to infusion pressure of cardiac preservation solutions. Previous studies have indicated that excess infusion pressure of cardioplegia and overdistension of vein grafts during coronary artery bypass grafting (CABG) procedure may cause endothelial damage, contributing to poor myocardial recovery.[90-92] Moreover, the perfusion pressure is critical during heart perfusion, as high or extremely low pressure may be detrimental to the endothelium, thus reducing cardiac protection. The coronary perfusion pressure may also affect the development of myocardial edema. Lower pressure but capable of generating enough

flow of the oxygenated perfusate for effective perfusion and preservation is associated with less edema formation.[20] Inadequate pressure may compromise myocardial oxygen delivery, while excessive pressure may damage the endothelium.[93]

Thus, these studies above suggest that perfusion pressure and presumably the flow rate affect optimal myocardial preservation. The optimal perfusion pressure or flow rate during machine perfusion needs further investigation.

1.2 Regulation of coronary blood flow

During heart preservation, maintenance of cardiac function relies on a proper myocardial blood supply through the coronary arteries. So, the regulation of coronary blood flow is an essential issue in heart preservation. It is generally accepted that coronary flow results from the interplay of coronary perfusion pressure, ventricular contraction, and coronary vascular tone.[94] Regulation of coronary blood flow involves multiple mechanisms that include extravascular compressive forces, coronary perfusion pressure, myogenic, local metabolic, endothelial, and neural and hormonal factors.[9] Due to the limited anaerobic capacity of the heart, coronary vascular resistance (CVR) is continuously regulated to deliver sufficient quantities of oxygen to meet any change in the demand of surrounding myocardial tissues.[95] This metabolism-perfusion matching is unique in the coronary circulation because of the heart's continuous pumping and high workload.[96] The heart is the highest per gram oxygen consumption of any organ. It also demonstrates a high myocardial O_2 extraction (MEO_2), requiring tight coupling of coronary blood flow to changing metabolic needs.[9, 97, 98] Therefore, to maintain normal myocardial functionality, the coronary vascular tone must be constantly modulated to ensure

appropriate myocardial perfusion. It is essential that mechanisms exist to assure that changes in myocardial metabolism (demand) are matched by concordant and proportional changes in oxygen delivery (supply).[9, 99-101]

1.2.1 Control of coronary blood flow by an autoregulatory mechanism

Autoregulation is the term used to describe the intrinsic ability of the arteries to adjust blood flow according to tissue needs. The heart has an inherent ability to maintain relatively constant coronary blood flow in the presence of alterations in perfusion pressure, termed coronary autoregulation.[102] Coronary pressure-flow autoregulation is accomplished by comparative changes in microvascular resistance. Reductions in perfusion pressure diminish coronary vascular resistance, while increases in perfusion pressure elevate coronary vascular resistance.

The mechanism of autoregulation can be explained by the metabolic hypothesis, which proposes that increased metabolism, reduced oxygen concentration, or decreased blood flow results in accumulation of vasodilatory chemicals in the vessel. The smooth muscle surrounding the vessel relaxes in response to the presence of the chemical, increasing vessel diameter. Also, the endothelial cells that line vessels are known to secrete a large body of relaxing and constricting factors, which may contribute to autoregulation.[103]

Coronary autoregulation adjusts to the level of myocardial metabolism.[94] The autoregulation theory states that as myocardial functional demand increases, coronary blood flow increases as well. Conversely, when the demands on the myocardium are reduced, coronary blood flow decreases in parallel, largely independent of the normal

range of driving pressure. However, our preliminary data reveals a poor correlation between hemodynamic stress and coronary blood flow, suggesting that this autoregulation phenomenon is disturbed during ESHP.[104] Finally, during ESHP, cardiac function and coronary blood flow trend in opposite directions, suggesting coronary artery function is disturbed.

1.2.2 Mechanisms underlying loss of coronary regulation during ESHP

Several mechanisms may be involved in the loss of coronary regulation during ESHP. Endothelial factors, oxygen carrier, neurohumoral factors, metabolic factors, redox mechanism, and the end effector, potassium channels' role in the regulation of coronary blood flow will be discussed.

1.2.2.1 Metabolic coronary flow regulation

The continuous provision of oxygen in quantities sufficient to support normal metabolism and the maintenance of transmembrane ion gradients is vital to the survival of any perfused organs. In case of the heart, it needs large amounts of energy to support contraction. Myocardial metabolism is the key regulator of coronary blood on every systolic event, from rest to maximal exercise.[105] Normally, coronary blood flow is tightly adjusted to the oxygen requirements of the myocardium.[9, 101, 106, 107] This matching termed metabolic dilation, or metabolic or active hyperemia, is critical to ensure adequate oxygen delivery for aerobic metabolism.[97] This tight coupling between oxygen supply and demand is necessary since the heart has little anaerobic metabolism capacity.[108] The local functional coupling of blood flow and myocardial metabolism is achieved by feedforward

and feedback control mechanisms, and it is believed that metabolic flow regulation plays a key role.[109]

Metabolites that control myocardial blood flow in a feed-forward manner must be produced at a rate proportional to oxidative metabolism. Such metabolites include carbon dioxide, which is generated in decarboxylation reactions of the tricarboxylic acid cycle (TCA cycle), and ROS formed in the respiratory chain in proportion to oxygen consumption.[110] Succinate is one of the TCA cycle metabolites that build up during ischemia.[111-114] After reperfusion, succinate is rapidly re-oxidized by succinate dehydrogenase (SDH), driving a burst of ROS production by mitochondrial complex I.[111] Some metabolites generated during normal heart metabolism are vasoactive mediators.[110] For example, both succinate and its derivative, fumarate, are vasodilators.[115, 116] Also, succinate can increase global Ca^{2+} transient in cardiomyocytes and generate vasorelaxant such as nitric oxide (NO), prostacyclin (PGI_2) and prostaglandin E_2 (PGE_2). It may be that the accumulation of metabolites of the TCA cycle like succinate and fumarate, may play a role in the coronary arteries vasodilation during isolated heart perfusion.

The heart also has a tight coupling between carbohydrate and fatty acid metabolism.[117] Free fatty acids are the key lipids oxidized in the myocardium. The heart's primary source of fatty acids is supplied by free fatty acids bound to albumin and very low-density lipoproteins.[117] Arachidonic acid (AA), a 20 polyunsaturated fatty acid, is naturally found incorporated in the cell membrane structure.[118] Cytochrome P450 (CYP) epoxygenase-derived metabolite of AA, 14, 15-epoxyeicosatrienoic acid (EET) can act directly on the vascular smooth muscle cell as a vasodilator.[119] It may be that 14, 15-EET,

a metabolite of free fatty acid, may accumulate in the perfusate and play a role in coronary vasodilation during ESHP.[118]

1.2.2.2 Redox mechanism in the regulation of coronary blood flow

Oxidative stress is a pivotal aspect of ischemia/reperfusion injury,[120] which may appear during donor heart procurement and the following reperfusion using the ESHP machine. Also, byproducts of normal mitochondrial metabolism and hemostasis include the buildup of the potentially dangerous level of ROS. They are generated either from the underlying myocardium tissues or within any vascular wall layer. Under physiological conditions, the balance between ROS generation and ROS scavenging is highly controlled. However, during ESHP, the lack of antioxidant systems would promote ROS accumulation and harm the myocardium and coronary arteries. For example, when the antioxidants in the perfusate have been exhausted, the heart may suffer from a direct ROS attack. The nature of ROS in and around the vasculature is consistent with an essential modulatory effect on vascular function.[121] Excess ROS production and/or impaired quenching of radical species leads to a redox imbalance that affects NO bioavailability.[122] Additionally, ROS can promote inflammation,[123] alter vasomotor function,[124] activate matrix metalloproteinases (MMPs), induce platelet activation,[125] and stimulate vascular smooth muscle proliferation.[126]

Hydrogen peroxide (H_2O_2) was first recognized as a hyperpolarizing factor (EDHF) by Matoba et al.[127] H_2O_2, dismutated from superoxide anion, hyperpolarizes and dilates human coronary arterioles.[128] It may be that the redox mechanism may play a role in the dilation of coronary arteries during ESHP, and H_2O_2 produced in the perfusion may contribute to the loss of coronary autoregulation.

1.2.2.3 The potential role of potassium (K^+) channels on the coronary arteries vasodilation (Effector mechanism)

Coronary resistance in any segment of the microcirculation represents the integration of local physical factors (e.g., pressure, flow), vasodilator metabolites (e.g., adenosine), autacoids, and neural modulation. Each of these mechanisms contributes to net coronary vascular smooth muscle tone, which may ultimately be controlled by opening and closing the potassium channel located on vascular smooth muscle.

Significant evidence supports the idea that K^+ channels function as critical downstream effectors, on which multiple signaling pathways converge to modulate coronary vasomotor tone.[129] Vascular smooth muscle cells contain K^+ channels that are sensitive to the intracellular energy charges and will react to many and vasoactive metabolites, like PGI_2, PGE_2, adenosine or NO, etc.[130-132] Open K^+ channels produce membrane hyperpolarization, inhibition of L-type calcium channels, and relaxation of coronary smooth muscle, thereby causing vasodilation.[133] There is a growing body of evidence supporting voltage-dependent potassium (K_V) channels as critical modulators in the local metabolic control of coronary blood flow.[134-136] It is likely that the coronary vasodilation and the loss of coronary regulation during ESHP is related to dysregulated metabolism and redox mechanism, which finally exert their effect on the activity of these channels.

Chapter Two: The Impact of Whole Blood Versus Dilute Whole Blood on the Coronary Vascular Tone Regulation During Ex Situ Heart Perfusion

This chapter is in preparation for submission to a peer-reviewed journal

Abstract

Background:

Evidence indicates that circulating chemical mediators in the blood are involved in the regulation of vascular tone. We aim to evaluate the effect of whole blood versus dilute whole blood perfusate on the regulation of coronary vascular tone during ESHP.

Methods:

Normal porcine hearts were perfused in working mode for 6 hours either by whole blood (n=6) or whole blood diluted 1:1 with modified Krebs-Henseleit solution. Cardiac functional parameters, coronary artery flow and metabolism were monitored continuously. Coronary vascular resistance (CVR) was calculated to evaluate coronary artery function. Pro-inflammatory, oxidative stress as well as vasoactive mediators in the perfusate were measured and compared between groups.

Results:

Coronary blood flow increased over time in dilute whole blood group and was significantly higher than whole blood group at each time point (T3 and T5). Heart perfused by dilute whole blood lost CVR and indicated uncoupling between cardiac work and coronary artery flow, while whole blood perfusion heart had significantly higher CVR at the end of 6 hours. Whole blood perfusion group had less cardiac and endothelial injury, indicated by troponin I, hyaluronan and thrombomodulin. Whole blood group has less platelet activation but higher leukocyte activation as well as oxidative stress. Higher concentration of serotonin, bradykinin, and histamine were found in the whole blood perfusate.

Conclusion:

The higher concentration of vasoactive mediators in whole blood perfusate appear to play a role in regulating coronary vascular tone, as indicated by relative higher vascular resistance and better relationship between cardiac work and coronary perfusion.

2.1 Introduction

The global crisis of shortage suitable donor hearts available to meet the increasing demand of end-stage heart failure patients has fostered great scientific interest and a rapidly expanding research field to find a solution. Preservation using *ex situ* machine perfusion has gained considerable interest as a method of increasing the utilization of cardiac grafts for transplantation. *Ex-Situ* heart perfusion (ESHP) allows metabolic resuscitation and functional assessment to give better short and long outcomes.[6, 7, 137] It has also emerged as a platform for reconditioning marginal donor hearts and consequently increasing the number and quality of donor organs available for transplant.[138, 139] Despite recent progress on ESHP, many questions remain on the optimal method for organ perfusion and preservation. For example, Ideal methods for maximally preserving cardiac function and graft quality are intensively sought after. The ideal approach for graft evaluation is yet to be established before considering for transplantation. Furthermore, to date we do not have a solid knowledge of quantifying the metabolic demands of *ex situ* perfused heart, especially the optimal amount of coronary circulation that is critical to address its metabolic needs.

It is widely accepted that both myocardial contraction and coronary perfusion are closely correlated. The ability of coronary resistance vessels to dilate in response to an increase in myocardial oxygen demand, as illustrated by the tight correlation between myocardial oxygen consumption and coronary blood flow, is critical for maintaining an adequate supply of oxygen to the myocardium.[9] A good balance between oxygen demand, as set by the correct perfusion temperature, and oxygen delivery, as set by appropriate coronary blood flow and capillary oxygen transfer capacity, is necessary to ensure organ

preservation and good clinical outcomes. However, many investigators have indicated that the *ex-situ* perfused heart loses the coronary vascular tone in a few hours.[140-142] Our preliminary results also suggest that coronary vasoregulation is disturbed during ESHP.[104] We believe the cause for this is multifactorial. The isolated perfused heart may lose complete systemic support as in the body, including metabolic substrate support and some paracrine factors released from erythrocytes or platelets, and circulating neurohormonal agonists, as well as local control through vascular shear stress. [143-145]

We hypothesized that some circulating chemical mediators in the blood might be essential for maintaining normal coronary vascular tone and capillary integrity of the heart. Therefore, the aims of this study are to compare the effects of whole blood perfusate and dilute whole blood perfusate on the coronary vascular tone regulation during isolated heart perfusion and find the potential mechanism for the loss of coronary vascular tone during ESHP.

2.2 Material and methods:

Animal care: The animals used in this study received humane care in accordance with the Canadian Council on Animal Care guidelines. The study protocol was approved by the Institutional Animal Care and Use Committee of the University of Alberta. Female domestic pigs (n=17) weighing 45-55 kg were used as heart and blood donors.

Ex situ heart perfusion and sample collection:

A schematic of the ESHP working heart apparatus is shown in **Fig. 1-1A**. The perfusion was done as previously described [146]. Following cardioplegic arrest in the donor animal, hearts were mounted on the ESHP system. Perfusion was initiated in Langendorff mode for 1 hour, during which the perfusate was warmed from room temperature to 37˚C. Working mode was initiated by raising left atrial pressure to 6mmHg, which was then maintained for 5 hours of perfusion. All hearts were paced with atrial wires at 100 beats/minute. In addition, Dobutamine (4 mcg/min) and Insulin (5 units/h) were infused continuously throughout the experiment. The detail methods are included in Supplementary Material.

Experimental groups

A schematic of the experimental design is seen in **Fig. 1-1B**. Donor animals were randomly assigned to the following groups:

Whole donor blood group (n=6): 1.6 liters of autologous whole blood were collected from the heparinized heart donor animal and used as the perfusate.

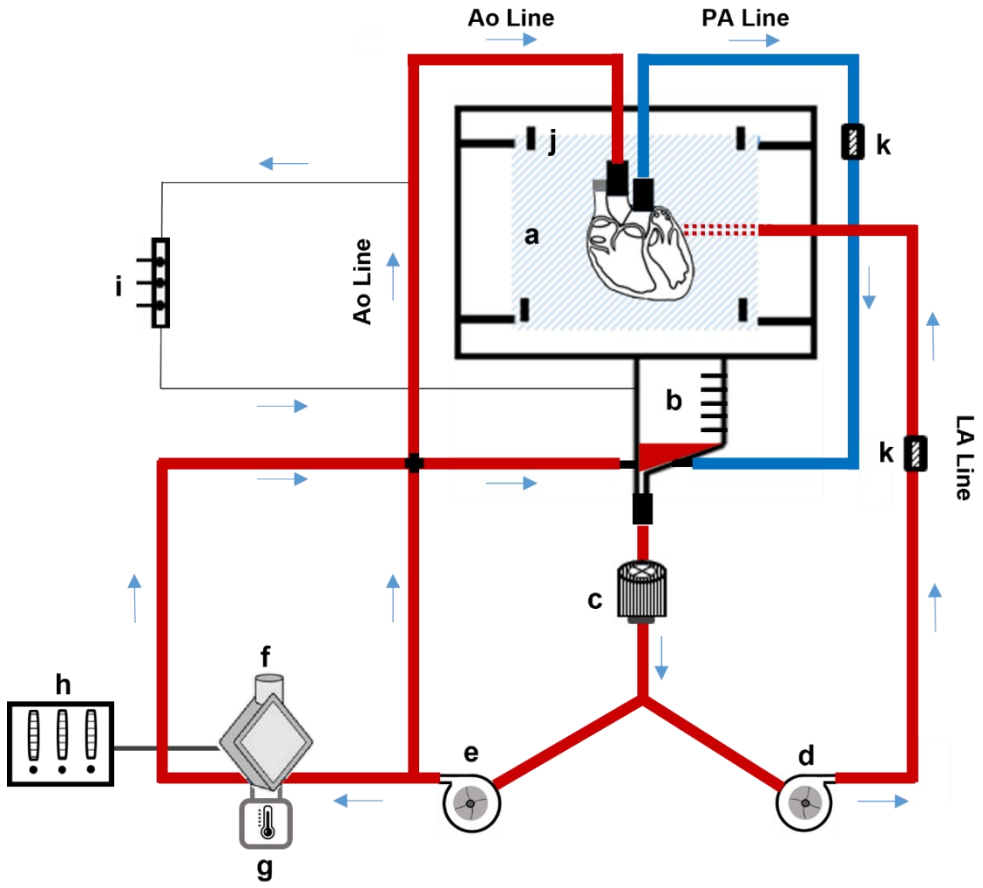
Dilute whole blood group (n=6): The 800 mL autologous whole blood was diluted 1:1 with modified Krebs-Henseleit solution containing 8% albumin. Total 1.6 liters of perfusate were used to achieve a hematocrit of 25% for the circuit priming.

***In situ* tissue samples (n = 5):** freshly procured, unmanipulated hearts were obtained to serve as *in situ* control. Left and right coronary artery tissues were isolated and stored immediately at -80 °C for subsequent analysis.

Functional and metabolic assessment of the *ex situ* perfused heart

Myocardial function parameters were assessed at 1h (T1), 3h (T3), and 5h (T5) after initiation of working mode as previously described[147] (LA pressure = 6 mmHg). dP/dt_{max} and dP/dt_{min} were acquired through a pigtail catheter (5F) placed in the left ventricle via the subclavian branch of the aortic arch. The cardiac index (CI) was determined by measuring the flow through the left atrial line divided by the heart weight ($mL \cdot minute^{-1} \cdot gram^{-1}$). Arterial and coronary sinus blood samples were collected at regular intervals during ESHP. Electrolyte, PO_2, PCO_2, hemoglobin concentrations, oxygen saturation of hemoglobin, pH, glucose, and lactate were measured using an ABL800 blood gas analyzer (Radiometer, Copenhagen, Denmark). The superior vena cava (SVC) and inferior vena caca (IVC) were ligated and coronary blood flow (CBF) was monitored by a Doppler flowmeter (Transonic System Inc., NY, USA) on the pulmonary artery cannula that was collecting effluent from the coronary sinus. The detailed methods of functional and metabolic evaluation are included in Supplementary Material.

A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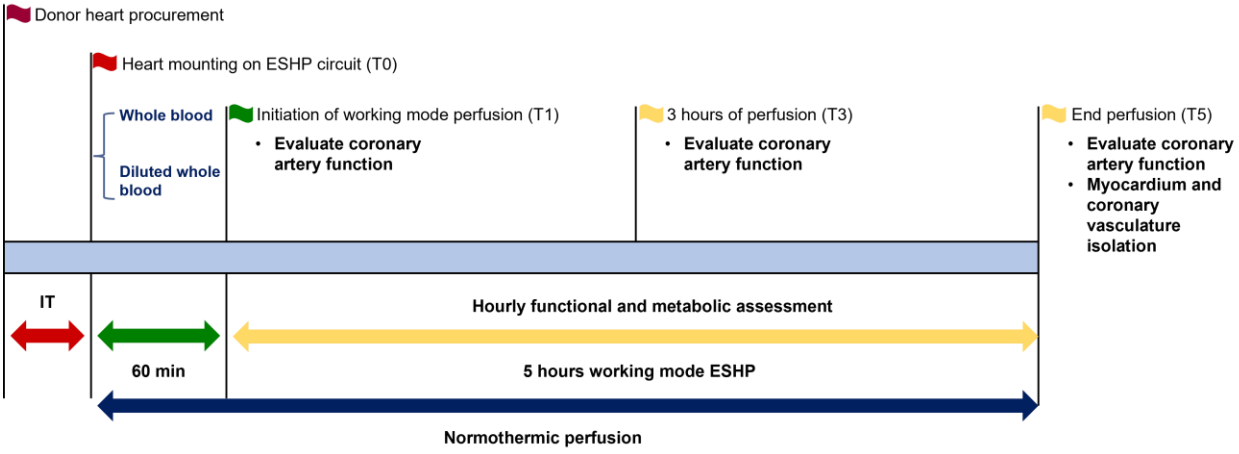

Donor heart procurement
Heart mounting on ESHP circuit (T0)
Whole blood
Diluted whole blood
Initiation of working mode perfusion (T1)
• Evaluate coronary artery function
3 hours of perfusion (T3)
• Evaluate coronary artery function
End perfusion (T5)
• Evaluate coronary artery function
• Myocardium and coronary vasculature isolation
IT
60 min
Hourly functional and metabolic assessment
5 hours working mode ESHP
Normothermic perfusion

Fig. 1-1 Schematic view of the perfusion system and experimental timeline

A, custom ESHP circuit. LA line connects one centrifugal pump and left atrium, providing the preload and Ao line connects another pump and the aorta, providing the afterload. PA line collects the effluent of the pulmonary artery to the reservoir. a, supportive membrane; b, reservoir; c, arterial filter; d and e, centrifugal pumps; f, oxygenator; g, heater; h, gas line; i, drug infusion line; k, flow probes. B, experimental timeline. Hearts were procured and, after a brief period of ischemic time (≤28 minutes) to allow mounting on the ESHP apparatus, were perfused in Langendorff mode for 1 hour and 5 hours of working mode normothermic perfusion. Hearts were allocated to either of the following three groups (n=6 each): (1) whole blood group, [20] Diluted whole blood group. Ao, aorta; PA, pulmonary artery; LA, left atrium; ESHP, *Ex situ* heart perfusion; IT, ischemic time.

Ex situ evaluation of the coronary artery function

The left ventricle preload challenge was induced by increasing left atrial pressure from 6 to 12 mmHg. CBF and LVSW were acquired at every systolic ejection event. The change of CBF was compared between the time points within each group as well as between the perfusion groups.

Leukocytes and platelet activation profiles

Proinflammation cytokines (TNF-α, IL-1β, IL-6, IL-8, IL-10, VCAM-1, MMP-9) and platelet activation marker (PF4, P-selectin, sCD-40L, β-TG) were measured in the perfusate using enzyme-linked immunosorbent assay (ELISA) techniques according to the manufacturer's instructions (R & D System Inc., USA; FineTest, Wuhan, China). MPO peroxidation activity was measure in the coronary artery tissue as well as the myocardium tissues (Biovision, USA). Coronary artery expression of p-ERK1/2 and ERK1/2 were detected by standard western blot techniques using monoclonal antibodies. Primary antibodies were used at a concentration of 1:1000 in a 5% bovine serum albumin solution. Secondary horseradish peroxidase-conjugated antibodies were used at a concentration of 1:1000. β-actin was used as a loading control for the blots. Full details of methods used are described in the Supplementary Material.

Myocardial and vascular injury

Perfusate concentration of cTnI was measured as a marker of myocardial injury using ELISA (Life Diagnostics, PA, USA). Perfusate hyaluronan, thrombomodulin and endothelin were measured by ELISA according to the manufacturer's instructions (R & D System Inc., MN, USA; FineTest, Wuhan, China).

Determination of oxidative stress and related modification

Lipid peroxidation was determined by measuring oxLDL in the perfusate by ELISA (MyBioSource Inc., CA, USA). MDA was determined in the coronary artery tissue by the thiobarbituric acid colorimetric method according to the manufacture's instruction (R & D System Inc., MN, USA). Protein modification was measured by a protein carbonyl content assay kit (Abcam, USA). GPx activity was determined in the myocardium according to the manufacture's instruction (Biovision, CA, USA)**.** Detailed methods are described in the Supplementary Material.

Determination of vasoactive mediators in the perfusate

The concentration of nitrate/nitrite, an index of NO formation, was determined in the perfusate according to the manufacturer's instruction (Biovision, CA, USA. Perfusate 5-HT, bradykinin and histamine were measured by ELISA (FineTest, Wuhan, China).

Statistics

All values were expressed as mean ± standard error of the mean. The significance of result differences was assessed using a two-way analysis of variance (ANOVA) method for repeated measurements, with additional post hoc tests. Student's t-test was applied for testing differences in continuous variables between the two groups. Values of $P < 0.05$ were considered statistically significant. Analyses were performed using IBM SPSS software version 25 (Chicago, IL, USA) for windows.

2.3 Results

2.3.1 Hemodynamic parameters and blood analysis

Hemodynamic parameters at T1, T3 and T5 were comparable between the two groups (**Table 1-1**). The dilute whole blood contained 50% of normal plasma and 50% of the average blood concentration of leukocytes and platelets (Supplementary figure). The hemoglobin concentration of whole blood perfusate was twice that in the dilute whole blood perfusate (84.3±3.0 vs. 42.7±1.0 g/L, $p<0.001$). Lactate was higher in the whole blood perfusate than dilute whole blood perfusate at T1 (3.9±0.3 vs. 2.7±0.1 mmol/L, $p<0.01$), and trended towards a lower level in both groups at T3 ($p<0.01$). Cell-free hemoglobin increased in the perfusate in both groups. Even though the absolute value of cell-free hemoglobin in the whole blood perfusate was higher, there was no statistically significant difference in red blood cell hemolysis percentage between groups. After 6 hours of perfusion, both groups had edema formation, with no statistical difference between groups.

Table 1-1

Variable	**Dilute whole blood (n=6)**	**Whole blood (n=6)**	**P value**
Characteristics			
Body weight, kg	46.5 ± 2.2	48.0 ± 1.7	0.60
Heart weight, g	249.5 ± 10.6	255.8 ± 12.4	0.71
Ischemic time, min	20.1 ± 0.9	20.3 ± 1.1	0.91
***Ex situ* heart perfusion**			
Systolic pressure, mmHg			
T1	111.5 ± 2.9	112.2 ± 3.4	0.89
T3	108.0 ± 3.6	107.5 ± 6.2	0.91
T5	104.2 ± 3.5$^{\#\#}$	101.8 ± 4.6$^{\#\#\#}$	0.70
Diastolic pressure, mmHg			
T1	39.7 ± 0.2	39.8 ± 0.2	0.55
T3	39.7 ± 0.2	39.8 ± 0.2	0.55
T5	39.7 ± 0.2	39.8 ± 0.2	0.55
Left atrium flow rate, mL/min			
T1	2105.0 ± 140.3	2285 ± 116.7	0.35
T3	1971.7 ± 145.9	2178.3 ± 114.4	0.29
T5	1735.0 ± 152.7	1888.3 ± 151.6$^{\#}$	0.49
Blood gas analysis			
Hemoglobin, g/L			
T1	42.7 ± 1.0	84.3 ± 3.0	<0.001**
T3	42.2 ± 1.5	85.8 ± 2.7	<0.001**
T5	40.7 ± 1.6	86.7 ± 2.6	<0.001**
Lactate, mmol/L			
T1	2.7 ± 0.1	3.9 ± 0.3	0.002*
T3	1.9 ± 0.4$^{\#}$	3.3 ± 0.3$^{\#}$	0.024*
T5	2.5 ± 0.2	3.4 ± 0.3	0.027*
Perfusate composition			
Plasma-free hemoglobin, mg/dL			
T0	1.34 ± 0.14	4.86 ± 0.42	<0.001**
T3	4.35 ± 0.35$^{\#\#\#}$	8.04 ± 0.80$^{\#\#\#}$	0.004*
T5	6.05 ± 0.49$^{\#\#\#}$	11.29 ± 0.91$^{\#\#\#}$	<0.001**
Red blood cell hemolysis, %			
T0	0.32 ± 0.03	0.58 ± 0.05	0.001*
T3	1.04 ± 0.09$^{\#\#\#}$	0.93 ± 0.09$^{\#\#\#}$	0.42
T5	1.46 ± 0.13$^{\#\#\#}$	1.30 ± 0.10$^{\#\#\#}$	0.34

Table 1-1: Hemodynamic parameter, blood gases and serum parameter

Data are expressed as mean ± SEM. *p<0.05, **p<0.001 for diluted whole blood group vs whole blood group; $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 for within group comparison between different time; n=6 each group

2.3.2 Functional and metabolic assessment in working mode ESHP

Heart function declined in both groups, indicated by the cardiac index and LVSW (**Table 1-2**). dP/dT_{max} was lower at T5 than T1 in the dilute whole blood group (1223.8±65.5 vs. 1492.3±157.2 mmHg/s, $p<0.05$). However, it was preserved in the whole blood group. dP/dT_{min} was lower at T5 than T1 in the whole blood group (-954.3±113.7 vs. -1235.8±158.9, $p<0.05$); however, no difference has been found in the dilute whole blood group. CBF increased significantly in both groups compared to the baseline (T5 vs. T1, 512.8±15.4 vs 253.1 ± 18.4 mL/min/100g in dilute whole blood group, 359.9±21.5 vs. 252.4±21.1 mL/min/100g in whole blood group). However, CBF was relatively lower in the whole blood group than the dilute whole blood group at T3 and T5 ($p<0.05$ and 0.01). CVR gradually declined during six hours of perfusion in both groups ($p<0.001$). However, in the whole blood group, CVR was higher than that in the dilute whole blood group at T5 (0.1±0.001 vs. 0.07 ± 0.002 mmHg·min/mL/100g, $p<0.05$). Consistent with decreased CVR, oxygen consumption decreased during perfusion in the dilute whole blood group (T5 vs. T1, 3.8±0.3 vs. 5.1±0.3 mL O_2/min/100g, $p<0.01$). However, no statistically significant difference in oxygen consumption has been observed in the whole blood group. As with the left ventricle workload challenge, CBF increased as well. However, at later time points, the change of CBF during the workload challenge decreased. Within each group, both T3 and T5 had significantly less change in CBF in response to increased left ventricle stroke work than at T1 ($p<0.001$).

Table 1-2

Parameter	**Dilute whole blood (n=6)**	**Whole blood (n=6)**	**P value**
Cardiac index ($mL \cdot min^{-1} \cdot g^{-1}$)			
T1	8.4 ± 0.7	9.1 ± 0.7	0.52
T3	8.0 ± 0.7	8.6 ± 0.6	0.53
T5	7.2 ± 0.8$^{###}$	7.5 ± 0.8$^{###}$	0.80
LVSW (mmHg·mL)			
T1	1729.5 ± 169.7	1704.3 ± 113.6	0.90
T3	1521.2 ± 135.7$^{#}$	1651.3 ± 107.6	0.47
T5	1295.5 ± 132.5$^{###}$	1329.5 ± 147.2$^{###}$	0.87
dP/dT_{max} ($mmHg \cdot s^{-1}$)			
T1	1492.3 ± 157.2	1226.2 ± 120.5	0.21
T3	1416.3 ± 111.3	1542.8 ± 92.9$^{#}$	0.40
T5	1223.8 ± 65.5$^{#}$	1418.3 ± 91.2	0.11
dP/dT_{min} ($mmHg \cdot s^{-1}$)			
T1	-(1298.7 ± 135.6)	-(1235.8 ± 158.9)	0.77
T3	-(1259.8 ± 136.8)	-(1248.2 ± 273.1)	0.95
T5	-(1229.2 ± 82.9)	-(954.3 ± 113.7)$^{#}$	0.08
CBF ($mL \cdot min^{-1} \cdot 100g^{-1}$)			
T1	253.1±18.4	252.4±21.1	0.98
T3	485.5±13.7$^{###}$	438.9±13.9$^{###}$	0.038*
T5	512.8±15.4$^{###}$	359.9±21.5$^{###}$	0.003**
CVR ($mmHg \cdot mL^{-1} \cdot min^{-1} \cdot 100g^{-1}$)			
T1	0.14±0.006	0.15±0.005	0.60
T3	0.07±0.002$^{###}$	0.08±0.004$^{###}$	0.17
T5	0.07±0.002$^{###}$	0.10±0.001$^{###}$	0.027*
MVO_2 ($mL\ O_2 \cdot min^{-1} \cdot 100g^{-1}$)			
T1	5.1±0.3	4.9±0.2	0.46
T3	4.1±0.5$^{##}$	4.6±0.1	0.39
T5	3.8±0.3$^{##}$	4.9±0.2	0.23
ΔCBF with ventricle workload challenge ($mL \cdot min^{-1} \cdot 100g^{-1}$)			
T1	189.8±20.4	155.4±12.2	0.19
T3	94.4±12.2$^{###}$	88.3±11.7$^{###}$	0.72
T5	64.9±10.5$^{###}$	41.4±8.2$^{###}$	0.11

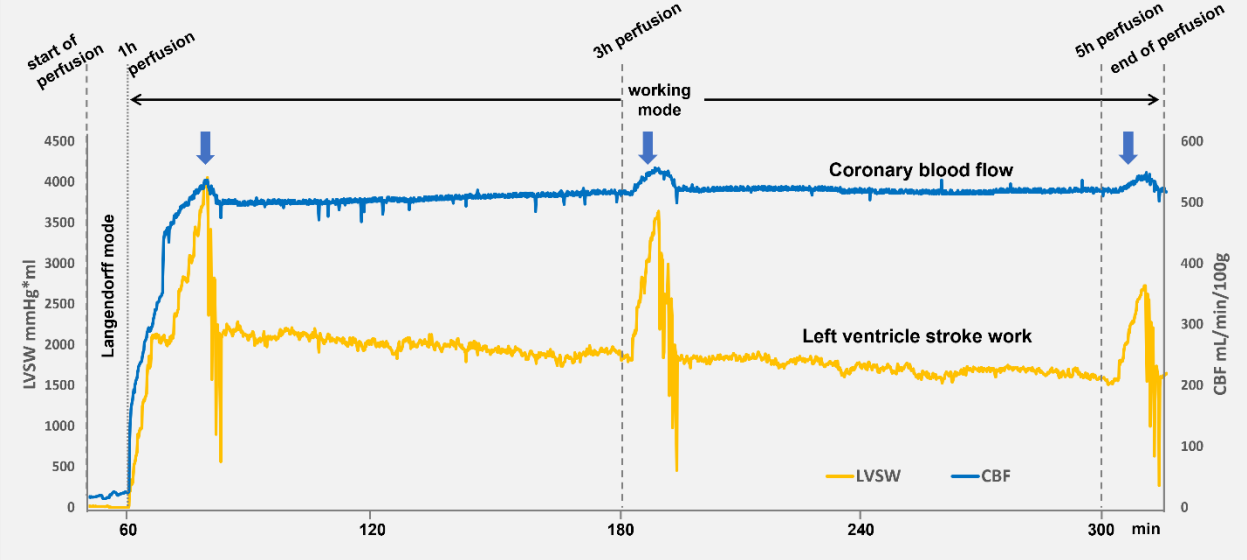
Table 1-2: Functional and metabolic parameters

Data are expressed as mean ± SEM. *p<0.05, **p<0.001 for diluted whole blood group vs whole blood group; $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 for within group comparison between different time; n=6 each group

2.3.3 Uncoupling between cardiac systolic function and coronary artery flow

Typical hemodynamic changes during the 6 hours of *ex situ* heart perfusion in both groups are depicted in **Figure 1-1**. There was a gradual decrease of LVSW, and an increase of CBF in the diluted whole blood group (**Figure 1-1A**). However, in the whole blood group, concurrently with decreasing ventricle stroke work during 6 hours of perfusion, CBF decreased (**Figure 1-1B**). The hemodynamic change of LVSW and CBF in the two groups showed a distinct pattern, suggesting uncoupling between cardiac function and coronary artery flow in dilute whole blood perfusion compared to whole blood perfusion.

A


start of perfusion
1h perfusion
3h perfusion
5h perfusion
end of perfusion
working mode
Langendorff mode
Coronary blood flow
Left ventricle stroke work
LVSW mmHg*ml
CBF mL/min/100g
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CBF
60
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180
240
300
min

B

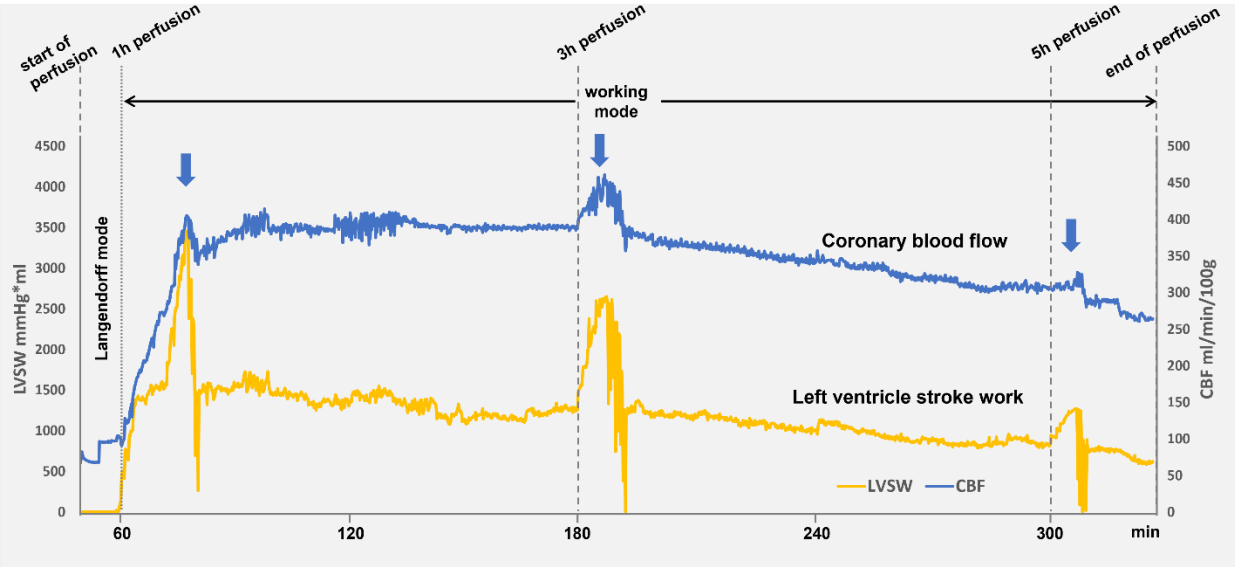

start of perfusion
1h perfusion
3h perfusion
5h perfusion
end of perfusion
working mode
Langendorff mode
Coronary blood flow
Left ventricle stroke work
LVSW mmHg*ml
CBF ml/min/100g
LVSW
CBF
60
120
180
240
300
min

Fig. 1-2 Typical hemodynamic changes during 6 hours *ex situ* heart perfusion in Langendorff and working mode.

Left ventricle stroke work (yellow) and coronary blood flow rate (blue) are depicted. The blue arrows indicate the workload challenge induced by manually increasing the left atrium pressure from 6 to 12 mmHg. After switching to working mode at 60 minutes, left ventricle stroke work and coronary blood flow rate reach a plateau after approximately 30 minutes. LVSW, left ventricle stroke work; CBF, coronary blood flow. Dilute whole blood perfusion (A) and whole blood perfusion (B).

2.3.4 Leukocyte and platelet were activated in both groups

Leukocyte and platelet were significantly activated in both groups during 6 hours of perfusion. TNF-α, IL-6, IL-1β, IL-8, IL-10 and MMP-9 increased compared to the baseline value in both groups ($p<0.001$, **Figure 1-3A**). The whole blood group has a lower IL-6 level than the dilute whole blood group at both T3 and T5 (2645.3±322.4 vs. 4719.0±283.8 pg/mL at T3, $p=0.001$; 6829.3±398.8 vs. 9912.9 pg/mL at T5, $p<0.001$). VCAM-1 significantly increased in the whole blood group, whereas there was no statistically significant difference in the dilute whole blood group. In the coronary artery tissue, there was significantly higher MPO peroxidation activity in the whole blood group than *in situ* group (0.47±0.13 vs. 0.11±0.06 mU/mg, $p=0.03$, **Figure 1-3C**), but no statistically significant difference was found between the two experimental groups. There was significantly higher activation of p44/42 mitogen-activated protein kinases (MAPK) pathway in the coronary artery tissue of both groups than the *in situ* control group ($p<0.05$, **Figure 1-3B**). Both experimental groups had increased platelet activation, indicated by the level of P-selectin, sCD40L and β-TG ($p<0.001$, **Figure 4**). The perfusate sCD40L and β-TG levels were significantly lower in the whole blood group at T5 compared with the dilute whole blood group ($p<0.01$). For PF4 and P-selectin, dilute whole blood perfusate indicates significantly higher platelet activation than whole blood at early time points (PF4 at T3 and P-selectin at T1). Altogether, these data suggest higher platelet activation in the dilute whole blood than whole blood perfusate during 6 hours ESHP.

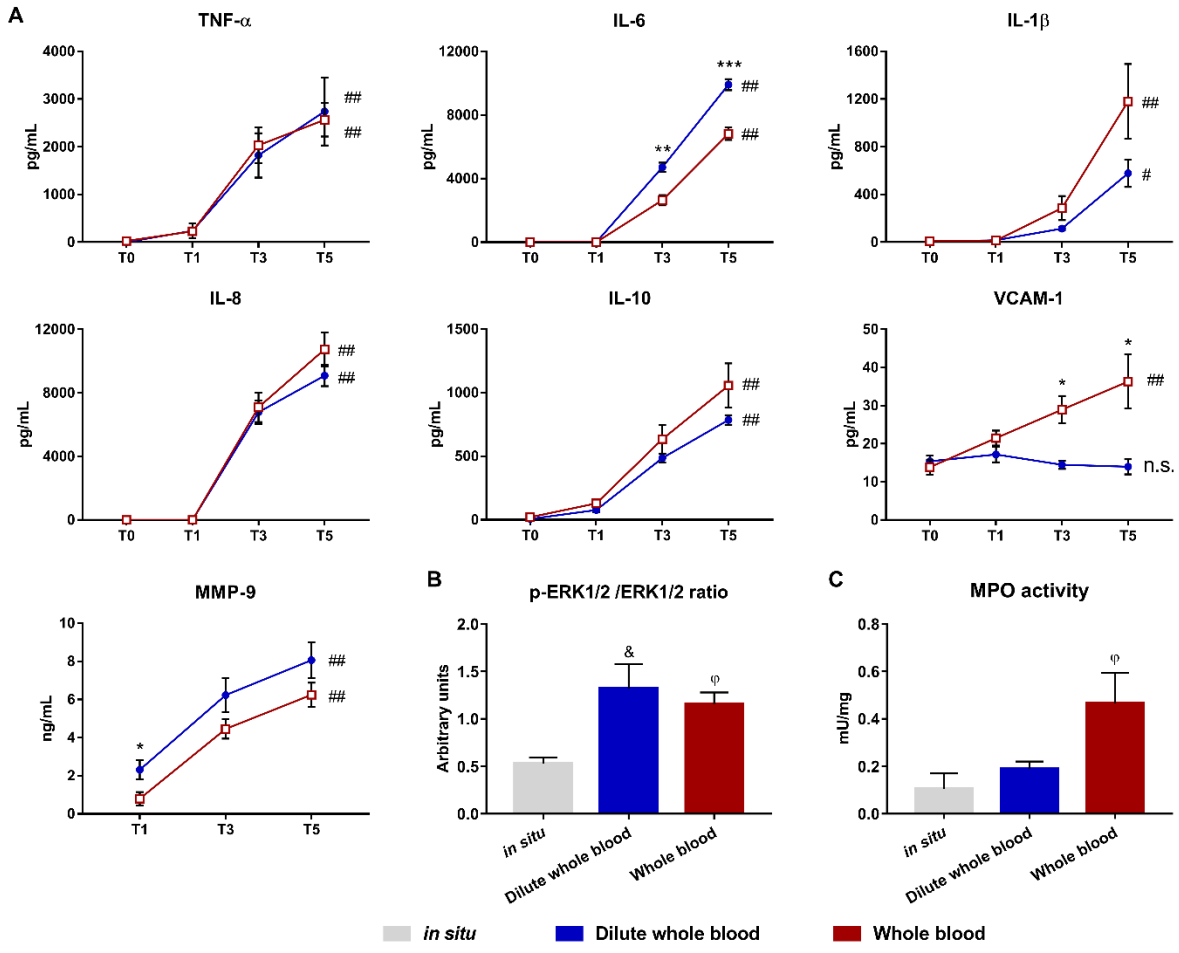

A
TNF-α
IL-6
IL-1β
IL-8
IL-10
VCAM-1
MMP-9
pg/mL
ng/mL
T0
T1
T3
T5
##
#
*
**
***
n.s.
B
p-ERK1/2 /ERK1/2 ratio
Arbitrary units
&
φ
C
MPO activity
mU/mg
in situ
Dilute whole blood
Whole blood

Fig 1-3 Leukocyte activation and related MAPK pathway activation

Pro-inflammatory and anti-inflammatory cytokines (A) were increased in both groups of perfusion hearts. The whole blood group had less perfusate IL-6 and higher VCAM-1 than the dilute whole blood group from T3. In the coronary artery tissue, MPO peroxidation activity was higher in the whole blood group than the *in situ* control group (C). Both groups had significantly higher activation of p44/42 MAPK pathway(B). TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; IL-1β, interleukin-1β; IL-8, interleukin-8; IL-10, interkeukin-10; VCAM-1, vascular cell adhesion molecule 1; MMP-9, matrix metallopeptidase 9; MPO, myeloperoxidase. (Comparison between groups, independent t-test, *p<0.05, **p<0.01, *p<0.001; compared within group, repeated measure ANOVA, $^{\#}$p<0.01, $^{\#\#}$p<0.001, dilute whole blood group vs. *in situ*: $^{\&}$p<0.05, whole blood group vs. *in situ*: $^{\phi}$p<0.05; n=6 for cytokines and n=5 for ERK and MPO activity)

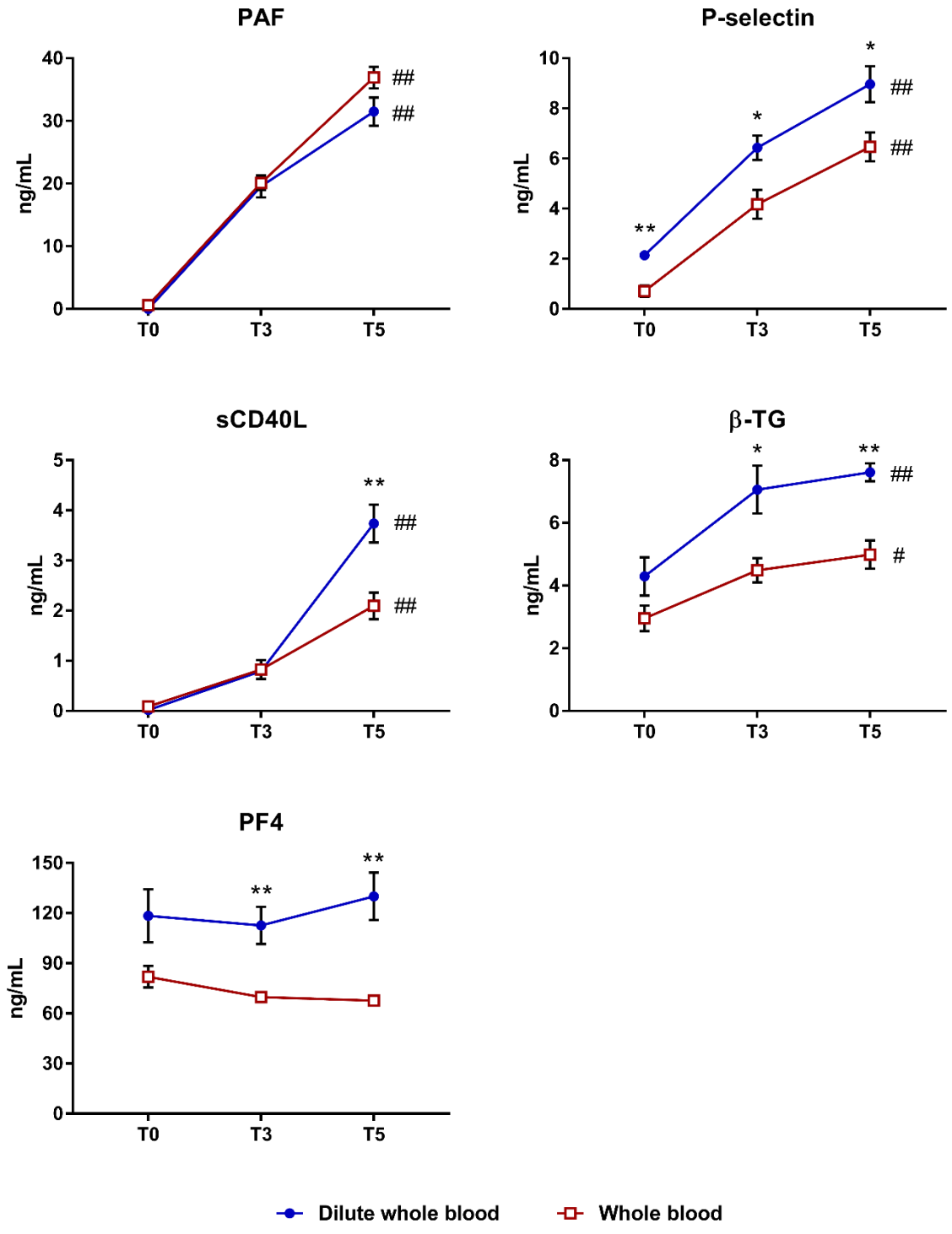

PAF
ng/mL
40
30
20
10
0
T0
T3
T5
##
##
P-selectin
ng/mL
10
8
6
4
2
0
T0
T3
T5
**
*
*
##
##
sCD40L
ng/mL
5
4
3
2
1
0
T0
T3
T5
**
##
##
β-TG
ng/mL
8
6
4
2
0
T0
T3
T5
*
**
##
#
PF4
ng/mL
150
120
90
60
30
0
T0
T3
T5
**
**
Dilute whole blood
Whole blood

Fig. 1-4 Platelet activation markers

Both groups had significantly increased platelet activation, indicated by P-selectin, sCD40L and β-TG and sCD40L. The whole blood group had less leukocyte activation than the dilute whole blood group, indicated by platelet activation marker, PAF, P-selectin, sCD40L, β-TG and PF4. PAF, platelet activating factor; sCD40L, Soluble CD-40 ligand; β-TG, β-Thromboglobulin; PF4, platelet factor 4. (Comparison between groups, independent t-test, *$p<0.05$, **$p<0.01$; compared within group, repeated measure ANOVA, $^{\#}p<0.01$, $^{\#\#}p<0.001$, n=6)

2.3.5 Dilute whole blood perfusion had a higher degree of cardiac and endothelial damage

Perfusate cTnI level increased significantly in both groups compared to baseline (p<0.001, Figure 4 A). The whole blood group had a lower degree of cardiac damage markers than the pressure control group starting from T3 (12.9±1.9 vs. 44.8±10.4 ng/mL, p=0.017 at T3, 39.7±9.9 vs. 77.8±13.0 ng/mL, p=0.048 at T5). Perfusate hyaluronan increased significantly in the dilute whole blood group (p<0.001, Figure 4B), while there was a non-significant increase in the whole blood group. At T3 and T5, hyaluronan was higher in the dilute whole blood perfusate than whole blood perfusate (330.8±75.2 vs. 54.4±8.7 ng/mL, p=0.018 at T3, 501.0±75.5 vs. 149.0±17.4 ng/mL, p=0.008 at T5), suggesting more damage of the glycocalyx in endothelial layers of the vasculature. At T5, the dilute whole blood group has significant higher thrombomodulin and endothelin than dilute whole blood group (6.7±0.9 vs. 3.4±0.8 ng/mL, p=0.022 for sTM and 121.3±2.9 vs. 88.1±7.0, p=0.007 for endothelin). Altogether, these results suggest less cardiac and endothelial injury in the whole blood perfusion than the dilute whole blood perfusion.

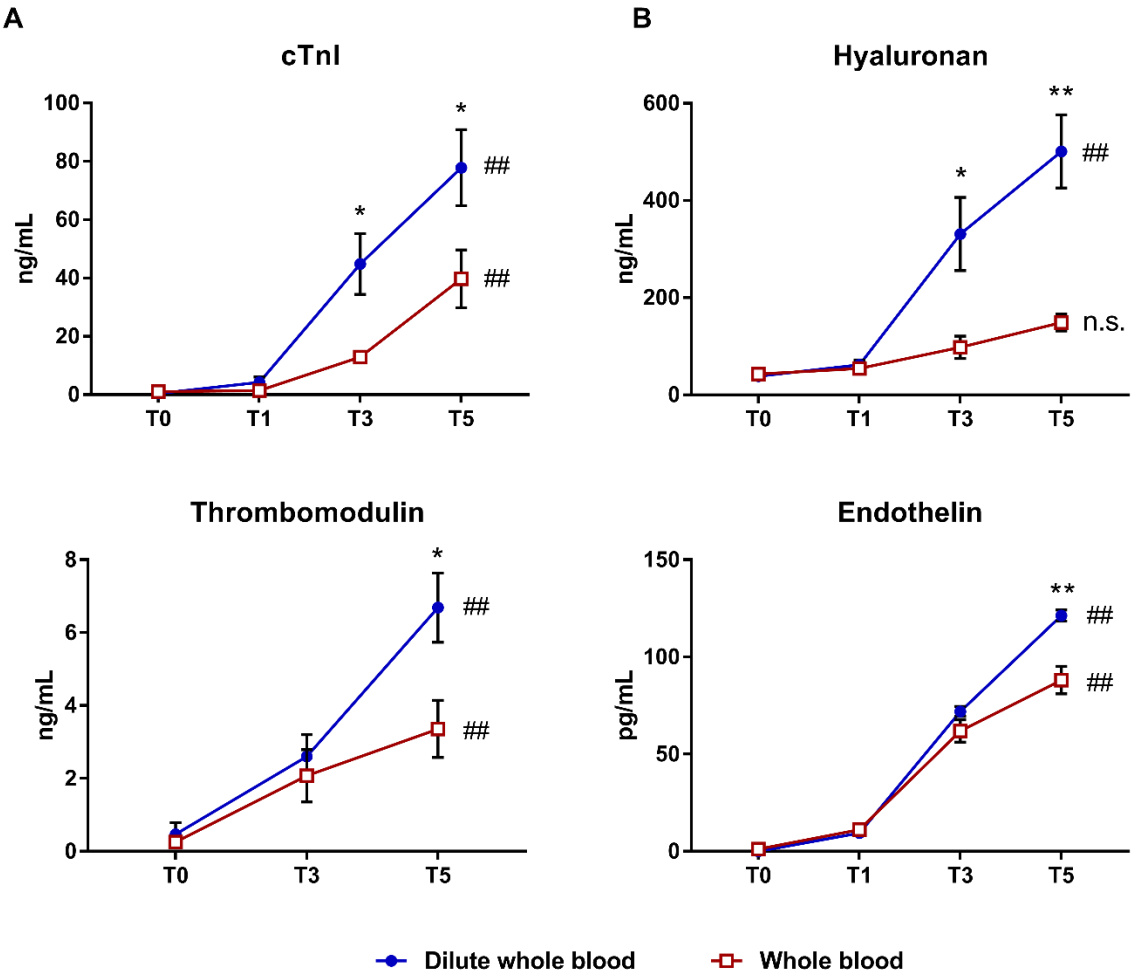


Fig. 1-5 Cardiac and endothelial cell damage

The whole blood group had less myocardial injury (A), endothelial injury (B) than the dilute whole blood group. The dilute whole blood group had significantly less perfusate than the whole blood group from T1. Both groups had a significant increase in perfusate endothelin and thrombomodulin than the baseline. cTnI, cardiac troponin I; vWF, von Willebrand Factor. (Comparison between groups, independent t-test, *p<0.05, **p<0.01; comparison within group, repeated measure ANOVA, $^{\#\#}p$<0.001, n=6)

2.3.6 Vasoactive substances were higher in whole blood perfusate

Whole blood perfusate has significantly higher NO, 5-HT, bradykinin and histamine from the start to the end of 6 hours perfusion. (Figure 5) Furthermore, there was a significant decrease of perfusate 5-HT, bradykinin and histamine in the whole blood perfusate. However, there were not any statistically significant differences in the dilute whole blood perfusate during perfusion.

2.3.7 Oxidative stress and related modification were higher in the coronary vasculature of the whole blood group

There was a significant increase of oxidative stress in both groups, indicated by lipid peroxidation markers of oxLDL in the perfusate and MDA in the coronary artery (Figure 6A, C), and oxidative stress-related modification of proteins in the coronary artery (Figure 6D). The whole blood group had significantly higher protein carbonyl content and nitrotyrosine in the coronary artery than the dilute whole blood group (Figure 6D, E), suggesting higher oxidative stress in this group.

Antioxidative enzyme system glutathione peroxidase was evaluated in the perfusate as well as in the myocardium tissue. Perfusate GPx1 increased significantly in both groups compared to the baseline value, and whole blood perfusate had significantly higher glutathione peroxidase 1 than the dilute whole blood perfusate at the start and end of perfusion (10.1±1.7 vs. 5.6 ±1.0 ng/mL at T5, p=0.046, Figure6B). Also, Gpx activity was measured in the myocardium. Both groups had a significantly lower GPx activity than *in situ* control hearts. However, it was higher in the whole blood group than the dilute whole blood group (14.9±1.4 vs. 5.7±1.3 U/mg protein, p=0.003, Figure6F).

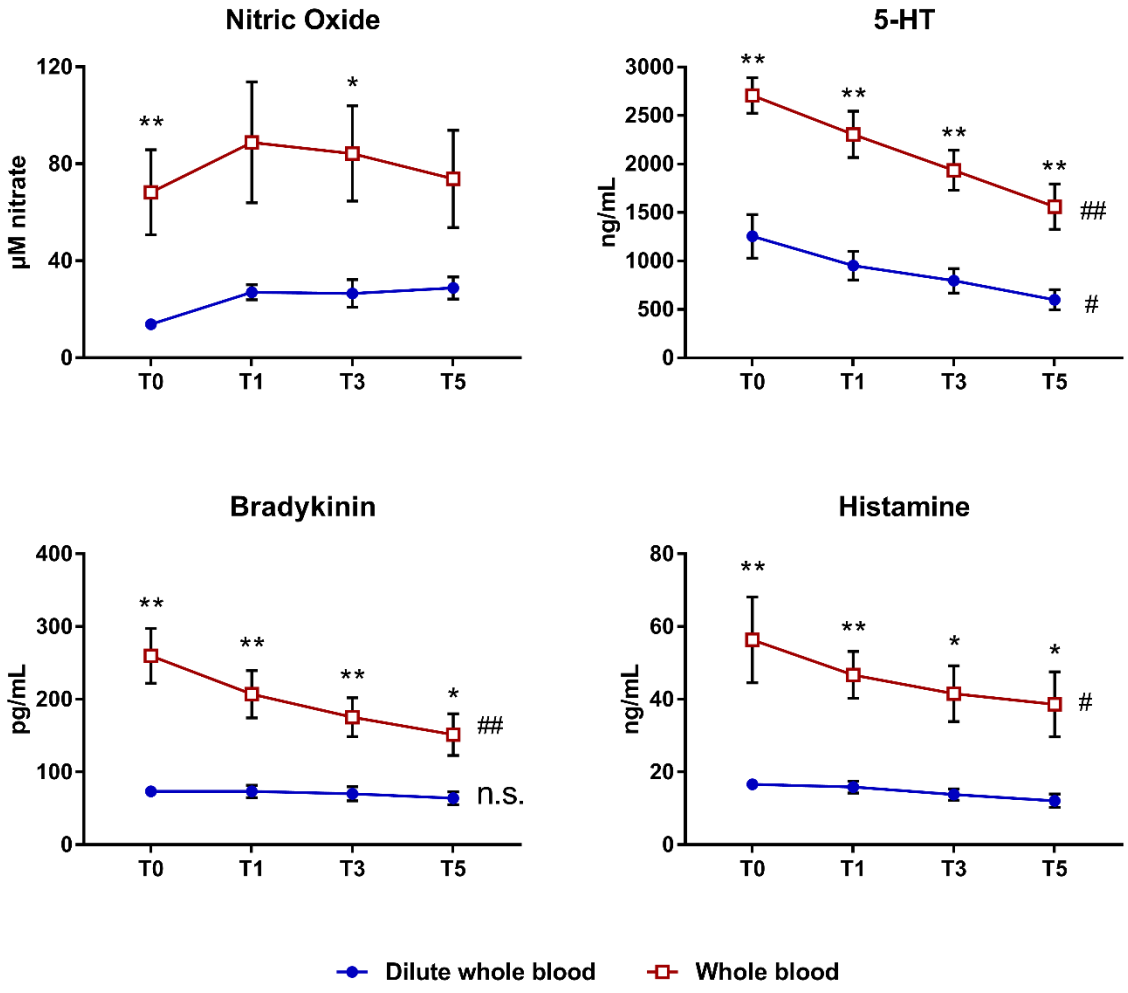
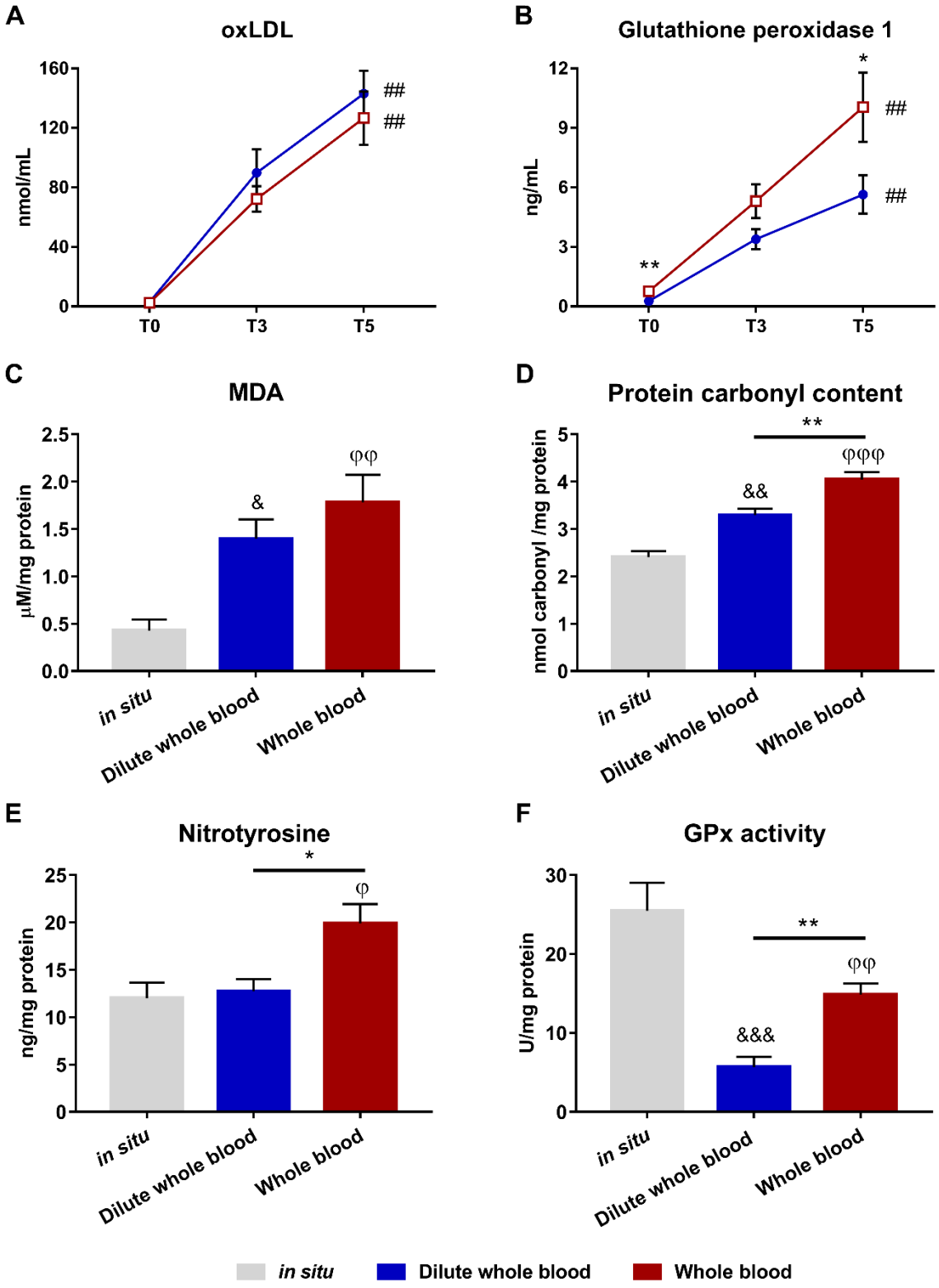


Fig. 1-6 Vasoactive compounds in the perfusate

(Comparison between groups, independent t-test, *p<0.05, **p<0.01; compared within group, repeated measure ANOVA, $^{\#}$p<0.01, $^{\#\#}$p<0.001, n=5)


A
oxLDL
nmol/mL
160
120
80
40
0
T0
T3
T5
##
##
B
Glutathione peroxidase 1
ng/mL
12
9
6
3
0
*
**
##
##
C
MDA
µM/mg protein
2.5
2.0
1.5
1.0
0.5
0.0
&
φφ
in situ
Dilute whole blood
Whole blood
D
Protein carbonyl content
nmol carbonyl /mg protein
5
4
3
2
1
0
**
&&
φφφ
in situ
Dilute whole blood
Whole blood
E
Nitrotyrosine
ng/mg protein
25
20
15
10
5
0
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φ
in situ
Dilute whole blood
Whole blood
F
GPx activity
U/mg protein
30
20
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in situ
Dilute whole blood
Whole blood
in situ
Dilute whole blood
Whole blood

Fig. 1-7 Oxidative stress and related modification

Both groups had significantly increased oxidative stress, indicated by lipid peroxidation markers of oxLDL in the perfusate and MDA in the coronary arteries (A and C), and oxidative stress related modification of proteins in the coronary arteries (E). The whole blood group had significantly higher protein carbonyl content and nitrotyrosine in the coronary arteries than the dilute blood group (D and E), suggesting higher oxidative stress in this group. The whole blood group had higher glutathione peroxidase 1 in the perfusate than the dilute whole blood group (B). oxLDL, oxidized low-density lipoprotein; MDA, malondialdehyde. (Comparison between groups, independent t-test, whole blood group vs. dilute whole blood group: $^{*}p<0.05$, $^{**}p<0.01$; Dilute whole blood group vs. *in situ*: $^{\&}p<0.05$, $^{\&\&}p<0.01$, $^{\&\&\&}p<0.001$; Whole blood group vs. *in situ*: $^{\phi}p<0.05$, $^{\phi\phi}p<0.01$, $^{\phi\phi\phi}p<0.0001$; comparison within group, repeated measure ANOVA, $^{\#\#}p<0.001$, n=6)

2.4 Discussion

To our knowledge, this is the first study comparing coronary vascular tone between whole blood and dilute whole blood perfusate in a large animal isolated heart perfusion model. This study shows that whole blood perfusion in working mode ESHP has better coronary vascular tone regulation, indicated by relative higher CVR at the end of perfusion and better coupling of cardiac work and coronary artery flow. However, there was a gradual loss of CVR irrespective of the two perfusion strategies, suggesting the change of vasoactive mediators or factors produced during ESHP may paly a part in the loss of CVR. This study focused on the perfusate components and coronary arteries to find the potential mechanism. We have tested the hypothesis that change of some circulating vasoactive mediators in the perfusate may play a role in loss of regulating coronary vascular tone during 6 hours ESHP. Also, the inflammation status and oxidative stress modification in the perfusate and coronary vasculature have been evaluated.

2.4.1 Whole blood based-perfusate strategy

Different perfusion solutions have been used for ESHP, including leukocyte-depleted blood, whole blood[73-78, 148], modified Krebs solutions[80, 81], and other asanguineous crystalloid perfusate[82, 83]. Even though crystalloid reperfusion negates the effect of several critical factors in the reperfusion injury (complement, leukocytes, platelets)[149], preservation using a whole blood-based perfusate may improve donor heart preservation compared to a solution in which the plasma component has been removed.[78] This improvement may be attributed to the antioxidant and anti-inflammatory properties of albumin and other plasma proteins, as well as the metabolic substrates present in the donor plasma.[105] However, using whole blood for ESHP has some limitations. Clinically,

autologous whole donor blood collection requires fine coordination with other organ procurement teams, and the volume may not be enough for multi-organ *ex-situ* perfusion. Secondly, the blood may carry high levels of circulatory catecholamines that are detrimental to graft function. Also, the stored donor blood is often unsuitable for machine perfusion because of the sensitivity of stored blood cells to hemolysis.[82]

This study used both whole blood perfusate and whole blood diluted 1:1 with modified Krebs-Henseleit solution with albumin. As defined by Krebs and Henseleit[150], this bicarbonate perfusion fluid was supposed to mimic the key ionic content of blood or plasma and have a pH of 7.4 at 37 °C, which was widely used by most studies in the literature. Also, the dilute whole blood perfusion strategy is commonly used in ESHP studies. Even though dilute whole blood has lower oxygen delivery than whole blood due to half of hemoglobin concentration and hematocrit, cardiac function was not compromised by this strategy, nor was there any evidence of inadequate oxygen delivery. Our observations were in agreement with previous studies[151, 152] that in the whole blood perfusion, there was relatively higher perfusate lactate from the start. Because the RBC can only have anaerobic glycolysis, the whole blood group had higher lactate than the diluted whole blood group due to higher concentration of RBC. However, in both group there was a gradual reduction of perfusate lactate, indicating the catabolism of heart. As our results showed, no statistical difference between the two groups has been observed regarding cardiac functional parameters. However, *ex-situ* perfused hearts in both groups had a functional decline, suggesting that the loss of cardiac function in both settings is not due to the shortage of oxygen delivery to the myocardium.

2.4.2 Uncoupling between cardiac function and coronary artery flow in the dilute whole blood perfusion

Hearts perfused with dilute whole blood lost CVR and indicated uncoupling between cardiac work and coronary artery flow. We have observed decreasing LVSW alongside increasing CBF in the dilute whole blood perfusion. At the initiation of working mode perfusion, as expected, CBF increased appropriately with increased LVSW, consistent with normal coronary autoregulation. However, LVSW and CBF trended in opposite directions during extended perfusion: while LVSW decreased over 5 hours of working mode perfusion, CBF increased continuously. The uncoupling of these two parameters indicates the loss of coronary autoregulation during perfusion. In comparison, coronary tone and autoregulation are better maintained in whole blood perfusion. Concurrently with decreasing ventricle stroke work during 6 hours of perfusion, CBF decreased in whole blood perfusion, suggesting better coupling between cardiac function and myocardial perfusion.

Compared to whole blood perfusion, the coronary artery in the dilute whole blood group was dilated to a supraphysiological level during 6 hours ESHP. Many previous studies have found that the hearts perfused with Krebs buffer are markedly vasodilated in an attempt to meet myocardial oxygen demand.[153, 154] Sutherland *et al.* also found that the coronary flow rate by blood-perfusate is much closer to the physiological range than that with the blood-free fluid perfused preparations.[105] This evidence leaves us to speculate that some mediators in the plasm may play a role in controlling of CVR during isolated heart perfusion.

2.4.3 Leukocyte and platelet activation induced myocardial and endothelial damage

Leukocyte, platelet and endothelial activation process are interrelated.[155, 156] The vascular endothelium is a large paracrine organ that secrets numerous factors regulating vascular tone, cell growth, platelet and leukocyte interactions and thrombogenicity.[157] We have observed leukocyte and platelet activation in both groups. Among these cytokines, IL-6 was significantly higher in the dilute whole blood perfusate than whole blood perfusate from early time points of perfusion and MMP-9 had a higher trend. IL-6 is secreted mainly from macrophages and endothelial cells while MMP-9 is from monocytes.[120, 158] Some previous studies have indicated that IL-6 induced the expression of MMP-9.[159, 160] Furthermore, expression of MMP-9 has been widely confirmed to be regulated by the activation of ERK1/2 in various pathologic conditions.[161-163] Poonam *et al.* also found that IL-6 mediated expression of MMP-9 is modulated on the activation of $MAPK^{ERK1/2}$ in macrophages.[164] Consistent with these findings, our results showed that ERK1/2 was significantly activated in the coronary vasculature of both perfusion groups, with no statistical difference found between the groups.

MPO is another indicator of leukocyte activation, most abundantly expressed in neutrophil granulocytes and released into the extracellular space during degranulation.[165] Here, we showed that MPO activity was significantly higher in the coronary vasculature of the whole blood group than *in situ* control group. VCAM-1 was also higher in the whole blood perfusate than dilute whole blood perfusate, suggesting higher endothelial activation. It is likely that leukocytes emigrated from perfusate to tissue and resided within the coronary vasculature. Higher numbers of leukocytes in whole blood perfusate may

contribute to the increased MPO activity and the activation of endothelial cells in this group.

In contrast to leukocyte activation, platelet activation was lower in the whole blood group. However, both groups had significantly higher platelet activation during 6 hours of perfusion, indicated by platelet activation factors as PAF, P-selectin, sCD40L, β-TG and PF4. Platelet activation is another manifestation of the interaction between leukocyte, platelet and endothelial cells. The leukocyte adhesion protein, P-selectin, is co-expressed with platelet activating factor by vascular endothelium within minutes of stimulation.[166] The combination of these two factors promotes the rolling and adhesion of unactivated leukocytes to microvessels as these cells traverse the microcirculation.[167] The membrane-spanning protein CD40 is up-regulated in leukocyte and endothelial activation[168, 169] and, after engagement with its natural ligand CD40L, amplify these events by further promoting cytokines release and adhesion of circulating leukocytes to the endothelium.[170]

Once adhered, leukocytes may activate and release oxygen radicals that amplify injury to both the vasculature and cardiac myocytes. Here we showed that compared to whole blood perfusion, dilute whole blood perfusion has greater myocardial and endothelial damage. TM, which is predominantly expressed on the endothelium, plays an essential role in maintaining vascular homeostasis by regulating the coagulation system. After the endothelial injury, TM is released into the intravascular space by proteolytic cleavage of the endothelium component. It can directly act as a natural regulator of inflammation by inhibiting leukocyte-mediated intravascular injury.[171] Hyaluronan belongs to the family of damage-associated molecular patterns (DAMPs), is produced upon

endothelial damage, and is involved in tissue repair.[172, 173] The accumulation of HA in the perfusate demonstrated endothelial glycocalyx shedding, leading to increased vessel permeability.[174] Our results showed that there was around 20% weigh gain in both perfusion groups, suggesting visible edema formation. That could contribute to myocardial and endothelial injury as well. When the coronary endothelium is damaged, leukocyte adhesion and activation can occur rapidly as a feed-forward mechanism.

2.4.4 Endothelium-dependent modulation of CVR

After activation, circulating mediators released from platelets, erythrocytes, leukocytes, and endothelial cells may affect coronary artery vasoactive responses. For example, histamine and bradykinin are autacoids involved in inflammatory processes that can exert powerful effects on vasomotor tone.[9] Whether their role is vasodilation or vasoconstriction depends on the distinct receptors in the vascular bed. For example, in coronary circulation, histamine can induce vasoconstriction via stimulation of H_1 receptors on vascular smooth muscle cells of large and small arteries. At the same time, it can mediate vasodilation by activation H_2 receptors located on vascular smooth muscle cells of arterioles.[175, 176] 5-HT is also a naturally occurring vasoactive substance in the platelet. It possesses both vasoconstrictor and vasodilator properties. It causes constriction in the epicardial arteries 2-3 mm in diameter.[177, 178] The constriction effect can be due to the direct activation of vascular smooth muscle.

We reported that the vasoactive substances were higher in the whole blood perfusate than dilute whole blood perfusate. Furthermore, there was a significant decrease of perfusate bradykinin and histamine in the whole blood group and a decrease of 5-HT in both groups. The correlation study between CBF and 5-HT, bradykinin and histamine

showed 5-HT had a higher correlation than others (5-HT, R^2=0.47; bradykinin R^2=0.35; histamine, R^2=0.32, Supplementary Result), suggesting 5-HT may very likely account for the loss of CVR in both groups compared to bradykinin and histamine. Also, the whole blood perfusate had a significantly higher 5-HT than the dilute whole blood group at T5 (1645.5±266.1 vs. 517.1±84.5 ng/mL, p=0.004), suggesting that 5-HT may account for the relatively high CVR in this group. Also, the decrease of CVR in the whole blood group may cause by a reduction of 5-HT in the perfusate. The decrease perfusate 5-HT in both groups may cause by it its disposition by endothelial monoamine oxidase and by re-uptake into platelets.[179, 180] Nitric oxide is another essential mediator capable of affecting vascular tone. Our results showed that nitrate was higher in the whole blood perfusate, suggesting that in our setting, the coronary vasodilation was not accompanied by the increase in nitrite outflow in the perfusate. These results indicated that the change of some vasoactive substances might play a role in the loss of CVR in both groups.

2.4.5 Hemolysis induced oxidative stress and related modification in the coronary vasculature

CBF increased significantly irrespective of two perfusate, suggesting that other factors may play a role in the loss of CVR in addition to the vasoactive substance. We have reported here that there was a significantly higher level of oxLDL in the perfusate and MDA in the coronary vasculature than baseline control, suggesting higher oxidative stress and lipid peroxidation in both groups. Also, protein carbonyl content in the coronary artery, which indicates ROS modification on the protein, was significantly higher in both groups than *in situ* control group. The whole blood group had higher protein carbonyl

content and nitrotyrosine than the dilute whole blood group, suggesting higher oxidative stress modification in this group.

Red blood cell hemolysis is very common and unavoidable on machine perfusion system.[82, 105] Hemolysis can release cytotoxic free hemoglobin and other mediators initiation pro-inflammatory cascade.[181] Hemolysis is also a prominent source of ROS. Oxidation of cell-free Hb produces methemoglobin.[182, 183] Sustained exposure of cell-free Hb to ROS can lead to the formation of ferrylHb[184, 185], which is an unstable oxidized form that can return to the ferric state and act as a redox center. Additionally, ferric iron can react with ROS, *e.g.,* H_2O_2 to produce ferryl iron capable of causing oxidative injury to endothelial cells.[186, 187]

Our results also showed that the glutathione antioxidative system, glutathione peroxidase, was significantly lower in both groups than *in situ* control group, which may explain the oxidative stress states we observed in the *ex-situ* perfused heart. The overproduction of ROS can cause endothelial injury and may cause the loss of CVR in the *ex-situ* perfused heart.

Supplementation

Donor heart procurement and preparation for *ex situ* heart perfusion

Animals were premedicated with intramuscular Atropine (0.02mg/kg) and Ketamine (20mg/kg). Anesthesia was induced and maintained using inhalational isoflurane through an endotracheal tube (end-tidal concentration: 1%-3.5%). A central venous line was introduced into a jugular vein for saline infusion. The porcine hearts were procured and prepared, as described before in detail [146]. Briefly, the heart was exposed by median sternotomy. After intravenous delivery of heparin (1000 U/kg), 800 ml or 1.6 liters of whole blood was retrieved through a two-stage venous cannula placed into the right atrium and was used to prime the ESHP circuit. Pig was then exsanguinated, and the heart was excised, weighed, and were immediately mounted on a custom ESHP system and perfused for 6 hours (n=12). Five freshly procured, unperfused hearts were used as normal controls.

Ex situ heart perfusion and sample collection:

The custom-designed software used here controlled the pump speed to maintain the desired aortic (Ao) and left atrial (LA) pressures during the perfusion enabling preservation and evaluation in different conditions Langendorff mode and working mode. Perfusion was initiated in Langendorff mode with isolated aortic root perfusion. The transition from empty beating non-working state into physiologic working mode was achieved with an automatic increase of left atrial pump RPMs upon changing the desired left atrial pressure of 0 mmHg, to 6 mmHg. During perfusion interval, the coronary sinus blood flow and LA flow were recorded with a TS410 Tubing Flow Module and flow meters

(Transonic, US). The perfusate was continuously infused with glucose (1 gram/hour), insulin (2 units/hour), and epinephrine (0.04 micrograms/minute) for metabolic and inotropic support of the heart during perfusion. Perfusate samples were collected from the aortic root at the baseline (T0), and then hour-1, 3, 5 (T1, 3, 5) after initiation of ESHP and the serum were frozen at -80°C for later analysis. Blood gas analysis of the samples (Electrolyte, PO2, PCO2, hemoglobin concentrations, oxygen saturation, pH, glucose, and lactate) was performed (ABL 800 analyzer, Radiometer, Copenhagen, Denmark). A pH: 7.35-7.45, and arterial partial pressure of oxygen (PaO_2): 100-150 mmHg was maintained during the perfusion. At the end of ESHP, samples were taken from the anterolateral wall of the left ventricle as well as left and right epicardial coronary arteries, were immediately snap frozen in liquid nitrogen and stored at -80˚c for later assessments.

Modified Steen solution

	mmol/L	g/L
Glucose	10	1.982
NaCl	85	4.967
KCl	4.6	0.343
$NaHCO_3$	25	2.100
NaH_2PO_4	1.2	0.144
$CaCl_2$	1.25	0.184
$MgCl_2$	1.2	0.296
Na-pyruvate	5	0.550
Bovine serum albumin (8%)	--	80

Functional and metabolic analyses

The ESHP software program collected atrial, ventricular and arterial pressure in a real-time pattern, and calculated the ventricular stroke volume (SV) and left ventricle

stroke work (LVSW). The SV was calculated as follows: SV (mL/beat) = atrial flow(mL/min) / heart rate (beats/min). The LVSW (mmHg*ml) was calculated as the product of LV developed pressure (mmHg): [mean arterial pressure (mmHg) – left atrial pressure (mmHg)] × SV. The cardiac index (CI) was determined by measuring the flow through the left atrial line divided by the heart weight (mL · minute^{-1} · gram^{-1}). To assess maximum (dP/dt $_{max}$) and minimum (dP/dt $_{min}$) rates of pressure change in the left ventricle, a 5F pigtail catheter was placed in the left ventricle through an introducer sheath placed in the subclavian artery with an orifice on the aorta arch. Coronary blood flow (CBF) was monitored by a Doppler fluxometer (Transonic System Inc., NY, USA) in the pulmonary artery cannula that collected from coronary sinus. Edema formation was calculated as the weight gained by the myocardial tissue at the end of perfusion.

Indexed coronary vascular resistance was calculated as follows:

CVR (mmHg*min/liter/100g) = diastolic pressure (mmHg)/coronary blood flow/100g heart weight.

Myocardial oxygen consumption (MVO_2) (mL O_2 · min^{-1} · 100 g^{-1}) will be determined by multiplying the coronary blood flow (CBF) by the arterial-venous difference in oxygen content (CaO_2 – CvO_2) as below:

MVO_2 = [CaO_2 - CvO_2 (mL O_2 · 100 mL^{-1})] × CBF (ml · min^{-1} · 100 g heart mass), where:

Arterial oxygen content (CaO_2) = [1.34 (mL O_2 · g Hb^{-1}) × Hb concentration (g · 100 mL^{-1}) × oxygen saturation (%)] + [0.00289 (mL O_2 · mm Hg^{-1} · 100 mL-1) × PaO_2 (mm Hg)]

Venous oxygen content (CvO_2) = [1.34 (mL O_2 · g Hb^{-1}) × Hb concentration (g · 100mL^{-1}) × oxygen saturation (%)] + [0.00289 (mL O_2 · mm Hg-1 · 100 mL-1) × PvO_2 (mm Hg)]

Cell-free hemoglobin analysis

Perfusate samples were centrifuged at 2200g for 10 minutes in 4 °C and a 40 µL aliquot was carefully removed and diluted in 1 mL of a modified Drabkin's reagent (potassium ferricyanide 0.61 mM, potassium cyanide 0.77 mM, potassium dihydrogen phosphate 1.03 mM). The sample was vortexed and incubated at room temperature in the dark for 5 min. A 200 µL aliquot was pipetted into the microplate and absorbance was measured spectrophotometrically at 540 nm using a SpectraMax Plus 384 (Molecular Devices, CA, USA). Hemolysis is calculated as the ratio of supernatant to total hemoglobin with hematocrit adjustment for the volume of supernatant in the sample.

SDS-PAGE and Western immunoblotting

Tissue powder obtained from frozen coronary artery sample was homogeneized in a RIPA buffer containing phenylmethyl sulfonyl fluoride (PMSF), proteinase inhibitor, and phosphatase inhibitor. Protein concentrations were measured by the Bradford assay (Thermo Scientific Pierce, IL, USA). Coronary artery tissue protein (40µg) was separated by the standard SDS-PAGE and transferred on nitrocellulose membranes. Western blotting was performed using anti-p-ERK1/2 and anti-ERK1/2 primary antibodies (1:1000 dilution, Cell Signaling Technology, MA, USA) at a concentration of 1:1000. Immunoblots were revealed using ECL Western Blotting Reagents (GE Healthcare). Signal were quantified using ImageJ software v1.48.

Myeloperoxidase (MPO) activity assay

To study the activity of myeloperoxidase (MPO), a peroxidase enzyme most abundant in neutrophil granulocytes, coronary arteries tissue was isolated free from myocardium at the end of perfusion. Small tissue sections (>10mg) were immediately snap frozen in liquid nitrogen and stored at -80 °C. MPO activity assay was conducted according to the manufacturer's instruction (Biovision, CA, USA). The reaction was carried out in a 96-well plate, and the samples were read in a fluorescence microplate reader (BioTek Instruments, Inc. Vermont, USA) at 400 nm excitation and 505 nm emission. The results were expressed in relative fluorescent units (RFU). The levels were corrected for the amount of protein (Bradford assay, Bio-Rad, Hercules, USA).

Glutathione peroxidase (GPx) activity assay

To study the activity of GPx in the myocardium. After perfusion, small tissue sections (>10mg) were immediately snap frozen in liquid nitrogen and stored at -80 °C. GPx activity assay was conducted according to the manufacturer's instruction (MyBioSource Inc., CA, USA). The reaction was carried out in a 96-well plate, and the samples were read in a fluorescence microplate reader (BioTek Instruments, Inc. Vermont, USA) at 340 nm. The results were expressed in the amount of enzyme that will cause the oxidation of 1.0 µmol of NADPH to NADP+ under the assay kit condition per minute at 25°C. The levels were corrected for the amount of protein (Bradford assay, Bio-Rad, Hercules, USA).

Chapter Three: The Evaluation of Constant Coronary Artery Flow versus Constant Coronary Perfusion Pressure During Normothermic Ex Situ Heart Perfusion

This chapter is in revision for the *Journal of Heart and Lung Transplantation*

Abstract

Background:

Evidence suggests that hearts that are perfused under *ex-situ* conditions lose normal coronary vasomotor tone and experience contractile failure over a few hours. We aim to evaluate the effect of different coronary perfusion strategies during *ex situ* heart perfusion on cardiac function and coronary vascular tone.

Methods:

Porcine hearts (n=6 each group) were perfused in working mode for 6 hours with either constant aortic diastolic pressure (40 mmHg) or constant coronary flow rate (500 mL/min). Functional and metabolic parameters, cytokine profiles, cardiac and vascular injury, coronary artery function and oxidative stress were compared between groups.

Results:

Constant coronary flow perfusion demonstrated better functional preservation and less edema formation (Cardiac index: flow control=8.33 versus pressure control=6.46 $mL \cdot min^{-1} \cdot g^{-1}$, p=0.016; edema formation: 7.92% versus 19.80%, p<0.0001). Pro-inflammatory cytokines, platelet activation as well as endothelial activation were lower in the flow control group. Similarly, less cardiac and endothelial injury was indicated by troponin I and hyaluronan in this group. The evaluation of the coronary artery function showed there was loss of coronary autoregulation in both groups. Oxidative stress was induced in the coronary arteries and was relatively lower in the flow control group.

Conclusions:

A strategy of controlled coronary perfusion flow during *ex situ* heart perfusion provides superior functional preservation and less edema formation, together with less myocardial damage, leukocyte, platelet, endothelial activation, and oxidative stress. There was loss of coronary autoregulation and decrease of coronary vascular resistance during ESHP. Inflammation and oxidative stress state in the coronary vasculature may paly a role.

3.1 Introduction

The clinical application of heart transplantation has expanded over time and outcomes have improved substantially.[1] Meanwhile, recipient waiting lists continue to grow worldwide due to the discrepancy between the demand and supply of suitable organs.[2] *Ex situ* heart perfusion (ESHP) allows for a shorter cold ischemic interval[7] and provides the opportunity to assess cardiac function and viability, and metabolism.[44] It also has the potential for reconditioning marginal donor hearts, thereby increasing the number and quality of donor organs available for transplant.[6]

Maintenance of cardiac function relies on adequate myocardial blood supply through the coronary arteries. The regulation of coronary blood flow is an important issue in heart preservation. Thus, coronary artery function should be monitored and preserved. The ability of coronary resistance vessels to dilate in response to increase in myocardial oxygen demand, as illustrated by the tight correlation between myocardial oxygen consumption and coronary blood flow, is critical for maintaining an adequate, but not excessive supply of oxygen to the myocardium.[9] Evidence indicates that perfused hearts may lose normal vasomotor tone and develop contractile dysfunction in a few hours.[140-142] Our preliminary data[104] also suggests that regulation of coronary artery function is disturbed during ESHP, leading to apparent excessive coronary blood flow over time.

Constant coronary artery flow and constant coronary artery perfusion pressure are two strategies widely used in the field of ESHP. Controlled perfusion utilizing a low coronary perfusion pressure would limit injury and preserve cardiac function.[148, 188-191] Although coronary perfusion pressure may affect the development of myocardial edema, inadequate flow may compromise myocardial oxygen delivery, while excessive pressure

may damage endothelium.[192] The optimal perfusion pressure in ESHP that minimizes microvascular fluid filtration and also ensures homogeneous myocardial perfusion to prevent ischemia has not been adequately determined. TransMedics Organ Care System (OCS™) targets the coronary flow in the 650-900 mL/min range, with the perfusion pressure 60-80 mmHg.[74, 139, 193-195] Our previous studies indicated that myocardial energy stores can be maintained with aortic pressure as low as 40 mmHg.[140] However, the best perfusion parameters (e.g. continuous flow vs. pulsatile flow, low perfusion pressure vs. high perfusion pressure) needed to achieve ideal results remain unclear.[196] Additionally, coronary flow results from the interplay of coronary perfusion pressure, myocardium contraction, and coronary vascular tone.[94] Determining the optimal coronary flow rate above which the oxygen demand of the heart is met is of critical importance to prevent the state of myocardial hypoxia and for better organ preservation. The purpose of this study was to examine the effect of coronary artery perfusion strategy on preservation of myocardial contractile function and coronary vascular tone during ESHP.

3.2 Material and methods:

All animal experimental procedures were approved and performed in accordance with the guidelines and regulations of the Institutional Animal Care and Use Committee of the University of Alberta. Female domestic pigs (n=17) weighing 45-55 kg were used as heart and blood donors.

Experimental design

Normal hearts were procured as previously described,[146] and normothermic ESHP was performed for six hours (Langendorff perfusion for one hour and 5 hours of working mode perfusion). The *ex situ* working heart apparatus, shown in **Figure 2-1A**, has been described in detail previously.[146] Briefly, two centrifugal pumps connecting left atrium (LA) and aorta, providing pre-load and afterload, respectively. This arrangement allows the left ventricle to perform work with pre-load (LA pressure=6 mmHg) and fixed afterload that permitted coronary perfusion. Automatic control of pump revolutions per minute (RPM) is achieved to maintain the pre-set diastolic and LA pressure by a custom designed software. A schematic of the experimental design is seen in **Fig 2-1B.**

Coronary pressure control group (n=6): Diastolic pressure was set at 40 mmHg during working mode perfusion at 1h (T1), maintaining the same pressure for the rest of the experiment.

Coronary flow control group (n=6): At one hour of perfusion**,** coronary flow rate of 500 mL/min was maintained by manually adjusting diastolic pressure. For functional assessment, to ensure comparable conditions to the pressure control group, diastolic pressure was set to 40 mmHg only at 1 h (T1), 3 h (T3), and 5 h (T5).

***In situ* heart control group (n=5):** Freshly procured, unmanipulated hearts were obtained to serve as *in situ* control.

Ex situ evaluation of myocardial function and coronary artery function

Myocardial function was assessed at T1, T3, and T5 in working mode.[146] Preload challenge was induced by increasing left atrial pressure from 6 to 12 mmHg. To correlate cardiac work with coronary flow and therefore estimate the integrity of coronary autoregulation, linear regression analyses were performed on coronary artery flow and LVSW at T1, T3 and T5 in each heart. The detailed methods are included in Supplementary Material.

Leukocytes and platelet activation profiles

Proinflammatory cytokines and platelet activation marker were measured in the perfusate (R & D System Inc., USA; FineTest, China). MPO peroxidation activity was measure in the coronary artery tissue (Biovision, USA). The detailed methods are included in the Supplementary Material.

Myocardial and vascular injury

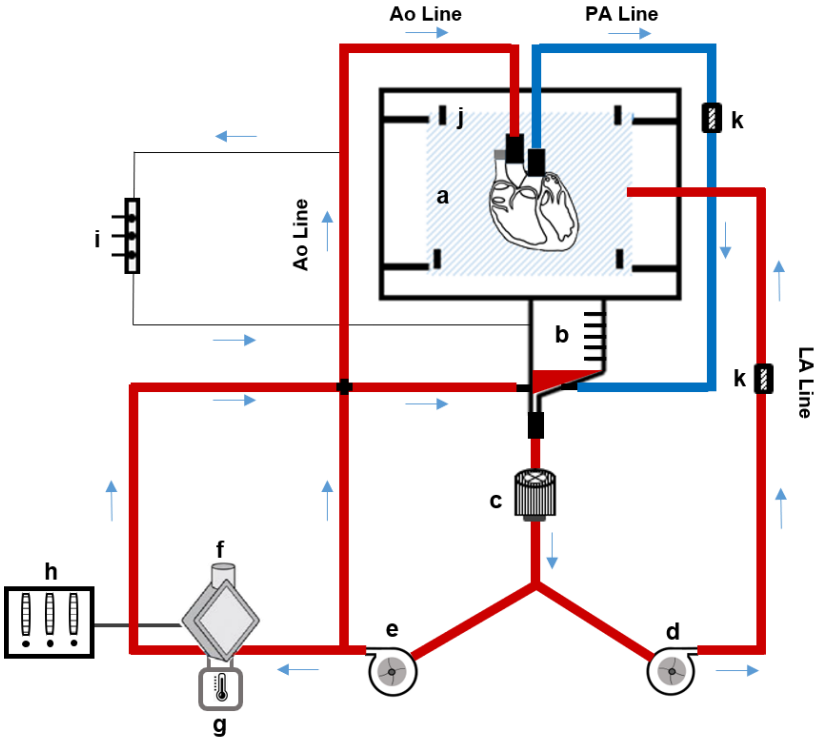
Myocardial injury was estimated by measuring cardiac troponin I in the perfusate using ELISA (Life Diagnostics, USA). Endothelial activation factor VCAM-1 (MyBioSources Inc., USA) and vascular injury marker vWF (FineTest, China) were evaluated in the perfusate as well. Coronary artery expression of ET_AR, ET_BR were detected by standard western blot techniques. Full details of methods used are described in the Supplementary Material.

Determination of oxidative stress related modification

Lipid peroxidation was determined by measuring of oxidized oxLDL in the perfusate by ELISA (MyBioSource Inc., CA, USA) and MDA in the coronary artery tissues (R & D System Inc., MN, USA). Oxidative protein modification was measured in the coronary arteries tissue by assessment of protein carbonyl formation (Abcam, Cambridge, UK). Detailed methods are described in the Supplementary Material.

Statistical analysis

The SPSS 25.0 (SPSS Inc., IL, USA) was used for statistical analysis. The data (mean ± standard error of the mean) were compared using the independent sample t-test or analysis of variance (ANOVA). Repeated measures ANOVA with paired t-test was used to identify trends over time within each group. LVSW and CBF were assessed by linear regression analysis, and R^2 was reported for the regression analyses.


Ao Line
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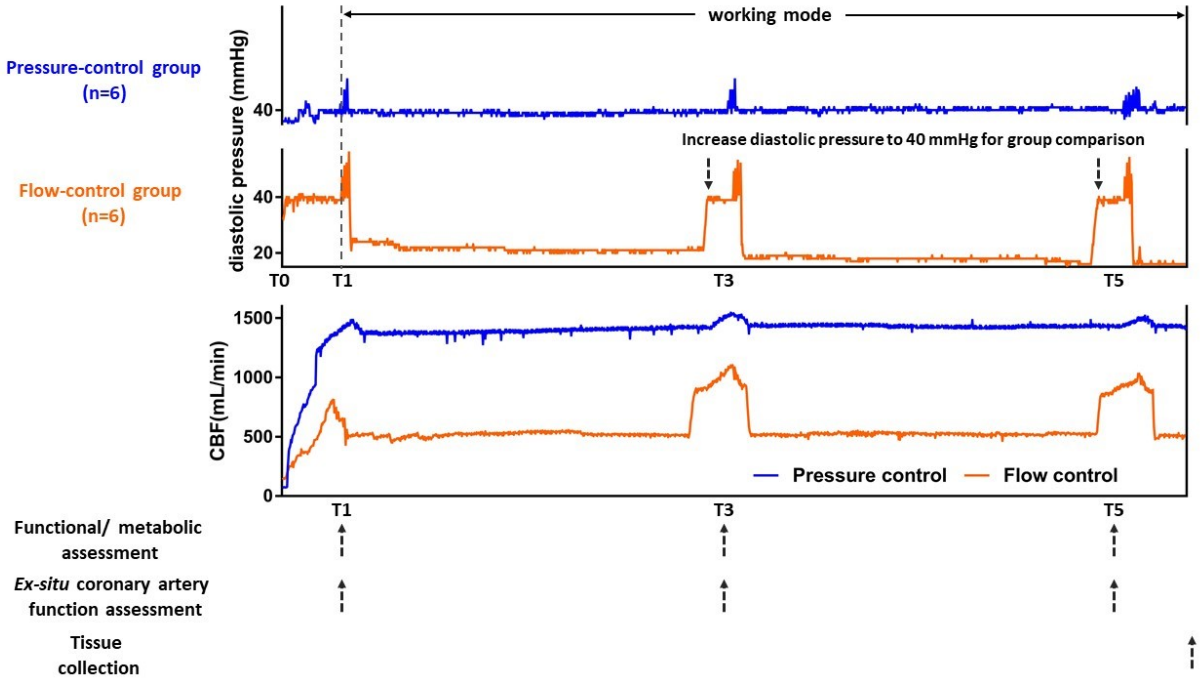

working mode
Pressure-control group (n=6)
Flow-control group (n=6)
diastolic pressure (mmHg)
40
Increase diastolic pressure to 40 mmHg for group comparison
40
20
T0
T1
T3
T5
CBF(mL/min)
1500
1000
500
0
Pressure control
Flow control
T1
T3
T5
Functional/ metabolic assessment
Ex-situ coronary artery function assessment
Tissue collection

Fig. 2-1 A, a schematic view of the perfusion system and B, experimental study design

A, custom ESHP circuit. LA line connects one centrifugal pump and left atrium, providing the preload and Ao line connects another pump and the aorta, providing the afterload. PA line collects the effluent of the pulmonary to the reservoir. a, supportive membrane; b, reservoir; c, arterial filter; d and e, centrifugal pumps; f, oxygenator; g, heater; h, gas line; i, drug infusion line; k, flow probes. Ao, aorta; PA, pulmonary artery; LA, left atrium. B. Experimental design. Hearts were allocated to either of the following 2 groups (n=6 each): (1) pressure-control group, diastolic pressure was adjusted to 40 mmHg or [20] Flow-control group, diastolic pressure was adjusted to make constant coronary blood flow of 500 mL/min. The hearts underwent 1 hour of Langendorff perfusion, followed by 5 hours working mode perfusion. Functional and metabolic evaluation, as well as *ex-situ* coronary artery function assessment were done at 1, 3, and 5 hours of working mode perfusion.

3.3 Results

3.3.1 Physiologic parameters and blood analysis

Physiologic parameters at T1, T3 and T5 were comparable between the two groups (**Table 2-1**). To achieve a coronary flow rate of 500 mL/min in the flow control group, the diastolic pressure was maintained at approximately 20 mmHg, which was half of that compared to the pressure control group (**Figure 2-1B**). The lactate level remained well below 2.5 mmol/L during perfusion in both groups. The calculated red blood cell hemolysis in the perfusate was increased from less than 0.5% at baseline to approximately 1.5 % at the end of perfusion in both groups (**Supplemental Figure 2-1**).

3.3.2 Reduced flow improves cardiac function and decreases edema formation

Typical hemodynamic changes during the 6 hours of pressure-controlled *ex situ* heart perfusion are depicted in **Figure 2-2**. Cardiac function was better preserved in the flow control group at T5, indicated by higher cardiac index, stroke work and dP/dt_{max} (**Figure 2-3A, 2-3B, and 2-3C**). At the end of perfusion, there was significantly lower edema formation in the flow control group (7.9±0.7% vs. 19.8±0.6%, $p<0.0001$; **Figure 2-3D**). Macroscopically, the myocardium in the pressure control group had evidence of epicardial hemorrhage and edema formation. However, the hearts in the flow control group showed higher gross similarity to the *in situ* appearance of the heart (**Supplemental Figure 2-2**).

Table 2-1

Variable	Pressure control (n=6)	Flow control (n=6)	*p*-value
Characteristics			
Body weight, kg	46.5 ± 2.2	47.7 ± 1.5	0.67
Heart weight, g	249.5 ± 10.6	238.2 ± 9.6	0.45
Ischemic time, min	20.17 ± 0.87	19.33 ± 1.0	0.55
***Ex situ* heart perfusion**			
Systolic pressure, mmHg			
60 min (T1)	111.5 ± 2.9	108.5 ± 5.1	0.62
180 min (T3)	108.0 ± 3.6	109.5 ± 3.9	0.79
300 min (T5)	104.2 ± 3.5	107.3 ± 4.5	0.59
Diastolic pressure, mmHg			
60 min (T1)	39.7 ± 0.2	39.8 ± 0.2	0.55
180 min (T3)	39.7 ± 0.2	39.8 ± 0.2	0.55
300 min (T5)	39.7 ± 0.2	39.8 ± 0.2	0.55
Left atrium flow rate, mL/min			
60 min (T1)	2105.0 ± 140.2	2165.0 ± 103.3	0.46
180 min (T3)	1971.7 ± 145.9	2061.7 ± 138.6	0.66
300 min (T5)	1735.0 ± 152.7	1895.3 ± 111.2	0.32
Blood gas analysis			
Hemoglobin, g/100 mL			
60 min (T1)	4.2 ± 0.2	4.7 ± 0.2	0.09
180 min (T3)	4.2 ± 0.2	4.7 ± 0.2	0.06
300 min (T5)	4.1 ± 0.2	4.7 ± 0.2	0.03
Lactate (mmol/L)			
60 min (T1)	2.67 ± 0.1	2.35 ± 0.1	0.08
180 min (T3)	1.87 ± 0.4	1.95 ± 0.5	0.90
300 min (T5)	2.50 ± 0.2	2.36 ± 0.3	0.73
Perfusate composition			
RBC, $\times 10^{12}$ cells/L			
0 min (T0)	2.70 ± 0.2	2.61 ± 0.1	0.74
180 min (T3)	2.75 ± 0.2	2.56 ± 0.1	0.43
300 min (T5)	2.67 ± 0.2	2.52 ± 0.1	0.58
WBC, $\times 10^{9}$ cells/L			
0 min (T0)	6.05 ± 1.0	6.90 ± 0.2	0.46
180 min (T3)	4.21 ± 0.6	3.89 ± 0.2	0.61
300 min (T5)	3.81 ± 0.6	3.54 ± 0.2	0.68
Platelet, $\times 10^{9}$ cells/L			
0 min (T0)	99.5 ± 16.0	103.8 ± 22.8	0.89
180 min (T3)	93.5 ± 16.9	95.8 ± 14.5	0.92
300 min (T5)	79.5 ± 14.4	89.3 ± 14.5	0.65

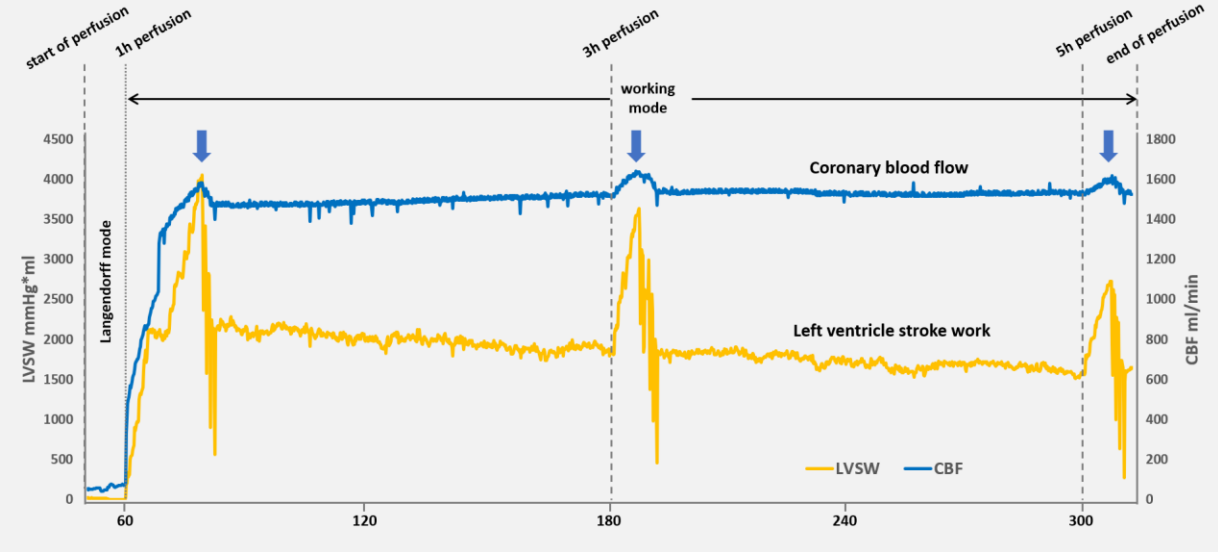


Fig. 2-2 Typical hemodynamic changes during 6 hours pressure control *ex situ* heart perfusion in Langendorff and working mode

Left ventricle stroke work (yellow) and coronary blood flow rate (blue) are depicted. The blue arrows indicate the workload challenge induced by manually increasing the left atrium pressure from 6 to 12 mmHg. After switching to working mode at 60 minutes, left ventricle stroke work and coronary blood flow rate reach a plateau after approximately 30 minutes. LVSW, left ventricle stroke work; CBF, coronary blood flow.

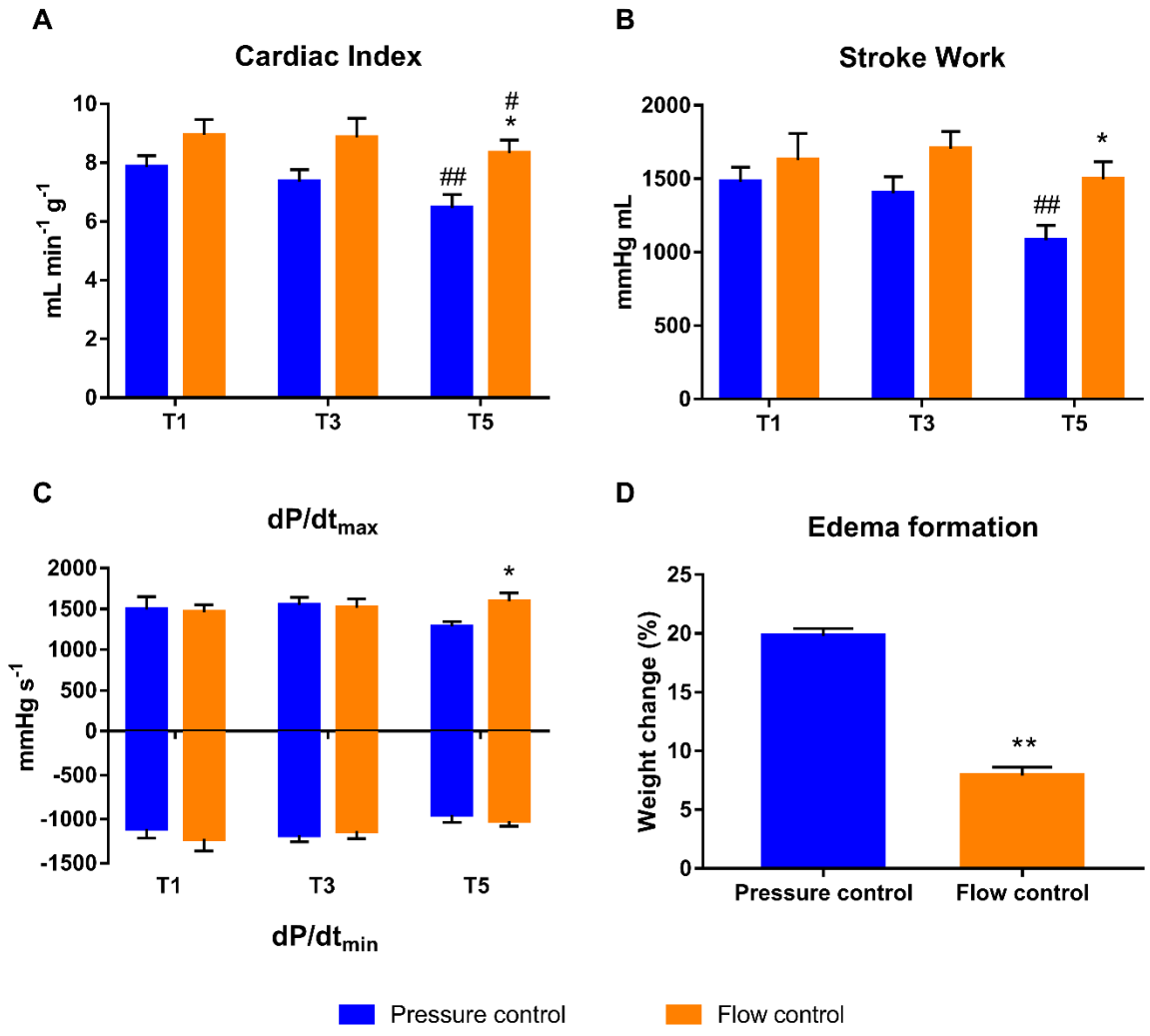


Fig. 2-3 Functional assessment and edema formation

Cardiac function was better preserved in the flow control group, indicated by cardiac index (A), stroke work (B). Contractility was better preserved in the flow control group, indicated by dP/dt_{max} (C). After perfusion, less edema formation was observed in the flow control group (D). (Compared between groups, independent t-test, $^{*}p<0.05$, $^{**}p<0.0001$; compared within group, repeated measure ANOVA, $^{\#}p<0.05$, $^{\#\#}p<0.001$, n=6 each group)

3.3.3 Assessment of coronary vascular resistance

In the pressure control group, coronary blood flow increased from less than 250 mL/min/100g to over 500 mL/min/100g within a few minutes after switching from Langendorff mode to working mode perfusion (**Fig. 2-2**). CBF in the flow control group at 3 (T3) and 5 (T5) hours was lower compared to the pressure control group (p=0.012 at T3, p=0.027 at T5, **Fig. 2-4A**). CVR gradually declined during six hours of perfusion in both group (p<0.05, **Fig. 2-4B**). However, in the flow control group, CVR was higher than that in the pressure control group at both T3 and T5 (p=0.009 at T3 and p=0.008 at T5). Consistent with decreased CVR, oxygen consumption and percentage of oxygen extraction decreased during perfusion in both groups as well (p<0.05, **Fig. 2-4C and 2-4D**). Oxygen consumption was higher in the flow control group at T3 and T5 (p=0.006 at T3, p=0.01 at T5), similar to oxygen extraction (p=0.004 at T3, p=0.047 at T5).

3.3.4 Leukocyte and platelet activation were induced for both groups

There was a significant increase of leukocyte and platelet activation compared to the baseline in both groups (evaluated by concentration of pro-inflammatory cytokines and platelet activation factors in the perfusate collected from the coronary sinus effluent, p<0.001, **Fig. 2-5A**). The flow control group had less pro-inflammatory cytokines compared to the pressure control group (p<0.05). In the coronary tissue, there was significantly higher MPO peroxidation activity in the pressure control group than both flow control group and *in situ* group (p=0.0001 vs. *in situ* and p=0.0003 vs. flow control group, **Fig. 2-5B**). Both groups had increased platelet activation, indicated by the level of β-TG and sCD40L (**Fig. 2-5C**). The β-TG level was significantly lower in the flow control group at T5 compared with the pressure control group (6.0±0.4 vs. 7.3±0.3 ng/mL, p=0.038).

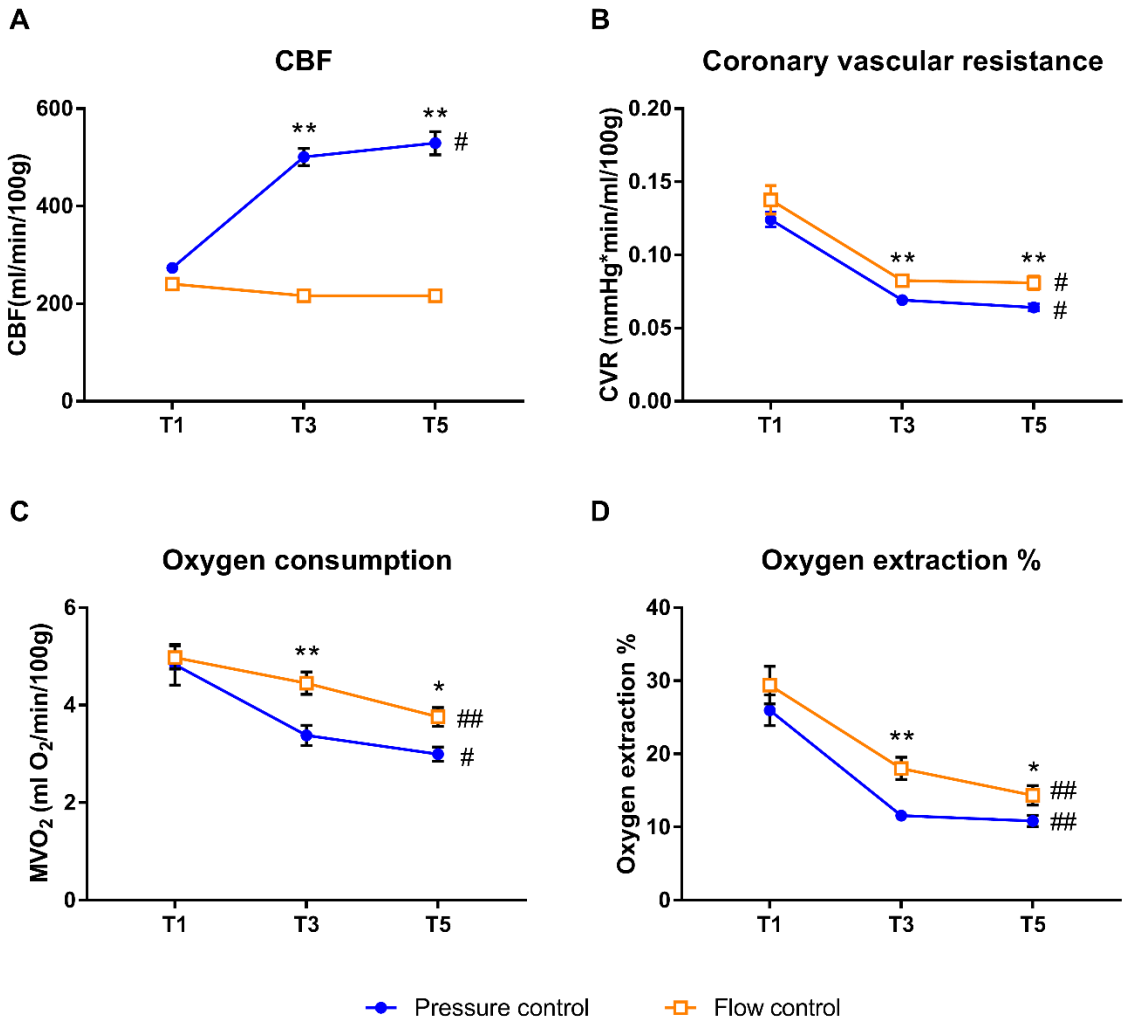


Fig. 2-4 Metabolic evaluation of *ex situ* heart perfusion

During 6 hours perfusion, there was a significant increase of coronary blood flow (A) over time in the pressure control group. Coronary vascular resistance (B) was gradually decreasing in both groups, with relative higher in the flow control group. Oxygen consumption (C) and oxygen extraction (D) both decreased significantly, with both values higher in the flow control group. (comparison between groups, independent t-test, *p<0.05, **p<0.01; compared within group, repeated measure ANOVA, $^{\#}p$<0.05, $^{\#\#}p$<0.01, n=6 each group)

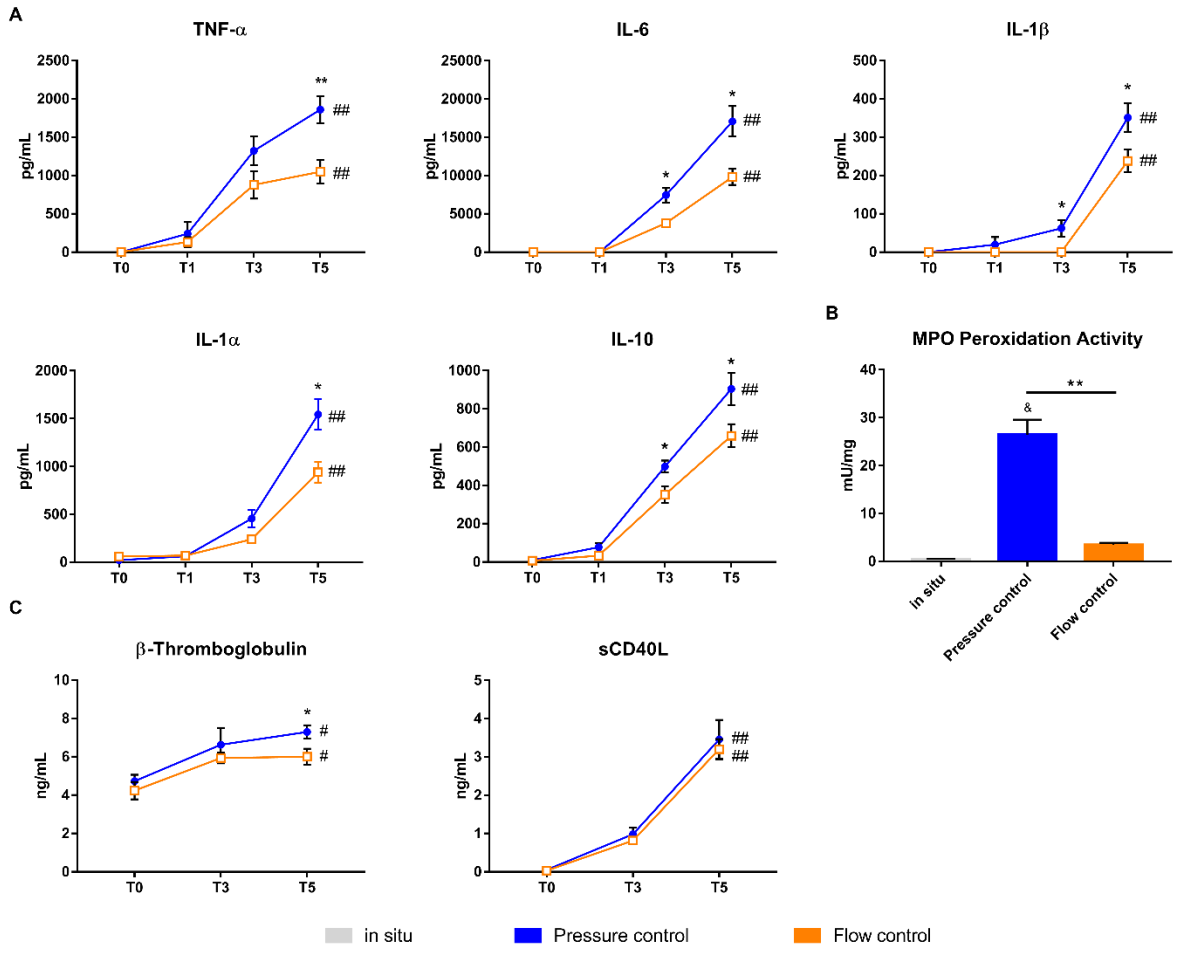


Fig. 2-5 Leukocyte and platelet activation markers

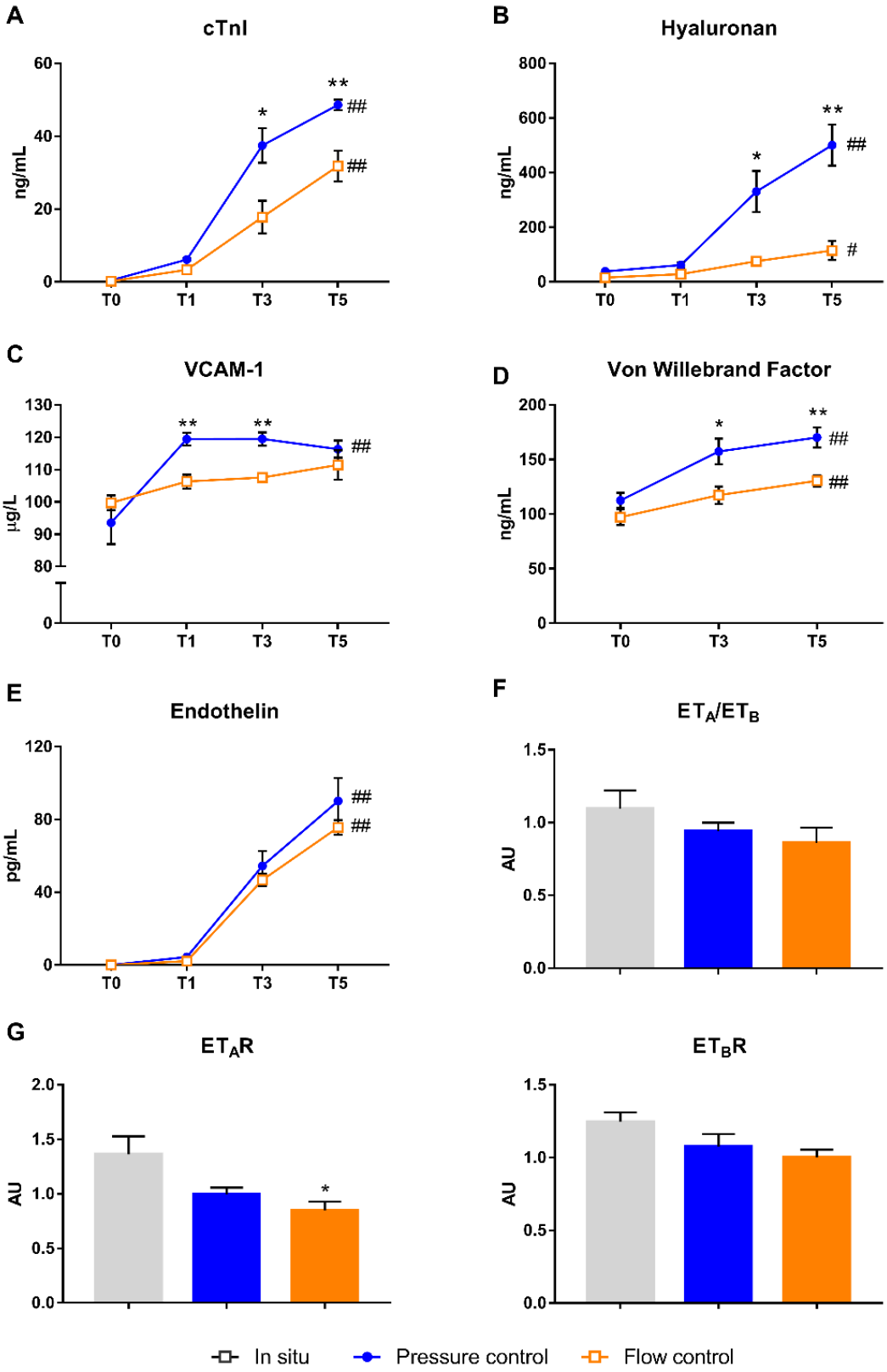
Pro-inflammatory and anti-inflammatory cytokines (A) were increased in both groups. The flow control group had less leukocyte activation than the pressure control group. Myeloperoxidase (MPO) peroxidation activity was higher in the pressure control group than both the flow control group and in situ group (B). Both groups had significantly increased platelet activation, indicated by β-Thromboglobulin and sCD40L (C). MPO, myeloperoxidase; n=5 (Comparison between groups, independent t-test, $^{*}p<0.05$, $^{**}p<0.001$. compared within group, repeated measure ANOVA, $^{\#}p<0.01$, $^{\#\#}p<0.001$, pressure group vs. *in situ*: $^{\&}p<0.001$, n=6 for cytokines and n=3 for MPO activity)

3.3.5 Cardiac and endothelial injury

Both perfusate cTnI and hyaluronan levels increased significantly compared to baseline (p<0.001, **Figure 2-6A and 2-6B**). The flow control group had a lower degree of cardiac damage markers than the pressure control group starting from T3 (cTnI, 17.8±4.5 vs. 37.5±4.7 ng/mL, p=0.017 at T3, 31.8±9.5 vs. 48.6±3.3 ng/mL, p=0.006 at T5; hyaluronan, 75.8±10.3 vs. 330.8±75.2 ng/mL, p=0.01 at T3, 115.1±34.6 vs. 501.0±75.5 ng/mL, p=0.002 at T5). In the pressure control group, VCAM-1 increased starting from T1 and was higher than that in the flow control group at T1 and T3 (p=0.002 and p=0.003, separately, **Figure 2-6C**). However, there was no significant difference in perfusate VCAM-1 value over time in the flow control group. The pressure control group had significantly higher values of vWF than those in flow control group at both T3 and T5 (117.4±8.0 vs. 157.4±11.7 ng/mL, p=0.018 at T3 and 170.3±9.2 vs. 130.6±5.2 ng/mL, p=0.004, **Figure 2-6D and 2-6E**). There were no significant differences in ET_AR/ET_BR ratio expression between groups (**Figure 2-6F, 2-6G, and 2-6H**).

3.3.6 Correlation for coronary blood flow versus left ventricle stroke work

At T1, T3 and T5, CBF and LVSW were recorded while increasing left atrial pressure from 6 to 12 mmHg, as depicted in **Figure 2-7A and 2-7B**. The slope of the correlation line for coronary blood flow versus left ventricle stroke work was higher in T1 than both T3 and T5 in both groups. At later time points, the change of CBF during the workload challenge decreased (**Figure 2-7C**). Within each group, both T3 and T5 had significantly less change in CBF in response to increased work than at T1 (pressure control group, p=0.0001 at T3 and T5; flow control group, p=0.0244 at T3, p=0.001 at T5). Altogether, these data suggest loss of coronary autoregulation during perfusion.


A
cTnI
ng/mL
60
40
20
0
T0
T1
T3
T5
*
**
##
##
B
Hyaluronan
ng/mL
800
600
400
200
0
T0
T1
T3
T5
*
**
##
#
C
VCAM-1
µg/L
130
120
110
100
90
80
0
T0
T1
T3
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**
##
D
Von Willebrand Factor
ng/mL
200
150
100
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##
E
Endothelin
pg/mL
120
80
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1.5
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ETAR
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2.0
1.5
1.0
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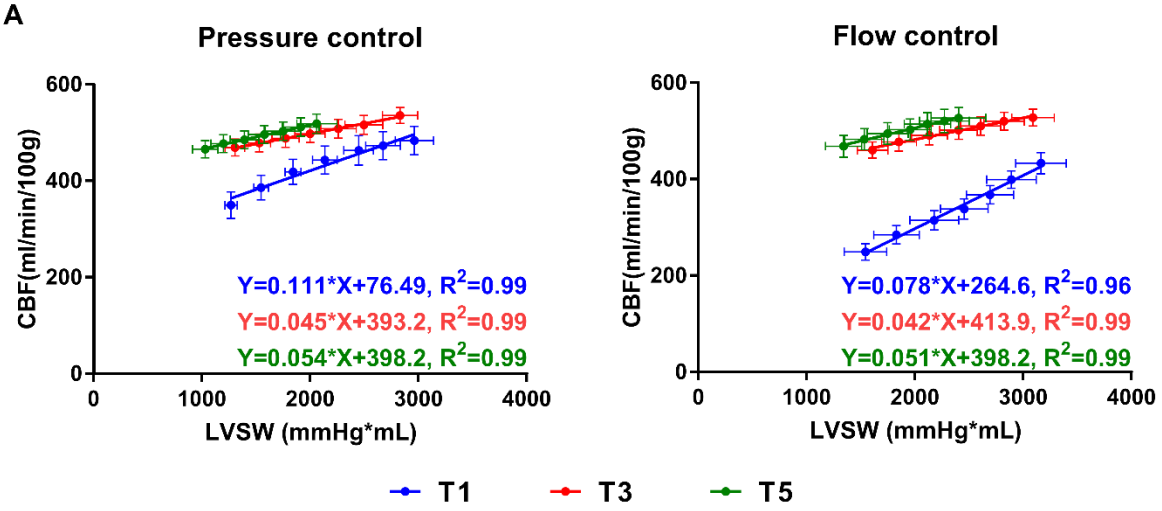
*
ETBR
AU
1.5
1.0
0.5
0.0
In situ
Pressure control
Flow control

Fig. 2-6 Cardiac and endothelial damage

The flow control group had less myocardial injury(A), endothelial injury (B) and endothelial activation (C) than the pressure control group. The flow control group had significantly less von Willebrand Factor (D). Both groups had a significant increase in endothelin in the perfusate. The flow control group had relatively less ET_AR than the *in situ* control coronary artery tissue (E). No difference was observed in ET_BR between groups (H). There were no significant differences in ET_AR/ET_BR ratio expression between groups. (Comparison between groups, independent t-test, $^{*}p<0.05$, $^{**}p<0.01$; comparison within group, repeated measure ANOVA, $^{\#}p<0.05$, $^{\#\#}p<0.01$, n=6)

A


Pressure control
Flow control
CBF(ml/min/100g)
LVSW (mmHg*mL)
Y=0.111*X+76.49, R2=0.99
Y=0.045*X+393.2, R2=0.99
Y=0.054*X+398.2, R2=0.99
Y=0.078*X+264.6, R2=0.96
Y=0.042*X+413.9, R2=0.99
Y=0.051*X+398.2, R2=0.99
T1
T3
T5

B

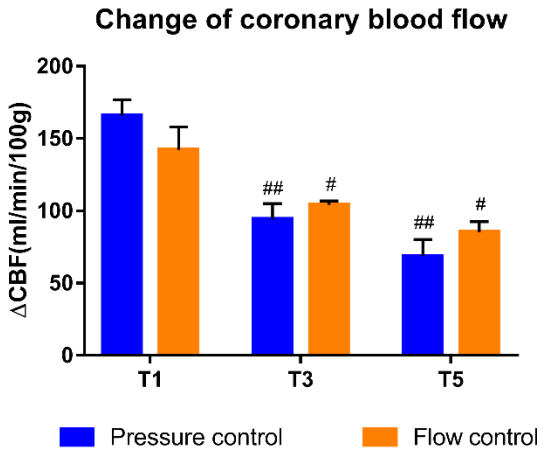

Change of coronary blood flow
ΔCBF(ml/min/100g)
##
#
##
#
T1
T3
T5
Pressure control
Flow control

Fig. 2-7 Correlation for coronary blood flow versus stroke work

Each dot in A and B is a different level of workload induced by increasing of left atrium pressure (LAP) from 6 to 12 mmHg. The corresponding values of left ventricle stroke work (LVSW) are on the X axis and coronary blood flow (CBF) is on the Y axis. Correlation was evaluated and a regression line was generated. The slope (correlation coefficient) of generated linear regression line was higher in T1 than T3 and T5 (A and B). CBF change (C) illustrates the difference in coronary blood flow from the low workload (left atrial pressure=6 mmHg) to the high workload (left atrial pressure=12 mmHg). At later timepoints, each group had a significant decrease in CBF change (C) in response to increased workload. (Comparison within groups, repeated measure ANOVA, $^{\#}p<0.05$, $^{\#\#}p<0.001$, n=6)

3.3.7 Oxidative stress related modification in the coronary arteries

There was a significant increase in oxLDL in the perfusate in both groups, compared with baseline (p<0.01, **Fig. 2-8A**), although it was lower in the flow control group at T5 compared to the pressure control group (76.4±27.0 vs. 143.1±37.5 nmol/mL, p=0.005). The pressure control group had significantly higher MDA in coronary tissue compared to both the *in situ* group and the flow control group (p<0.01), whereas there was a non-significant increase in the flow control group (**Fig. 2-8B**). These data suggest lower lipid peroxidation was induced in the flow control group. Moreover, the pressure control group had significantly higher protein carbonylation compared to both *in situ* group (p=0.0005), and the flow control group (p=0.046, **Fig. 2-8C**).

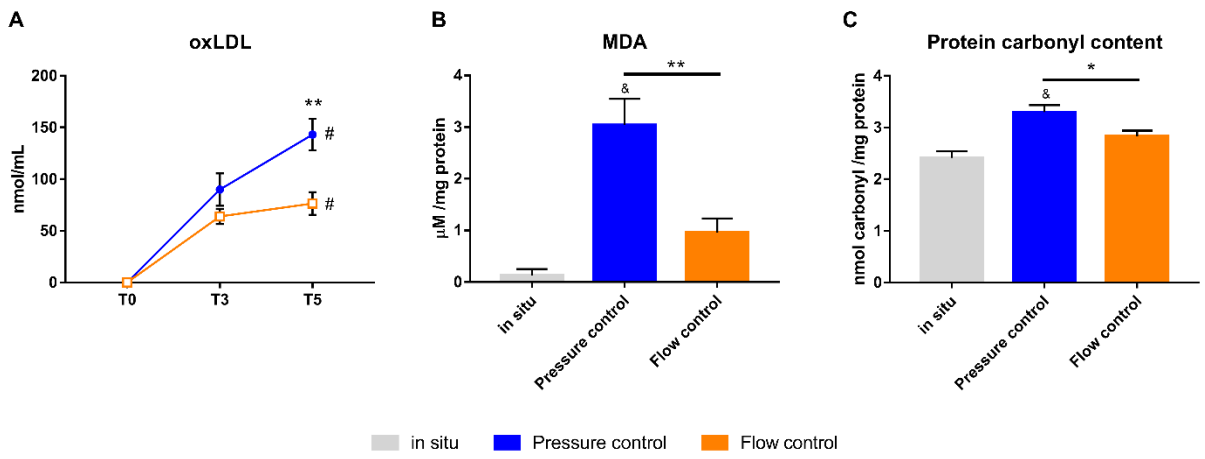


Fig. 2-8 Less oxidative stress and related modification in the flow control group

A, oxLDL (oxidized low-density lipoprotein). B, MDA (Malondialdehyde). C, Protein carbonyl content. The pressure control group had significantly higher lipid peroxidation than the pressure control group (A and B) and higher oxidative stress related modification of proteins in the coronary arteries (C). (Comparison between groups, independent t-test, pressure vs. flow group: $^{*}p<0.05$, $^{**}p<0.01$; Pressure group vs. *in situ*: $^{\&}p<0.001$; comparison within group, repeated measure ANOVA, $^{\#}p<0.001$, n=6)

3.4 Discussion

In this study, we have shown that controlled coronary flow during working mode ESHP provides superior functional preservation and lower edema formation compared to pressure-controlled coronary perfusion. The most striking finding was the gradual loss of coronary vascular resistance and coronary autoregulation during ESHP irrespective of the two strategies. This study focused on the coronary arteries in order to evaluate the benefits of the controlled coronary flow strategy over the pressure-controlled strategy. We have reported here the potential advantage of using low coronary flow perfusion in the *ex situ* setting, including better ventricular functional preservation and attenuated leukocyte and platelet activation, less myocardial damage and endothelial activation and less oxidative stress in the vasculature. The benefits of utilizing the low flow perfusion strategy in organ preservation has also been suggested before,[197-200] where targeting a low flow perfusion resulted in better function and structure of cardiomyocytes and endothelium.

3.4.1 Controlled coronary blood flow *ex situ* heart perfusion provides superior functional preservation and less edema formation.

In this study, the pressure controlled perfusion was performed with a target diastolic pressure of 40 mmHg based on the physiological arterial pressure of juvenile pigs.[201] This strategy is widely used in ESHP studies. However, in this study, we compared this approach with constant coronary flow perfusion, targeting a coronary flow rate of 500 mL/min, approximately 200 mL/min/100g heart weight. The value 500mL/min flow rate is closer to the physiological value, in which the coronary blood flow accounts for 5 % of cardiac output.[202] To keep this flow rate, the diastolic pressure was reduced to 20mmHg.

In the pressure-controlled strategy, we have observed a gradual increase in CBF, concurrently with decreasing ventricle stroke work during 6 hours of perfusion. CBF increased to over 1400 mL/min, which was almost three times of that in the coronary flow-controlled group. By comparison, we have demonstrated that controlled coronary blood flow perfusion provides superior preservation of cardiac function and less edema formation. Qazi *et. al*[199] demonstrated that higher flow can cause a higher degree of cardiac edema and related diastolic dysfunction, and general impairment of contractility of the heart. Cardiac edema can result from endothelial damage that leads to increased vascular permeability due to the loss of intracellular junctions. Lower cTnI and hyaluronan in the flow control group suggests less cardiac damage and endothelial injury in this group. The accumulation of Hyaluronan in the perfusate suggest endothelial glycocalyx shedding which may lead to increased vessel permeability.[174] The glycocalyx damage could also greatly enhance the adherence of leukocytes to the endothelium.[203] Plasma hyaluronan level has also been proposed as a biomarker of myocardial damage.[204] In our study, the flow control group had lower hyaluronan and correspondingly less edema formation.

3.4.2 Leukocyte, platelet and endothelial activation

Leukocyte, platelet and endothelial activation process are interrelated.[155, 156] The endothelial lining of the coronary vasculature forms the physical barrier between the blood and underlying myocardial tissues. The vasculature is also pivotal for a range of other homeostatic functions relating to the circulation such as hemostasis, lipid transport, and immune surveillance.[157] During ESHP the coronary vasculature resides in directly contacting with the circulating mediators in the perfusate, so it is prone to the direct attack. We have reported here that leukocyte, platelet and endothelial activation were all

significantly lower in the flow control group than the pressure control group. The up-regulation of the expression of adhesion molecules in the vascular endothelium is the initiating event and allows leukocytes and monocytes to adhere to the endothelial cell surface.[205] Leukocytes and monocytes penetrate into the sub-endothelial environment, where TNF-α, IL-6 and other cytokines are released, resulting in recruitment of additional circulating cells. The membrane-spanning protein CD40 is also up-regulated in leukocyte and endothelial activation[168, 169] and, after engagement with its natural ligand CD40L, amplify these events by further promoting cytokines release and adhesion of circulating cells to the endothelium.[170] VWF plays a pivotal role in platelet adhesion and aggregation at sites of high shear rates.[206, 207] Plasma levels of VWF are increased in different states of endothelial damage and have been proposed as markers of endothelial dysfunction.[208] Our results indicated that lower coronary flow reduces leukocyte and platelet activation and therefore may maintain a healthier regulation of the vasculature and better endothelial protection during ESHP.

3.4.3 Potential mechanism of decreased coronary vascular resistance and loss of coronary autoregulation

The decreasing LVSW alongside increasing CBF indicates uncoupling between cardiac function and myocardial perfusion. At the initiation of working mode perfusion, as expected, CBF increased appropriately with increased LVSW, consistent with normal coronary autoregulation. However, over extended perfusion, LVSW and CBF trended in opposite directions: while LVSW decreased over 5 hours of working mode perfusion, CBF increased continuously. The uncoupling of these two parameters indicates the loss of coronary autoregulation during perfusion. Here we showed that with the preload

challenge at T1, T3 and T5, there was further increase of CBF together with increasing of the LVSW, suggesting that the coronary autoregulation phenomenon was not completely lost. However, the LVSW-related changes of CBF at T3 and T5 were lower than that at T1. These observations suggest that at six hours of ESHP, CBF may approach its maximum capacity, and that coronary artery vasomotor response to increased myocardial demand was decreasing. The exact mechanism underlying these observations remains unclear. However, we evaluated inflammation as well as oxidative stress state in the coronary artery tissue. ROS and RNS play a role in altered vascular reactivity and breakdown of the vascular barrier and promote cellular injury during various pathophysiological events.[156] For example, ROS can cause fragmentation of the glycocalyx.[209, 210] We have evaluated ROS in the perfusate, as well as in the coronary vasculature. The flow control group had a relatively lower level of oxLDL in the perfusate, and MDA in the vasculature, suggesting less oxidative stress and lipid peroxidation in this group. There was also lower protein carbonyl content in the flow control group, indicating less ROS modification on the protein.

Our results suggest that the oxidative stress environment of the perfusate may play an important part in the activation of the coronary endothelial cells and facilitate protein modification, affecting normal endothelial function, leading to coronary artery vasodilation, increased permeability, and loss of coronary autoregulation. Furthermore, increased flow in the coronary arteries may increase the shear-stress on the vascular wall, which may facilitate more ROS production in the vasculature as a feed-forward mechanism,[136, 205] leading to supraphysiological dilation of the vessel through flow-mediated mechanism.[211-213] Leukocytes are another potential source of ROS during ESHP. We have previously

reported the infiltration of neutrophils in the myocardium.[214] Here we have shown that controlled coronary flow leads to lower MPO activity. This may potentially contribute to the lower level of oxidative stress in this group. It is also known that the mitochondrial respiratory chain is a significant source of ROS in the myocardium. Thus, myocardial ROS production may be the predominant source of oxidative stress during ESHP, leading to coronary vascular dysfunction in addition to myocardial contractile dysfunction.

3.5 Conclusion

Herein we have demonstrated that coronary flow-controlled perfusion during ESHP is superior compared to pressure control with respect to myocardial contractile function and edema formation. Flow-controlled ESHP is associated with lower myocardial tissue injury and leukocyte, platelet and endothelial activation as well as lower induction of oxidative stress. The loss of coronary autoregulation and physiologic coronary vascular resistance during normothermic ESHP may be attributed to the oxidative stress state developed in the coronary vasculature and may be at least partially prevented with flow-controlled perfusion.

Supplementation

Donor heart procurement and preparation for *ex situ* heart perfusion

Animals were intubated and ventilated, and hemodynamics were monitored invasively using a micro-tip pressure transducer. A heat mat was used to maintain body temperature between 36.5 °C and 37.5 °C. The porcine hearts were procured and prepared, as described before in detail. Briefly, a standard median sternotomy was performed under general anesthesia, and the heart was exposed. After intravenous delivery of heparin (1000 U/kg) and Ringer's lactate solution (1000 mL), The right atrial appendage was cannulated using a 34 F venous cannula, thereby allowing approximately 800 ml of donor whole blood to prime the ESHP circuit. 500 ml blood cardioplegia (20 mmol/l [K^+], one part blood and four parts crystalloid) was administered to induce electromechanical quiescence. Pig was then exsanguinated, and the heart was excised, weighed, and were immediately mounted on a custom ESHP apparatus and perfused for 6 hours (n=12). Five freshly procured, unmanipulated hearts were obtained to serve as normal control.

Perfusion apparatus for ESHP

The perfusion apparatus shown in Figure 1B has been described in detail previously and was comprised of a circuit containing perfusate reservoir, two centrifugal pumps (International Biophysics, TX, USA), a membrane oxygenator and an arterial line filter (Sorin Group, Italy). Other parts including the perfusion-controlling and data-collecting software were custom designed and manufactured.

***Ex situ* heart perfusion and sample collection:**

A combination (1:1) of whole donor blood and modified Krebs-Henseleit solution containing 8% albumin was used for the perfusate. The ESHP apparatus was constructed as previously described. [140] The custom-designed software used here controlled the pump speed to maintain the desired aortic (Ao) and left atrial (LA) pressures during the perfusion, enabling preservation and evaluation in different conditions of Langendorff mode and working mode. Perfusion was initiated in Langendorff mode with isolated aortic root perfusion. The transition from empty beating non-working state into physiologic working mode was achieved with an automatic increase of LA pump RPMs upon changing the desired LA pressure of 0 mmHg, to 6 mmHg. During the perfusion interval, the coronary sinus blood flow and LA flow were recorded with a TS410 Tubing Flow Module and flow meters (Transonic System Inc., NY, US). The perfusate was continuously infused with glucose (1 gram/hour), insulin (2 units/hour), and dobutamine (0.04 micrograms/minute) for metabolic and inotropic support of the heart during perfusion. Perfusate samples were collected from the aortic root at the baseline (T0), and then hour-1, 3, 5 (T1, T3, T5) after initiation of ESHP and the serum were frozen at -80°C immediately for further analysis. Fresh blood was analyzed using a Radiometer ABL 800 analyzer (Radiometer, Copenhagen, Denmark). Hemoglobin, hematocrit, electrolytes, metabolites, acid-base, and blood gases were measured and group compared. pH: 7.35-7.45, and arterial partial pressure of oxygen (PaO_2): 100-150 mmHg was maintained during the perfusion. Blood counting was done using an hematology analyzer (ADVIA 2120i Hematology System, Siemens, Germany). At the end of ESHP, samples were taken from the anterolateral wall of the left ventricle as well as left and right epicardial coronary

arteries, were immediately snap frozen in liquid nitrogen and stored at -80 °C for later assessments.

Functional and metabolic analyses

The ESHP software program collected atrial, ventricular and arterial pressure in a real-time pattern, and calculated the ventricular stroke volume (SV) and stroke work (SW). The SV was calculated as follows: SV (mL/beat) = atrial flow(mL/min) / heart rate (beats/min). The SW (mmHg*ml) was calculated as the product of LV developed pressure (mmHg): [mean arterial pressure (mmHg) – left atrial pressure (mmHg)] × SV. The cardiac index (CI) was determined by measuring the flow through the left atrial line divided by the heart weight ($mL \cdot minute^{-1} \cdot gram^{-1}$). To assess maximum ($dP/dt_{max}$) and minimum ($dP/dt_{min}$) rates of pressure change in the left ventricle, a 5F pigtail catheter was placed in the left ventricle through an introducer sheath placed in the subclavian artery with an orifice on the aorta arch. Edema formation was calculated as the weight gained by the myocardial tissue at the end of perfusion.

Indexed coronary vascular resistance was calculated as follows:

CVR (mmHg*min/liter/100g) = diastolic pressure (mmHg)/coronary blood flow/100g heart weight.

Myocardial oxygen consumption (MVO_2) ($mL\ O_2 \cdot min^{-1} \cdot 100\ g^{-1}$) will be determined by multiplying the coronary blood flow (CBF) by the arterial-venous difference in oxygen content ($CaO_2 – CvO_2$) as below:

$MVO_2 = [CaO_2 - CvO_2\ (mL\ O_2 \cdot 100\ mL^{-1})] \times CBF\ (ml \cdot min^{-1} \cdot 100\ g\ heart\ mass)$, where:

Arterial oxygen content (CaO_2) = [1.34 (mL O_2 · g Hb^{-1}) × Hb concentration (g · 100 mL^{-1}) × oxygen saturation (%)] + [0.00289 (mL O_2 · mm Hg^{-1} · 100 mL-1) × PaO_2 (mm Hg)]

Venous oxygen content (CvO_2) = [1.34 (mL O_2 · g Hb^{-1}) × Hb concentration (g · 100mL^{-1}) × oxygen saturation (%)] + [0.00289 (mL O_2 · mm Hg-1 · 100 mL-1) × PvO_2 (mm Hg)]

Cell-free hemoglobin analysis

Perfusate samples were centrifuged at 2200g for 10 minutes in 4 °C and a 40 µL aliquot was carefully removed and diluted in 1 mL of a modified Drabkin's reagent (potassium ferricyanide 0.61 mM, potassium cyanide 0.77 mM, potassium dihydrogen phosphate 1.03 mM). The sample was vortexed and incubated at room temperature in the dark for 5 min. A 200 µL aliquot was pipetted into the microplate and absorbance was measured spectrophotometrically at 540 nm using a SpectraMax Plus 384 (Molecular Devices, CA, USA). Hemolysis is calculated as the ratio of supernatant to total hemoglobin with hematocrit adjustment for the volume of supernatant in the sample.

Coronary artery tissue sample preparation

Left and right coronary artery tissue were rapidly dissected out (free of epicardial fat and myocardium) and were snap frozen in liquid nitrogen and stored at −80 °C for later analyses.

Protein preparation and Western blotting assays

In the 24-hr preservation study, grafts were harvested at the endpoint of preservation without reperfusion and then homogenized in RIPA buffer (Abcam, Cambridge, UK) containing Protease Inhibitor Cocktail and Phosphatase Inhibitor Cocktail 2 (Sigma-Aldrich). Total proteins (40 µg) were resolved on 4-15% SDS-PAGE and then transferred to a PVDF membrane (Bio-Rad Laboratories, UK). The membrane was blocked with 5%

bovine serum albumin (BSA) for 1 hr, and then incubated with the primary antibodies diluted at 1:1000 overnight at 4 °C. The following antibodies were used in this test as primary antibodies: ETAR, ETBR (Santa Cruz Biotechnology, Texas, USA), and β-actin (Cell Signaling, Danvers, MA, USA). After being washed, the membrane was incubated in the secondary antibody, HRP-linked anti-rabbit IgG (Cell Signaling, Danvers, MA, USA) (1:1000 dilution) for 1 hr at room temperature. The membrane was then incubated with ECL Plus (GE Healthcare, UK) for 1 min, and was assessed using an ImageQuant LAS 4000 (Bio-Rad Laboratories, UK).

Myeloperoxidase (MPO) activity assay

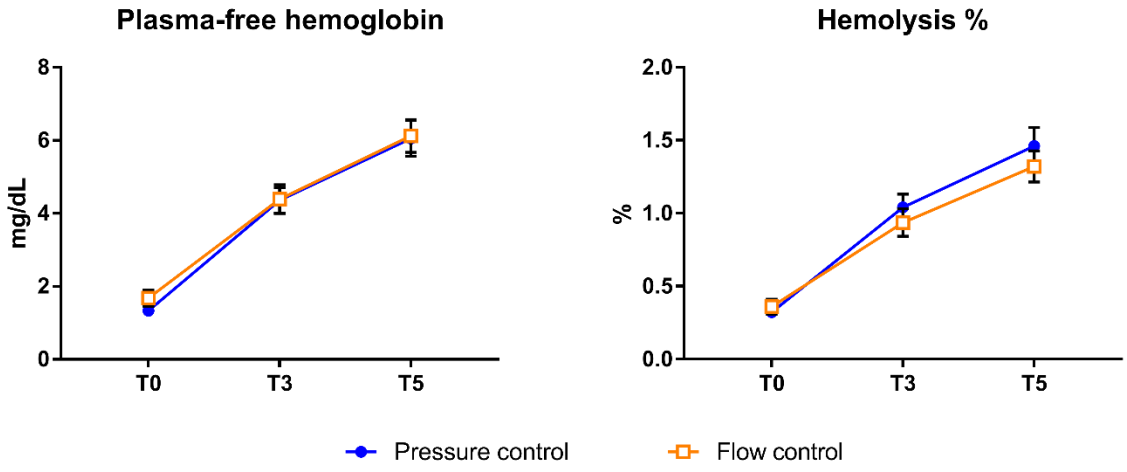
To study the activity of myeloperoxidase (MPO), a peroxidase enzyme most abundant in neutrophil granulocytes, coronary arteries tissue was isolated free from myocardium at the end of perfusion. Small tissue sections (>10mg) were immediately snap frozen in liquid nitrogen and stored at -80 °C. MPO activity assay was conducted according to the manufacturer's instruction (Biovision, CA, USA) A. The reaction was carried out in a 96-well plate, and the samples were read in a fluorescence microplate reader (BioTek Instruments, Inc. Vermont, USA) at 400 nm excitation and 505 nm emission. The results were expressed in relative fluorescent units (RFU). The levels were corrected for the amount of protein (Bradford assay, Bio-Rad, Hercules, USA).

Statistical analysis

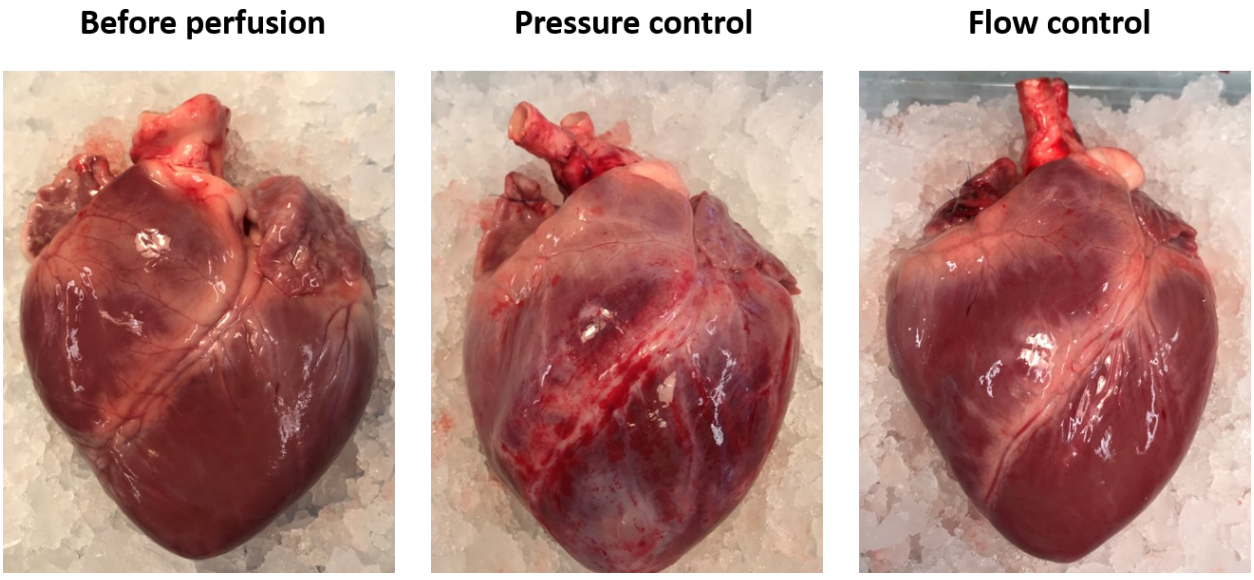
The SPSS 25.0, (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The data (mean ± standard error of the mean) were compared using the independent sample t-test or analysis of variance (ANOVA). The Welch analysis was used when the assumption of equal variance was not met. The parameters were analyzed using a

repeated-measures ANOVA to identify the trends over time within each group. Data regarding the relationship between coronary blood flow and left ventricle stroke work were analyzed by linear regression and analysis of covariance (ANCOVA). A p-value < 0.05 was considered statistically significant. Spearman correlation analyses were used to determine the correlation between LVSW and CBF.

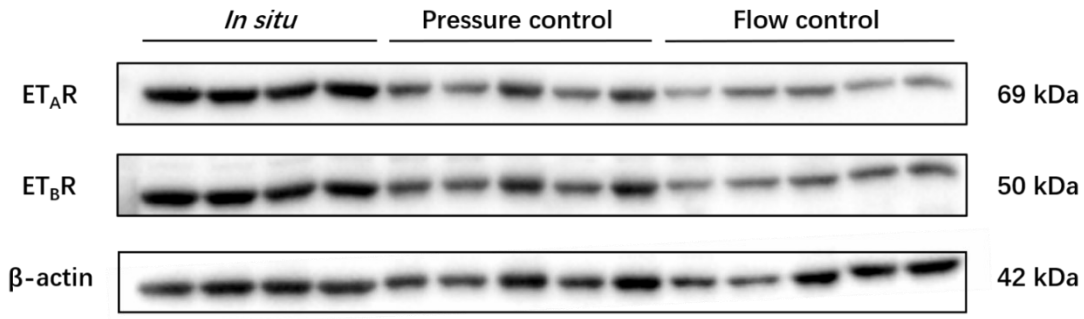
Supplementary figures



Supplemental Fig. 2-1: Red blood cell hemolysis in the perfusate



Supplemental Fig. 2-2: Before and after perfusion appearance



Supplemental Fig. 2-3: Endothelin receptor A (ETAR) and Endothelin receptor B (ETBR) in the coronary vasculature

Chapter Four: The Pleiotropic Effects of Oxygen-derived Free-radical Scavengers on the Graft During Normothermic Ex Situ Heart Perfusion

This chapter is in preparation for submission to a peer-reviewed journal

Abstract

Background:

Oxidative stress is a pivotal aspect during ESHP. The lack of antioxidant enzymes would promote the accumulation of reactive oxygen species (ROS) and negatively affect the graft and functional recovery. We aim to evaluate the use of oxygen-derived free-radical scavengers on cardiac function preservation and the antioxidative stress effect during ESHP.

Methods:

Using a porcine heart perfusion model, we compared the effect of different oxygen-derived free radical scavengers on the graft. Hearts were perfused in working mode for 6 hours either in control group (n=6) or adding oxygen derived free radical scavengers polyethylene glycol (PEG)-catalase (10,000U/L, n=6) and PEG-superoxide dismutase (SOD) (550U/L, n=6) at the initiation of perfusion. Contractile function, myocardial injury and endothelial integrity were determined. ROS was directly detected in LV biopsies. The oxidative modification was determined in the myocardium and coronary vasculature.

Results:

Cardiac function and endothelial integrity were better preserved in the catalase group ($p<0.05$). Catalase and SOD both decreased myocardial injury. Compared to SOD, catalase reduced ROS production and oxidatively modified protein in the myocardium and coronary vasculature. The antioxidant enzymes activity decreased among groups. However, adding catalase and SOD can restore the enzyme activity, respectively.

Conclusions:

The antioxidant enzyme activity decreased in the *ex situ* perfused heart. Catalase better preserved cardiac function and coronary endothelium than SOD, likely resulting from distinct oxidative stress states in the graft. Catalase and SOD play the opposite direction in hydrogen peroxide detoxification, which may create a pleiotropic effect on the graft preservation during ESHP.

4.1 Introduction

An insufficient number of donor organs currently limits heart transplantation (HTx).[215] The ability to prolong the safe storage of donor organs would greatly expand the donor pool and increase the number of cardiac allografts available for transplantation. In addition, improved methods of graft preservation may enhance functional recovery after heart transplantation and reduce operative morbidity and mortality rates.[216, 217] *Ex situ* heart perfusion (ESHP) allows functional and metabolic resuscitation of donor hearts before transplantation, thus potentially expanding the donor pool. Also, prolonged ex situ perfusion is desirable for pretransplant graft evaluation and therapeutic interventions such as gene and cell-based therapies, thus potentially increasing donor organ quality.[151] Although there has been significant progress on ESHP, many questions remain on the optimal method for organ perfusion and preservation.

Ex situ perfused heart loses vasomotor tone and fails in a few hours, which may be partially ascribed to lost endothelial integrity and function.[218] Several reports have indicated that oxidative stress may play a part.[219, 220] Oxidative stress is a pivotal aspect during isolated organ perfusion,[221] especially in the setting of donation after circulatory death (DCD).[218] Oxygen-derived free radicals and other cytotoxic substances are released by activated neutrophils, resulting in graft injury[222], while myocardial metabolism necessary for maintenance of contractile function also generates considerable reactive oxygen species (ROS). For example, the interaction of oxygen-derived free radicals with lipids and other proteins in the cell membrane may contribute to myocardial or endothelial damage during heart preservation.[69, 223]

Because of the potential of reactive oxygen species to induce significant biological damage, cells and tissues have an abundance of antioxidant systems to scavenge or otherwise eliminate them. These include antioxidant enzymes such as catalase[224], superoxide dismutase (SOD), and glutathione peroxidase (GPx)[225] as well as low-molecular-weight compounds such as vitamins C and E[226] and reduced glutathione (GSH). However, these defense systems may be overwhelmed during ESHP since the heart lacks support from other organs. We hypothesized that applying oxygen-derived free radical scavengers during ESHP would control ROS, helping to preserve graft function. The objective of the present study was to evaluate the use of oxygen-derived free-radical scavengers on cardiac functional preservation andoxidative stress effect during ESHP.

4.2 Material and methods

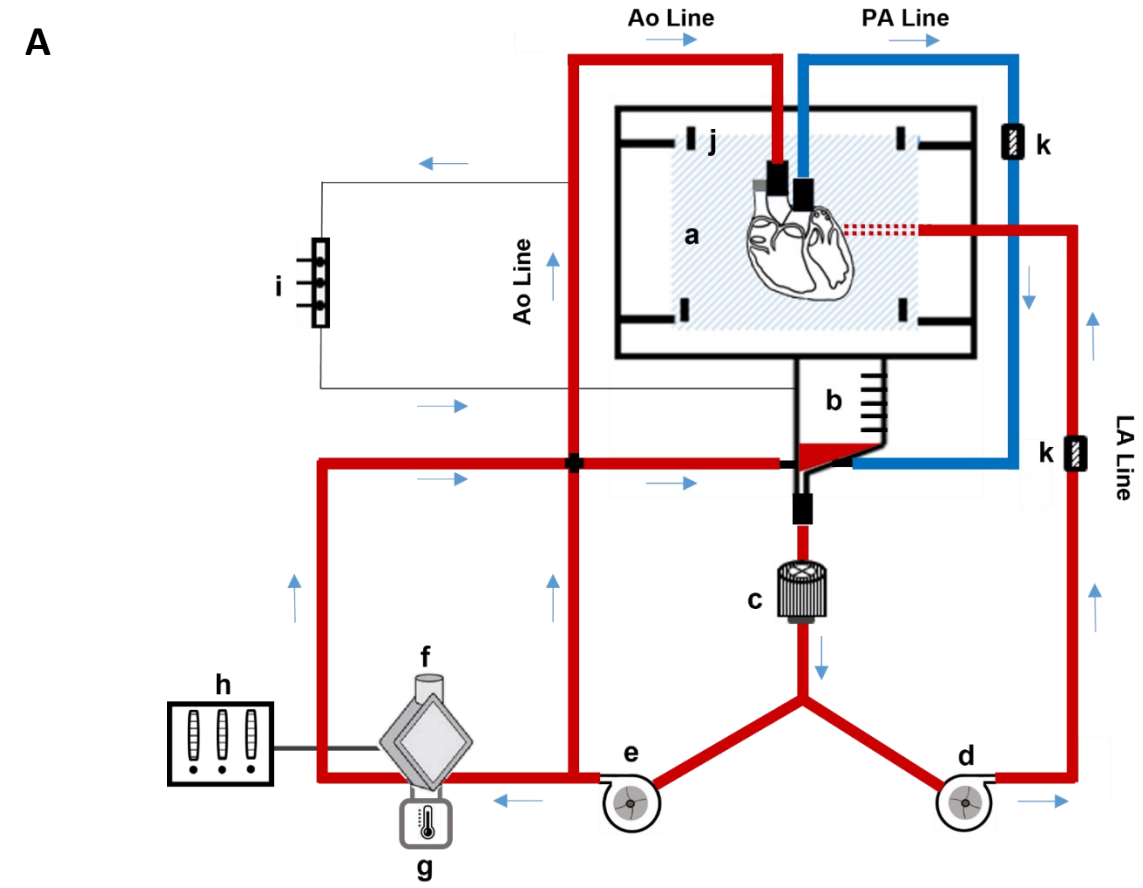
Animal care:

All animals used in this study received humane care in compliance with the Canadian Council on Animal Care guidelines. The study protocol was approved by the Institutional Animal Care and Use Committee of the University of Alberta. Female domestic pigs (n=24) weighing 45-55 kg were used as heart and blood donors.

Ex situ heart perfusion and sample collection:

The ESHP apparatus was constructed as illustrated in **Figure 3-1A**, and perfusion was conducted as previously described.[146] Following cardioplegic arrest in the donor animal, hearts were mounted on the ESHP system. 1.6 liters of combination (1:1) of autologous whole blood (800 mL) and modified Krebs-Henseleit solution (800 mL) containing 8% albumin were used for the perfusate to achieve a hematocrit of 15 % for the circuit priming. Perfusion was initiated in Langendorff mode for 1 hour, during which the perfusate was warmed from room temperature to 39. Working mode was initiated by raising left atrial pressure to 6 mmHg, then maintained for 5 hours of perfusion. All hearts were paced with atrial wires at 100 beats/minute. In addition, Dobutamine (4 mcg/min) and Insulin (5 units/h) were infused continuously throughout the experiment. A graphical description of the experimental overview is provided in **Figure 3-1B**. The detailed methods are included in the Supplementary Material.

A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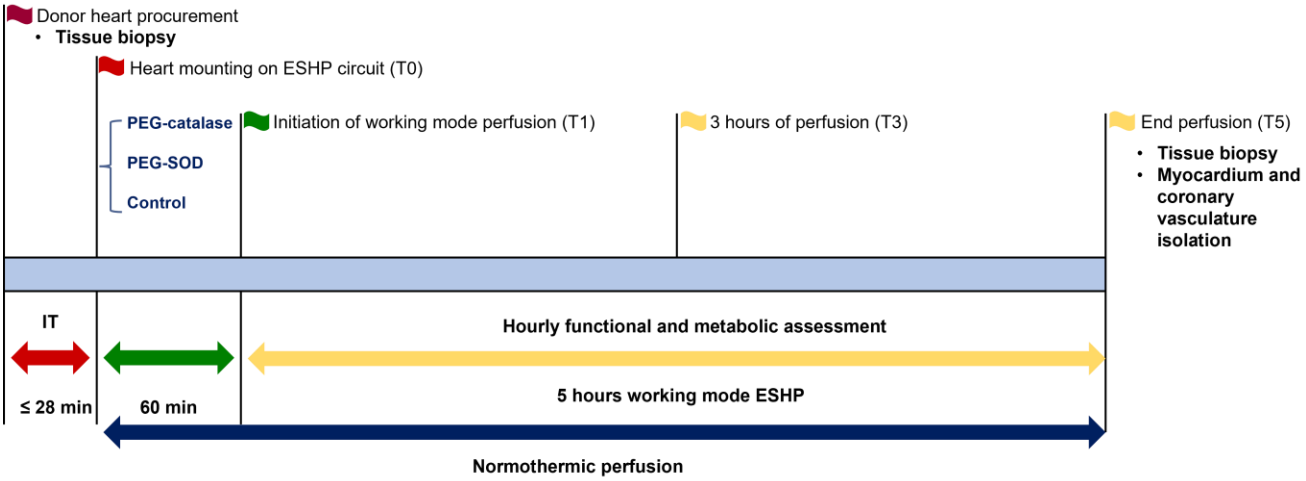

Donor heart procurement
• Tissue biopsy
Heart mounting on ESHP circuit (T0)
PEG-catalase
PEG-SOD
Control
Initiation of working mode perfusion (T1)
3 hours of perfusion (T3)
End perfusion (T5)
• Tissue biopsy
• Myocardium and coronary vasculature isolation
IT
≤ 28 min
60 min
Hourly functional and metabolic assessment
5 hours working mode ESHP
Normothermic perfusion

Fig. 3-1 Schematic view of the perfusion system and experimental timeline

A, custom ESHP circuit. LA line connects one centrifugal pump and left atrium, providing the preload and Ao line connects another pump and the aorta, providing the afterload. PA line collects the effluent of the pulmonary artery to the reservoir. a, supportive membrane; b, reservoir; c, arterial filter; d and e, centrifugal pumps; f, oxygenator; g, heater; h, gas line; i, drug infusion line; k, flow probes. B, experimental timeline. Hearts were procured and, after a brief period of ischemic time to allow mounting on the ESHP apparatus, were perfused in Langendorff mode for 1 hour and 5 hours of working mode normothermic perfusion. Hearts were allocated to either of the following three groups (n=6 each): (1) control group, [20] Catalase group, [148] SOD group. The different drugs were delivered through the coronary artery before the initiation of perfusion. Ao, aorta; PA, pulmonary artery; LA, left atrium; ESHP, Ex situ heart perfusion; IT, ischemic time.

Experimental groups:

Donor animals were randomly assigned to the following groups:

Catalase group (n=6): Before the initiation of perfusion, polyethylene glycol-conjugated catalase (PEG-catalase) was administrated in 1.6 liters of perfusate, final concentration 10,000U/L.

SOD group (n=6): PEG-SOD was administrated in the perfusate before the initiation of perfusion, with final concentration of 550U/L.

Control group (n=6): 3 mL of saline was delivered in the perfusate as the control group.

In situ tissue samples (n = 6): freshly procured, unmanipulated hearts were obtained to serve as in situ control. Left ventricle myocardium, Left and right coronary artery tissues were isolated and stored immediately at -80 °C for subsequent analysis.

Drugs: The following drugs were used in this study: PEG-catalase and PEG-SOD (Signa-Aldrich, Inc., MI, USA). All drugs were dissolved in normal saline before administration.

Functional and metabolic assessment of the *ex situ* perfused heart

Myocardial function parameters were assessed at 1h (T1), 3h (T3), and 5h (T5) after initiation of working mode as previously described [147] (LA pressure = 6 mmHg). dP/dt_{max} and dP/dt_{min} were acquired through a pigtail catheter (5F) placed in the left ventricle via the subclavian branch of the aortic arch. The cardiac index (CI) was determined by measuring the flow through the left atrial line divided by the heart weight ($mL \cdot minute^{-1} \cdot gram^{-1}$). Arterial and coronary sinus blood samples were assayed for the partial pressure of oxygen (PO_2), carbon dioxide (PCO_2), pH, hemoglobin concentration [227], oxygen saturation (SaO_2), electrolyte, glucose, and lactate using a blood gas analyzer

(ABL 800 analyzer, Radiometer, Copenhagen, Denmark). The SVC and IVC were ligated, and coronary blood flow (CBF) were monitored by a Doppler flowmeter (Transonic System Inc., NY, USA) on the pulmonary artery cannula that was collecting effluent from the coronary sinus. The detailed methods of functional and metabolic evaluation are included in the Supplementary Material.

Myocardial and vascular injury

Perfusate concentration of cTnI was measured as a marker of myocardial injury using ELISA (FineTest, Wuhan, China). Perfusate hyaluronan, Syndecan-1, thrombomodulin, and vWF were measured by ELISA according to the manufacturer's instructions (R & D System Inc., MN, USA; FineTest, Wuhan, China).

Live tissue oxidative stress detecting in LV biopsies

3-5 mm length needle biopsies in the apex of the heart were acquired before heart procurement (*in situ* baseline), and 5 hours of perfusion (T5). ROS were measured by staining tissue with CellROX® oxidative stress staining kit (Invitrogen, MA, USA). Detail methods are described in the Supplementary Material.

Detection of oxidative modification and antioxidant activity in the myocardium and coronary artery tissue

The oxidatively modified lipid and protein were evaluated by MDA and protein carbonylation, respectively. Lipid peroxidation was determined by measuring MDA in the myocardium by the thiobarbituric acid colorimetric method according to the manufacture's instruction (R & D System Inc., MN, USA). Total myocardial protein carbonylation was measured using the protein carbonyl content assay kit (Abcam, Cambridge, UK). Nitrotyrosine was determined in the coronary artery tissue by ELISA (MyBioSource Inc.,

CA, USA). Catalase, SOD and GPx activity were determined in the myocardium and coronary artery tissue according to the manufacture's instruction (BioVision Inc., CA, USA). Detailed methods are described in the Supplementary Material.

Statistics

All values were expressed as mean ± standard error of the mean (SEM). The significance of result differences between groups was assessed using a two-way analysis of variance (ANOVA) method for repeated measurements, with additional post hoc tests. Student's t-test was applied for testing differences in continuous variables between the two groups. Values of $P < 0.05$ were considered statistically significant. Analyses were performed using IBM SPSS software version 25 (Chicago, IL, USA).

4.3 Results

4.3.1 Hemodynamic parameters and blood gas analysis

There were no differences in body weight, heart weight, and total ischemic time between the groups (**Table 3-1**). Hemodynamic parameters were comparable between the groups. The diastolic pressure was kept constant at 40 mmHg during perfusion. SOD group had a significant decline of ventricle systolic pressure at T5 compared to T1 (106.5±4.1 vs. 119.2±13.6 mmHg, $p=0.0001$). Both groups had decreased cardiac index at T5 compared to T1 (catalase group, $p<0.05$; control group, $p<0.01$, SOD group, $p=0.0001$). The blood gas analysis indicated that there was a trend of decreasing perfusate lactate levels in all groups. The difference had been observed in T3 and T5 compared to T1 in the control group ($p<0.01$); and T3 with T1 in the catalase group ($p<0.05$). Coronary sinus oxygen saturation increased in all groups compared to the baseline ($p=0.0001$). Within-group comparison, the SOD group had a significantly higher venous oxygen saturation than the catalase group at T5 (92.7±1.2% vs. 87.6±1.5%, $p=0.017$).

4.3.2 Functional and metabolic analysis

Heart function declined in all groups, indicated by cardiac index and LVSW (**Figure 3-2A and 3-2B**). Percent change from baseline value shows that left ventricular function was better preserved in the catalase group. On average, there was an initial decrease of the cardiac index to 93.4% in the catalase group, 87.9% in the control group, and 80.9% in the SOD group, between T5 and T1. LVSW declined in all groups with 86.8% in the catalase group, 81.9% in the control group, and 73.7% in the SOD group at T5 compared to baseline. No statistically significant difference in dP/dt_{max} and dP/dt_{min} was observed

between groups. Neither were there statistically significant differences in edema formation. There was an average weight gain of 19.6% in the control group, 17.8% in the SOD group, and 14% in the catalase group.

CBF increased significantly in all the groups compared to the baseline (**Figure 3-3A**, $p<0.0001$). However, CBF was relatively lower at the end of perfusion in the catalase group than the other groups ($p=0.030$ vs. SOD group, $p=0.027$ vs. control group). Similarly, CVR declined during perfusion in all groups (**Figure 3-3B**, $p<0.0001$). At T5, the catalase group had higher CVR than the other two groups ($p=0.002$ vs. SOD control group, $p=0.001$ vs. control group). MVO_2 was relatively higher in the SOD group than the other two groups at the start of perfusion (**Figure 3-3C**, SOD vs. catalase group, $p<0.0001$; SOD vs. control group, $p=0.0024$). In both the SOD and control groups, MVO_2 dropped during perfusion ($p<0.0001$), while there was no statically significant difference in the catalase group. The glucose utilization rate increased in all groups compared to the baseline (**Figure 3-3D**, $p<0.0001$). No statically significant difference has been observed among groups.

Variable	Control group (n=6)	CAT group (n=6)	SOD group (n=6)	P value
Characteristics				
Body weight, kg	46.8 ± 1.1	44.4 ± 1.8	43.3 ± 2.1	0.355
Heart weight, g	228.8 ± 10.9	218.8 ± 10.0	217.8 ± 12.3	0.743
Ischemic time, min	26.7 ± 1.4	25.0 ± 0.8	25.5 ± 0.3	0.462
Ex situ heart perfusion				
Systolic pressure, mmHg				
60 min (T1)	107.3 ± 2.1	106.8 ± 2.9	119.2 ± 13.6	0.063
180 min (T3)	107.3 ± 2.8	108.8 ± 3.7	114. ± 13.4	0.424
300 min (T5)	102.7 ± 3.3	102.2 ± 2.9	106.5 ± 4.1$^{\#\#}$	0.640
Diastolic pressure, mmHg				
60 min (T1)	39.3 ± 0.2	39.8 ± 0.2	39.5 ± 0.3	0.381
180 min (T3)	39.3 ± 0.5	39.2 ± 0.2	39.2 ± 0.3	0.848
300 min (T5)	39.7 ± 0.2	39.8 ± 0.2	39.5 ± 0.3	0.651
Left atrium flow rate, mL/min				
60 min (T1)	2070.0±126.8	2085.0±157.4	2576.7±324.4	0.215
180 min (T3)	2056.6±115.1	2078.3±173.8	2446.7±313.5	0.385
300 min (T5)	1858.3±133.7$^{\#\#}$	1926.7±161.4$^{\#}$	2083.3±285.5$^{\#\#}$	0.732
Blood gas analysis				
Lactate, mmol/L				
60 min (T1)	3.4 ± 0.1	3.0 ± 0.2	2.8 ± 0.3	0.188
180 min (T3)	1.7 ± 0.4$^{\#\#\#}$	2.0 ± 0.5$^{\#}$	2.4 ± 0.4	0.388
300 min (T5)	2.3 ± 0.2$^{\#\#}$	2.7 ± 0.2	2.8 ± 0.3	0.249
Coronary sinus oxygen saturation, %				
60 min (T1)	67.4 ± 2.2 %	66.5 ± 4.2 %	66.3 ± 2.6 %	0.966
180 min (T3)	86.6 ± 0.6 %$^{\#\#\#}$	85.4 ± 1.0 %$^{\#\#\#}$	85.8 ± 0.8 %$^{\#\#\#}$	0.568
300 min (T5)	88.9 ± 0.5 %$^{\#\#\#}$	87.6 ± 1.5 %$^{\#\#\#}$	92.7 ±1.2 %*$^{\#\#\#}$	0.015

Table 3-1: Hemodynamic parameter and blood gases

Data are expressed as mean ± SEM. *p<0.05 for SOD group vs Catalase group; $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p=0.0001 for within group comparison between different time; n=6 each group.

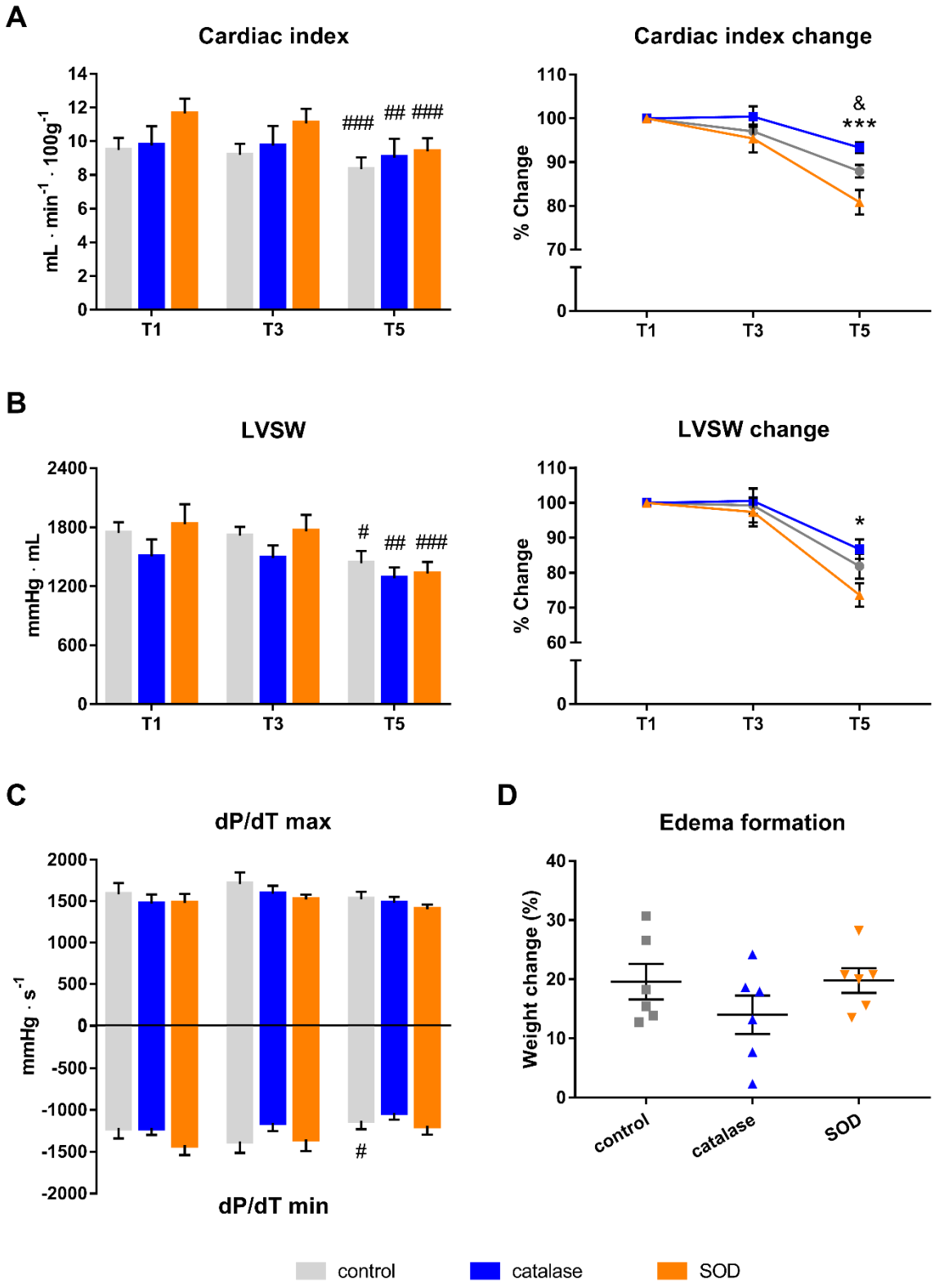

A
Cardiac index
mL · min-1 · 100g-1
14
12
10
8
6
4
2
0
T1
T3
T5
###
Cardiac index change
% Change
110
100
90
80
70
0
T1
T3
T5
& ***
B
LVSW
mmHg · mL
2400
1800
1200
600
0
T1
T3
T5
###
LVSW change
% Change
110
100
90
80
70
60
0
T1
T3
T5
*
C
dP/dT max
mmHg · s-1
2000
1500
1000
500
0
-500
-1000
-1500
-2000
#
dP/dT min
D
Edema formation
Weight change (%)
40
30
20
10
0
control
catalase
SOD
control
catalase
SOD

Fig. 3-2 Functional assessment and edema formation

Left ventricular function was better preserved in the catalase group, indicated by cardiac index (A), left ventricle stroke work (B). There was no statistically significant difference in dP/dt_{max} and dP/dt_{min} between groups (C). No significant difference had been observed in edema formation between groups (D). LVSW, left ventricle stroke work; dP/dt_{max}, maximum rates of left ventricle pressure change; dP/dt_{min}, minimum rates of left ventricle pressure change. (Compared between groups, one-way ANOVA. Catalase group vs. SOD group: *$p<0.05$, ***$p<0.0001$; SOD group vs. control group, $^{\&}p<0.01$. compared within group, repeated measure ANOVA, $^{\#}p<0.05$, $^{\#\#}p<0.001$, $^{\#\#\#}p<0.0001$, n=6 each group)

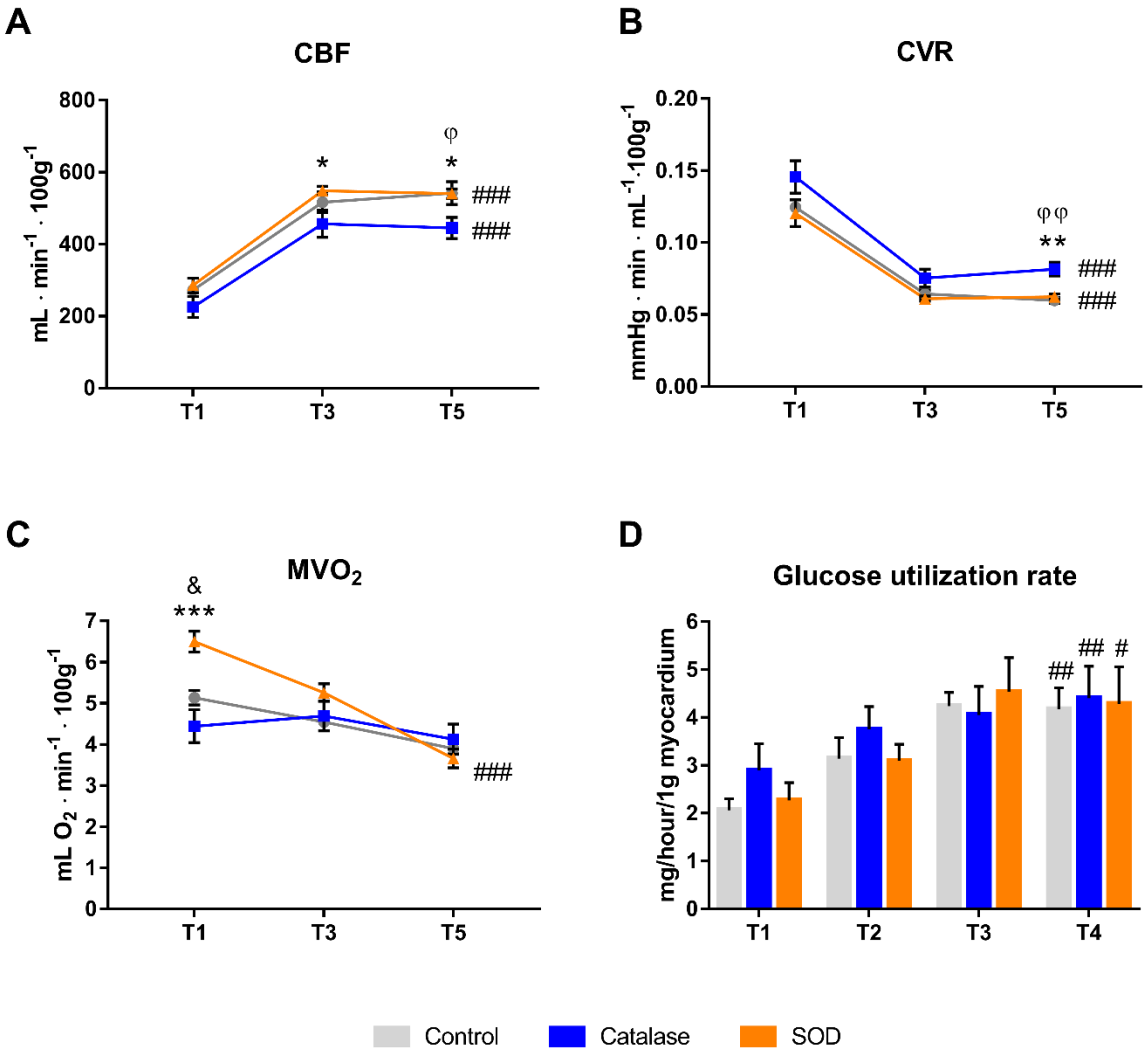

A
CBF
mL · min-1 · 100g-1
800
600
400
200
0
T1
T3
T5
*
φ
*
###
###
B
CVR
mmHg · min · mL-1·100g-1
0.20
0.15
0.10
0.05
0.00
T1
T3
T5
φφ
**
###
###
C
MVO2
mL O2 · min-1 · 100g-1
7
6
5
4
3
2
1
0
T1
T3
T5
&
***
###
D
Glucose utilization rate
mg/hour/1g myocardium
6
5
4
3
2
1
0
T1
T2
T3
T4
##
##
#
Control
Catalase
SOD

Fig. 3-3 Metabolic evaluation of *ex situ* heart perfusion

During 6 hours perfusion, there was a significantly increased CBF over time, with relative lower in the catalase group (A). CVR gradually decreased in both groups, with a relatively higher in the catalase group (B). MVO_2 decreased significantly in the SOD group and the control group. However, no significant different change has been seen in the catalase group (C). CBF, coronary blood flow; CVR, coronary vascular resistance; MVO_2, myocardial oxygen consumption. (Compared between groups, one-way ANOVA, Catalase group vs. SOD group: *p<0.05, **p<0.01, ***p<0.0001; Catalase group vs. control group: $^{\phi}$p<0.05, $^{\phi\phi}$p<0.01; SOD group vs. control group, $^{\&}$p<0.05. compared within group, repeated measure ANOVA, $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.0001, n=6 each group)

4.3.3 Myocardial injury

In all groups, there was a significant increase in perfusate cTnI and CK-MB (**Fig. 3-4A**, $p<0.0001$). Both the catalase group and the SOD group had a statistically significant lower level of perfusate cTnI than the control group at T5 (catalase group: 1105.9±108.6 pg/mL vs. the control group: 1647.7±154.8 pg/mL, $p=0.006$; SOD group: 996.0±85.7 pg/mL vs. the control group, $p=0.002$). There were also significant differences in CK-MB level at T5 between the groups. The SOD group had the lowest level of CK-MB (SOD group: 2.6±0.4 ng/mL vs. catalase group: 3.9±0.4 ng/mL vs. control group: 5.2±0.7ng/mL).

4.3.4 Loss of endothelial integrity and glycocalyx shedding

Perfusate syndecan-1 (CD138) and hyaluronan increased in all groups (**Fig. 3-4B**, $p<0.0001$). The SOD group had a significantly higher level of syndecan-1 than other groups from T3 ($p<0.0001$). At T5, the perfusate level in the SOD group was almost three times higher than that in the catalase group and control group (SOD group: 708.9±70.1 ng/mL vs. catalase group: 253.3±40.9 ng/mL vs. control group: 299.7±38.4 ng/mL). Similarly, perfusate hyaluronan in the SOD group was significantly higher than other groups from T3. In all groups, there was increased perfusate vWF and thrombomodulin ($p<0.0001$), indicating damage of the endothelial layer (**Fig. 3-4C**). However, the catalase group had significantly lower vWF than the other two groups at both T3 and T5. There was no significant difference among groups in perfusate thrombomodulin levels. Taken together, these results suggest less endothelial injury in the catalase group and more damage of endothelial glycocalyx in the SOD group.

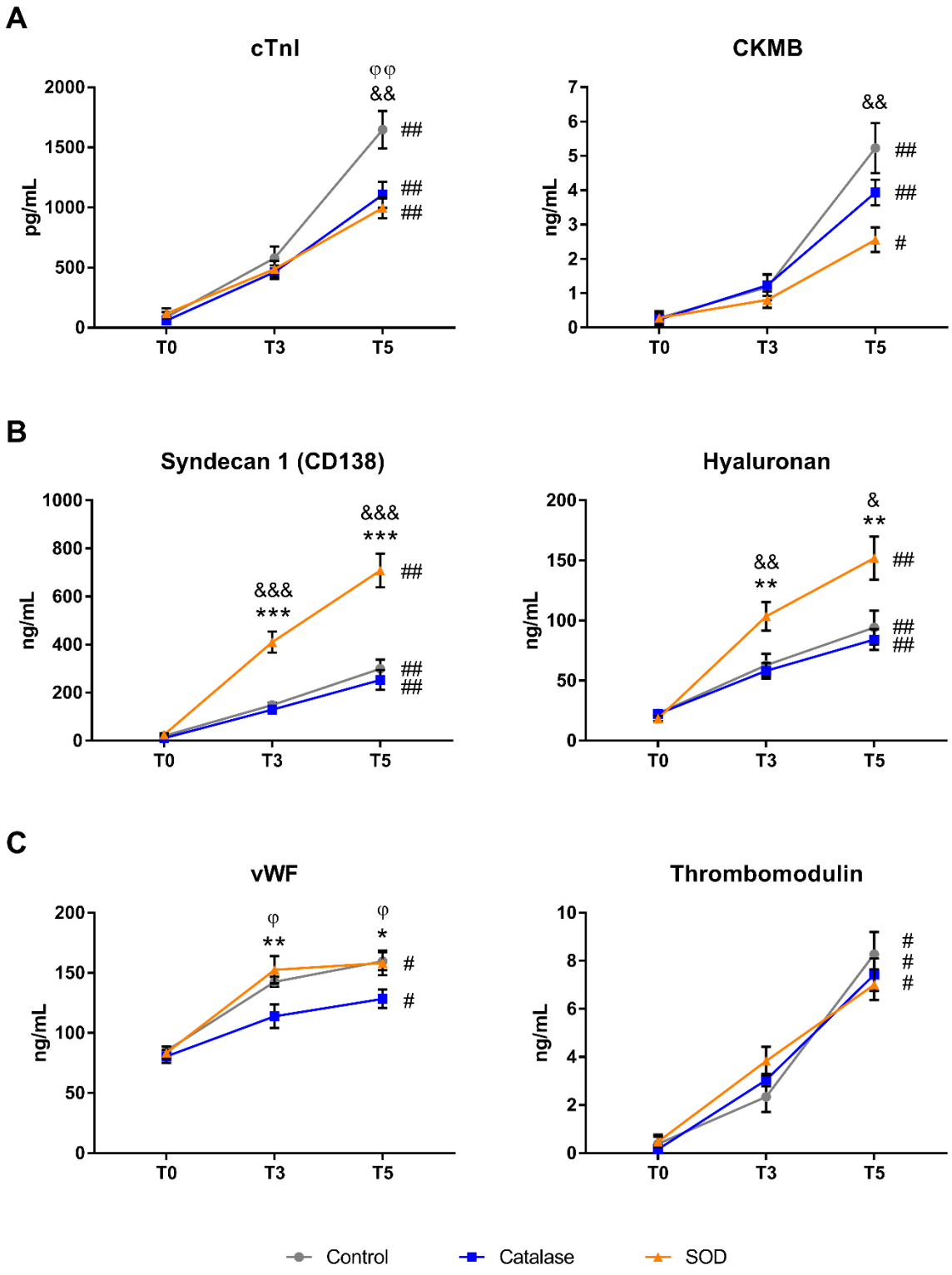

A
cTnI
pg/mL
2000
1500
1000
500
0
T0
T3
T5
φφ
&&
##
##
##
CKMB
ng/mL
7
6
5
4
3
2
1
0
T0
T3
T5
&&
##
##
#
B
Syndecan 1 (CD138)
ng/mL
1000
800
600
400
200
0
T0
T3
T5
&&&
***
&&&
***
##
##
##
Hyaluronan
ng/mL
200
150
100
50
0
T0
T3
T5
&
**
&&
**
##
##
##
C
vWF
ng/mL
200
150
100
50
0
T0
T3
T5
φ
**
φ
*
#
#
Thrombomodulin
ng/mL
10
8
6
4
2
0
T0
T3
T5
#
#
#
Control
Catalase
SOD

Fig. 3-4 Cardiac and endothelial damage

The catalase group and SOD group had less myocardial injury (A). Both groups had a significant increase in perfusate glycocalyx compared to baseline. The SOD group had the highest level of perfusate syndecan-1 and hyaluronan (C). The catalase group had less endothelial injury than other groups (C). cTnI, cardiac troponin I; CK-MB, creatine kinase myocardial band; vWF, von Willebrand Factor. (Comparison between groups, one-way ANOVA, catalase group vs. SOD group: *p<0.05, **p<0.01, ****p*<0.0001; Catalase group vs. control group: $^{\phi}$p<0.05, $^{\phi\phi}$p<0.01; SOD group vs. control group, $^{\&}$p<0.05, $^{\&\&}$p<0.01, $^{\&\&\&}$p<0.0001. compared within group, repeated measure ANOVA, $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.0001, n=6 each group)

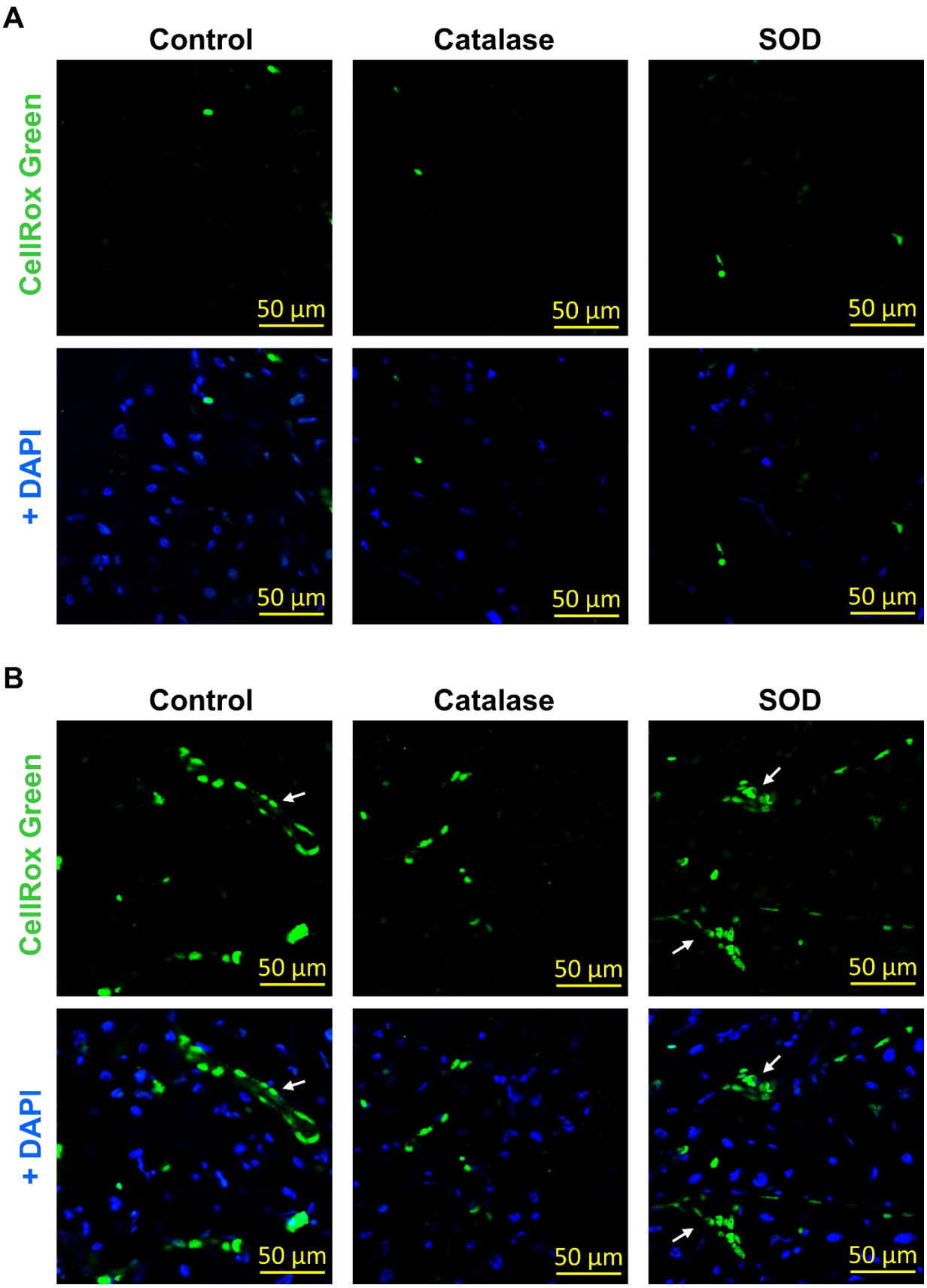
4.3.5 Less oxidative stress and related modification was induced in the catalase group

At baseline (*in situ*), the fluorescence of CellRox green was very low in all groups (**Figure 3-5A**). After 5 hours of perfusion, the green fluorescence had increased significantly in all three groups ($p<0.0001$), indicating increased oxidative stress (**Figure 3-5B**). The catalase group had lower fluorescence than the other two groups at T5 (Catalase group vs. SOD group, $p=0.005$, catalase group vs. the control group, $p<0.0001$, **Figure 3-5C**).

In addition**,** the catalase group had a lower myocardial protein carbonyl content and coronary artery nitrotyrosine content than the SOD group (**Figure 3-6A** and **3-6C**), suggesting less oxidative modification in this group. However, the lipid modification marker MDA was not statistically significantly different among the groups (**Figure 3-6B**).

4.3.6 Antioxidant enzyme activity

There was a significant decrease in the myocardial and coronary artery glutathione peroxidase activity compared to in vivo control (**Figure 3-7A** and **3-7B**). Similarly, each perfusion group had decreased catalase activity in the myocardium (**Figure 3-7C**). However, the catalase activity in the catalase group was significantly higher than both the SOD group and control groups. Similarly, the SOD group had higher SOD activity than the other two perfusion groups (**Figure 3-7D**). There was no significant difference between the SOD group and *in* vivo control.


A
Control
Catalase
SOD
CellRox Green
+ DAPI
50 µm
B
Control
Catalase
SOD
CellRox Green
+ DAPI
50 µm

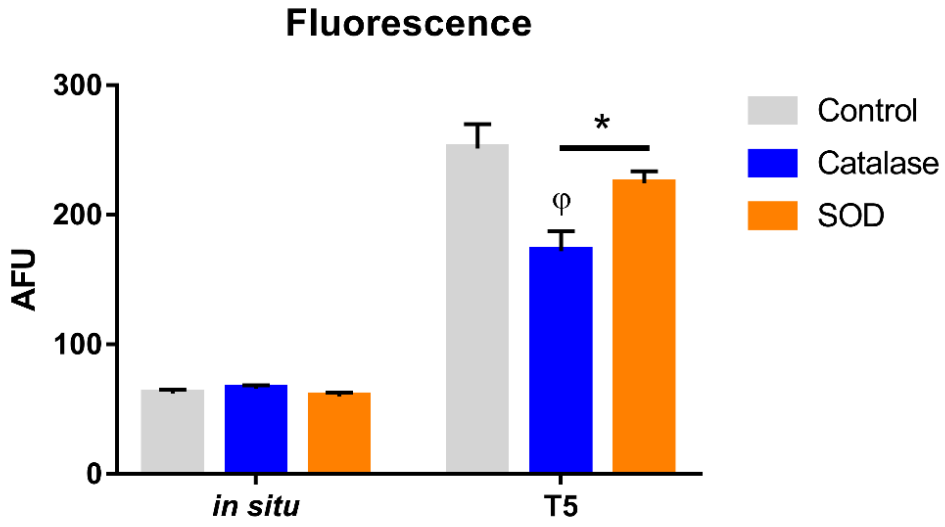


Fig. 3-5 Live tissue oxidative stress detecting

At the baseline (*in situ*), the green fluorescence was very low in all the groups (A). After 5 hours of perfusion, the green fluorescence had increased significantly in all three groups ($p<0.0001$), indicating the increase of oxidative stress (B). The catalase group had lower CellRox green fluorescence than the other two groups at T5. In both the control group and SOD group, the localization of fluorescence was in the vasculature area (white arrow). AFU, absolute fluorescence units. (Comparison between groups, one-way ANOVA, Catalase group vs. SOD group: $*p<0.01$; Catalase group vs. control group: $^{\phi}p<0.0001$)

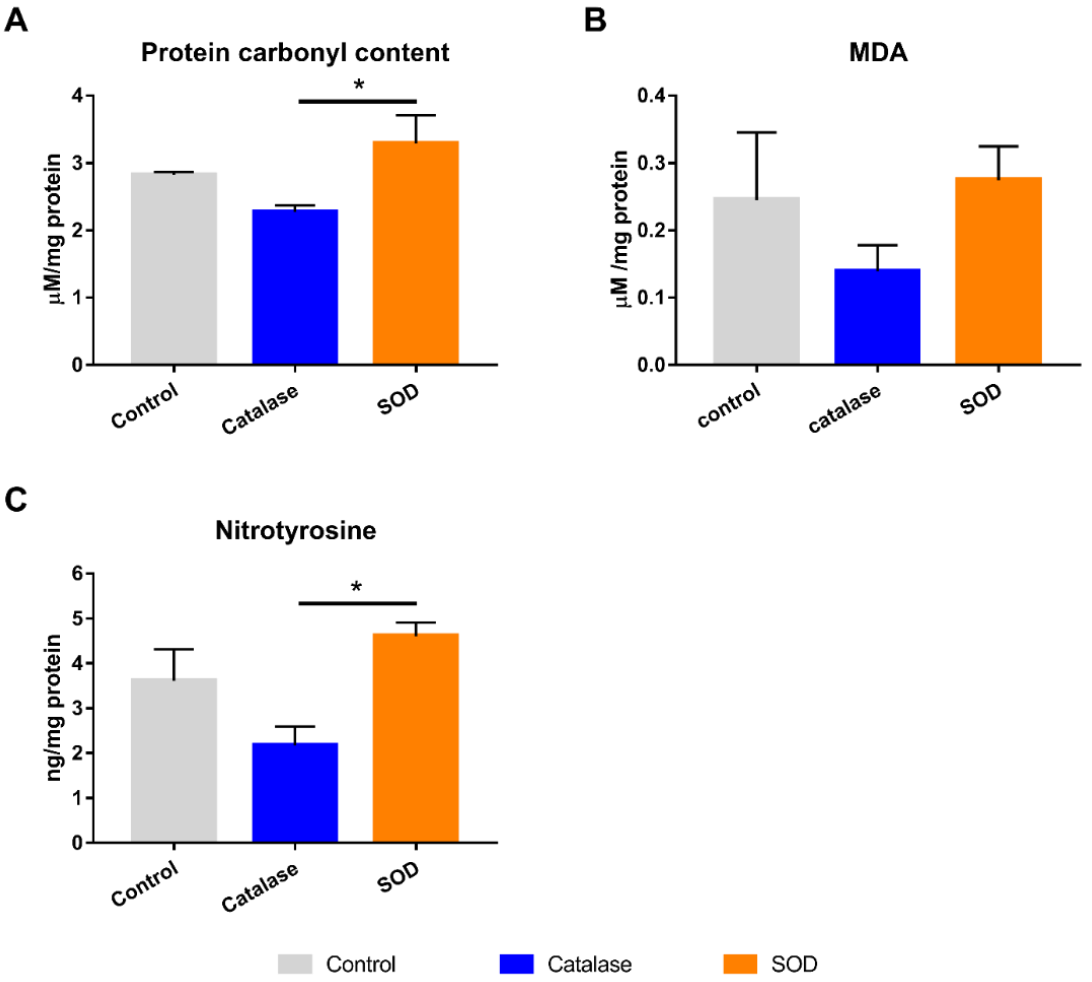


Fig. 3-6 Oxidative stress-related modification

The catalase group had significantly lower protein carbonyl content in the myocardium and nitrotyrosine in coronary arteries than the SOD group (A and C). There was no statistically significant difference in the myocardial MDA between groups (B). MDA, malondialdehyde. (Comparison between groups, one-way ANOVA, catalase group vs. SOD group: *p<0.05, **p<0.01; SOD group vs. control group: $^{\&}$p<0.05; SOD group vs. *in situ* group: $^{\sigma}$p<0.05, n=6)

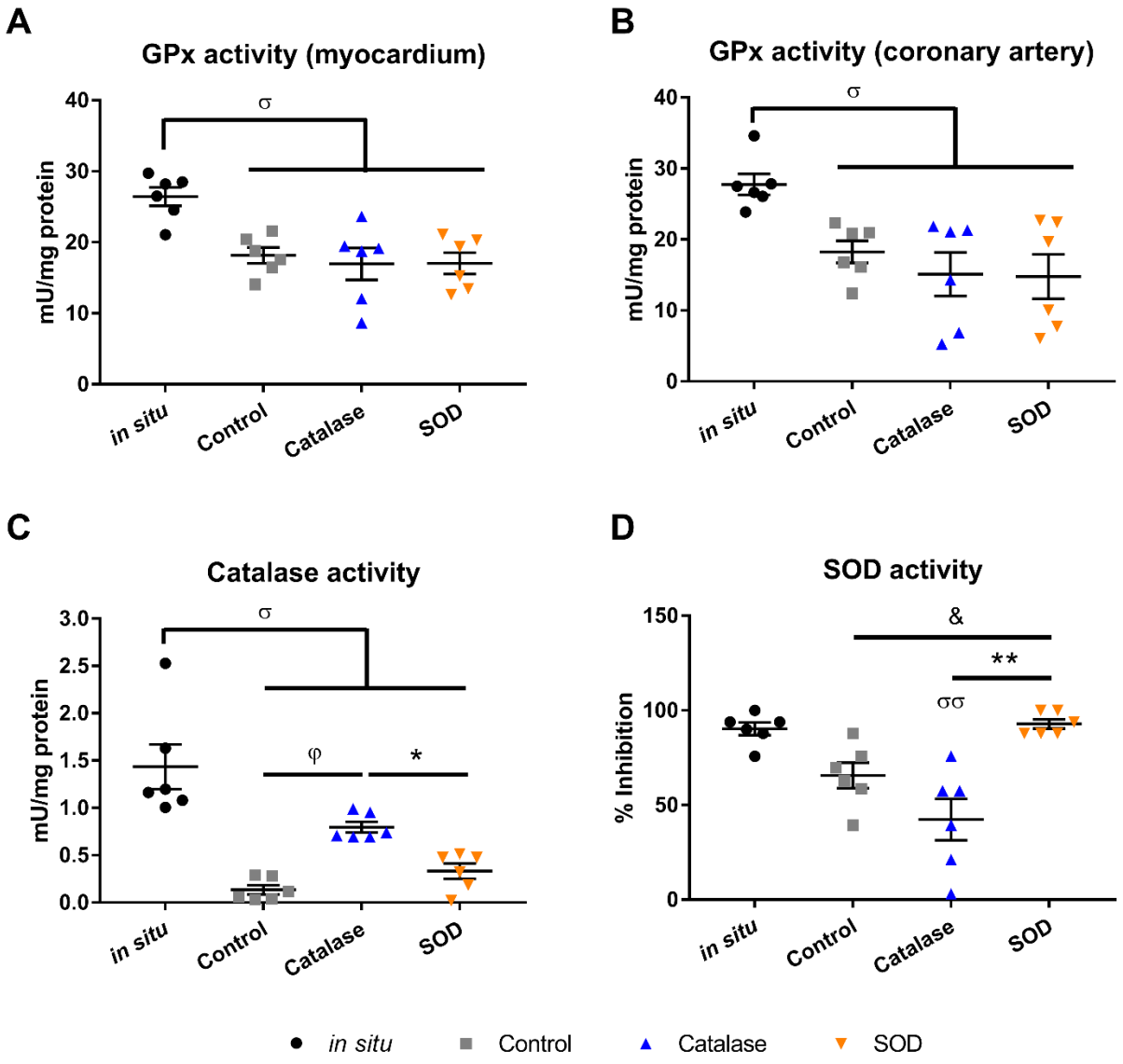

A
GPx activity (myocardium)
mU/mg protein
40
30
20
10
0
σ
in situ
Control
Catalase
SOD
B
GPx activity (coronary artery)
mU/mg protein
40
30
20
10
0
σ
in situ
Control
Catalase
SOD
C
Catalase activity
mU/mg protein
3.0
2.5
2.0
1.5
1.0
0.5
0.0
σ
φ
*
in situ
Control
Catalase
SOD
D
SOD activity
% Inhibition
150
100
50
0
&
**
σσ
in situ
Control
Catalase
SOD
in situ
Control
Catalase
SOD

Fig. 3-7 Antioxidant enzyme activity

There was a decrease in myocardial and coronary artery glutathione peroxidase activity compared to *in situ* control (A and B). Each perfusion group had a decrease in catalase activity in the myocardium. The catalase group had significantly higher catalase activity than the SOD group and control group (C). The SOD group had significantly higher SOD activity than the other two perfusion groups. However, there was no significant difference between the SOD group and *in situ* group (D). (Comparison between groups, one-way ANOVA, catalase group vs. SOD group: *$p<0.05$, **$p<0.001$; Catalase group vs. control group: $^{\phi}p<0.01$; SOD group vs. control group: $^{\&}p<0.05$; other groups vs. *in situ* group: $^{\sigma}p<0.05$, $^{\sigma}p<0.001$, n=6)

4.4 Discussion

The focus of this study is oxidative stress, this biological process during isolated normothermic heart perfusion. Oxidative stress can be assessed by measuring oxidatively modified biomolecules like lipid peroxidation and DNA and protein oxidation, or by using staining techniques such as CellRox. We employed these methods in this study to determine the effectiveness of exogenous catalase and SOD in protecting the *ex situ* perfused heart from excess ROS. The present results suggested that different antioxidants had a distinct impact on myocardial function as the antioxidative capacity of *ex situ* perfused heart declines. Also, it had a different effect on endothelial preservation and coronary vasomotor tone. Accumulating evidence indicates that the *ex situ* perfused heart loses normal vasomotor tone and develops heart failure in a few hours.[142, 228, 229] We hypothesize that augmented intracellular accumulation of reactive/nitrogen species may play a key role.

4.4.1 Antioxidant enzyme activity decreases in normothermic *ex situ* perfused heart

We used two major oxygen-derived free radical scavengers, catalase and SOD; both are primary intracellular protective antioxidant enzymes in the body. They compose a chain reaction in scavenging the ROS. Dismutation of superoxide ($O_2^{\bullet-}$) by SOD produces hydrogen peroxide (H_2O_2), which is then converted enzymatically into H_2O by catalase and glutathione peroxidase (GPx). Considering the short half-life of both catalase and SOD, we employed catalase and SOD conjugated to PEG, which has a half-life greater than 30 hours.[230, 231] Also, it has been reported to associate with vascular endothelial cells, potentially increasing cellular enzyme activity in a manner that protects against the cytotoxic effects of $O_2^{\bullet-}$.[231-234] The dose of PEG-Catalase chosen in the present study

was 10,000 IU/L, and the corresponding PEG-SOD dose was 550 IU/L for the ESHP, considering previous research.[230, 235]

We measured catalase, SOD, and GPx activity, which is the most critical parameter determining the biological effect of the antioxidant enzymes. The present study provides evidence that administration of PEG-catalase and PEG-SOD at the given doses can increase the catalase activity and SOD activity after 6-hour ESHP compared to the control group, respectively. However, without the administration of catalase and SOD, there was a decrease in the activity of these enzymes. Our results also showed that GPx activity decreased in both the myocardium and the coronary vasculature of the perfused hearts. GPx is a crucial antioxidant enzyme involved in preventing the harmful accumulation of intracellular H_2O_2.[236] It has been found to be even more effective than catalase at scavenging intracellular peroxides under many physiological conditions.[237] The decrease in antioxidant enzyme activity may explain the oxidative stress states we observed in the *ex-situ* perfused heart.

4.4.2 Oxidative stress was induced during ESHP

Under normal circumstances, there is a well-managed balance between the formation and neutralization of ROS. Oxidative stress occurs when this balance of oxidant formation exceeds the ability of the antioxidant system to scavenge ROS, such as occurs when immune cells (e.g., neutrophils and macrophages) are activated by pro-inflammatory stimuli to undergo an “oxidative burst” by exposure to a foreign agent.[238] Under these conditions, biomolecules become subjected to attack by excess ROS and significant molecular and physiological damage can occur. Inflammatory activation was induced during normothermic ESHP, which may be a significant source of ROS in isolated heart

perfusion.[220] Inflammation has a reciprocal relationship with oxidative stress.[239] ROS produced physiologically by leukocytes and macrophages as a host defense mechanism can damage surrounding tissue.[240] For example, oxidants that are produced by enzymes contained in certain intracellular granules release myeloperoxidase (MPO). On neutrophilic activation, it can catalyze a reaction between H_2O_2 and chloride to produce hypochlorous acid (HOCl), a highly potent oxidant that can lead to tissue injury.[241, 242] Our previous results (not published) indicate an increase in MPO and MDA in the coronary vasculature of *ex situ* perfused heart, which may indicate neutrophil infiltration and activation related vascular redox state.

Another source of ROS during isolated organ perfusion may come from the oxidation of hemoglobin. Red blood cell hemolysis is very common and potentially unavoidable in machine perfusion systems.[82, 105] Hemolysis can release cytotoxic free hemoglobin and other mediators initiation pro-inflammatory cascade.[181] On the other hand, hemolysis is also a prominent source of ROS.[243] This results in the release of large amounts of hemoglobin and free heme into circulation. The primary source of intracellular ROS in the red blood cell is autoxidation of oxyhemoglobin, which generates superoxide and, through dismutation, produces H_2O_2.[244] Our live tissue oxidative stress staining results indicate that some of the green fluorescence was located in the vascular area. We propose that part of this came from the red blood cell. There are two major antioxidant enzymes in the red blood cell, catalase, and GPx, which can neutralize H_2O_2 and protect heme degradation.[245]

4.4.3 Oxidative stress-related modification in the myocardium and coronary vasculature

Cardiovascular tissue is also a rich source of ROS, including $O_2^{\bullet -}$, H_2O_2, and hydroxyl radical ($^{\bullet}OH$).[221] They can induce biochemical alterations and cause oxidative damage to cellular macromolecules, such as proteins, carbohydrates, lipids, and DNA, and are thus cytotoxic.[246-248] Under ideal circumstances, the production rate of an oxidatively modified cellular component should be comparable to that of its removal or repair. However, the lack of an antioxidative system may lead to the accumulation of oxidatively modified biomolecules.

The present results showed that the administration of catalase and SOD had an opposite effect on the myocardial protein carbonyl content. Carbonylation is likely to result from severe oxidative stress. This result was inconsistent with the live tissue oxidative stress staining. The catalase group had higher myocardial protein carbonylation than the SOD group. It may negatively affect the cardiac function since myocardial structural protein oxidation could contribute to the contractile impairment and loss of myocardial viability.[249]

The present study also investigated protein modification by peroxynitrite ($ONOO^-$) in the coronary vasculature. The catalase group had higher nitrotyrosine than the SOD group, suggesting higher oxidatively modified proteins in the coronary artery. $ONOO^-$, generated from nitric oxide reacting with $O_2^{\bullet -}$, oxidizes proteins by the preferential nitration of tyrosine to form nitrotyrosine.[250, 251] Previous research has shown that oxidation of the protein may cause vascular alteration and dysfunction that leads to

vascular disease.[252] The oxidatively modified protein in the coronary vasculature may affect the coronary artery function during ESHP.

4.4.4 Myocardial function was better preserved in the catalase group

It is well recognized that myocardial function declines during normothermic ESHP.[142, 228, 253, 254] also demonstrated in the present study. However, the catalase group had better cardiac function preservation than both the SOD and control groups. Consistent with the decreased myocardial function, the MVO_2 was significantly reduced in the SOD and control groups. The distinct impact on functional and metabolic preservation may be attributed to the different redox states in each group, as oxidative stress appears to be involved in cardiac remodeling, mechanoenergetic uncoupling, and altered calcium sensitivity, thus contributing to contractile impairment.[255, 256]

Both the catalase group and SOD group had a lower degree of myocardial injury than the control group, which suggests that augmenting antioxidant capacity can protect the myocardium from damage. Previous research also showed that SOD provides sustained protection against myocardial injury.[257]

4.4.5 Pleiotropic effects of antioxidants on coronary vasomotor tone and endothelial integrity

Accumulating evidence indicates that *ex situ* perfused hearts gradually lose coronary vasomotor tone.[104, 142, 253, 258] The present study showed a significant decrease in CVR compared to the baseline in all the groups. CBF increased to a supraphysiologic level. However, there was a relatively lower CBF in the catalase group from an early time point, suggesting relatively preserved CVR. We believe the loss of CVR during ESHP is multifactorial, and the redox state in the coronary vasculature may play a part. H2O2 can

induce vasorelaxation of the coronary artery.[259] The present results showed that catalase could partially decrease ROS production and oxidatively modify proteins, which may help to preserve the coronary artery function. However, the pattern of decreased CVR across all the groups was very similar, suggesting other factors may also be involved.

There was an increase of perfusate vWF and thrombomodulin in all the groups, suggesting endothelial damage. However, the catalase group had lower perfusate vWF than the other groups, indicating the protection of catalase on the endothelial layer. In addition, the SOD group had a significantly higher perfusate syndecan-1 and hyaluronan than the catalase group and control group, suggesting severe endothelial glycocalyx (EG) shedding in this group. The glycocalyx plays an important role in preserving endothelial cell integrity and is required for normal endothelial function.[260] Many factors can cause EG damage. ROS are capable of directly destroying components of the glycocalyx.[261-263] The glycocalyx shedding may affect the coronary endothelial function in the SOD group. These results suggested that catalase and SOD had a distinct effect on endothelial integrity.

4.4.6 Potential mechanism of the pleiotropic effects of different antioxidants on the graft during ESHP

Catalase and SOD work in the opposite direction in H_2O_2 detoxification. SOD plays a major role in the formation of H_2O_2, whereas GPx and catalase play a role in the enzymatic catabolism of this ROS.[236] Under the circumstances of decreased GPx and catalase activity, the H_2O_2 produced by SOD dismutation would accumulate, thus paradoxically increasing ROS. This may contribute to the different redox states in the graft during ESHP.

4.5 Conclusion

The administration of catalase and SOD had a distinct effect on cardiac function preservation. In addition, it had a different impact on endothelial preservation and coronary vasomotor tone. Cardiac function and endothelial integrity were better preserved by adding catalase. However, there was a paradox effect of SOD on the myocardium and endothelium protection. It decreased myocardial injury. On the other hand, it causes more damage to the endothelial glycocalyx. The relatively higher oxidatively modified protein in the myocardium and coronary vasculature suggest SOD may paradoxically increase ROS under the circumstance of decreased enzyme activity of GPx and catalase during ESHP.

Supplementation

Live tissue oxidative stress staining

The ROS were measured by staining myocardium biopsy tissue using CellROX® oxidative stress staining kit (Invitrogen, MA, USA). 3-5 mm needle biopsy of myocardium in the apex of heart was acquired before initiation of perfusion (T0), 3 hours of perfusion (T3), and 5 hours of perfusion (T5). The tissue was kept in the same modified KH solution before staining. All the staining was done within 30 minutes of tissue collection. After several washes with the medium, the tissue was stained with 5μM CellROX® green reagent and Hoechst 33342 by adding the probe to the complete media and incubating at 37°C for 30 minutes. The tissue was then washed with PBS three times and imaged in the modified KH solution on a Zeiss LSM 710 inverted confocal microscope (Carl Zeiss Jena GmbH, Germany). After Imaging the tissue was fixed in 4% paraformaldehyde in PBS for 15 minutes at room temperature and rinsed with PBS. Tissue was then immediately frozen at -80 °C for later use.

Glutathione peroxidase (GPx) activity assay

To study the activity of GPx in the myocardium and coronary vasculature. After perfusion, small tissue sections (>10mg) were immediately snap frozen in liquid nitrogen and stored at -80 °C. GPx activity assay was conducted according to the manufacturer's instruction (MyBioSource Inc., CA, USA). The reaction was carried out in a 96-well plate, and the samples were read in a fluorescence microplate reader (BioTek Instruments, Inc. Vermont, USA) at 340 nm. The results were expressed in the amount of enzyme that will cause the oxidation of 1.0 μmol of NADPH to $NADP^+$ under the assay kit condition per

minute at 25°C. The values were corrected for the amount of protein (Bradford assay, Bio-Rad, Hercules, USA).

Catalase activity assay

To determine the activity of catalase in the myocardium. small tissue sections (0.1 g) were immediately snap frozen in liquid nitrogen and stored at -80 °C. catalase activity assay was conducted according to the manufacturer's instruction (MyBioSource Inc., CA, USA). The reaction was carried out in a 96-well plate, and the samples were read in a fluorescence microplate reader (BioTek Instruments, Inc. Vermont, USA) at 570 nm. The results were expressed in the amount of enzyme that decomposes 1.0 μmol of H_2O_2 per minute at pH at 25°C. The values were corrected for the amount of protein (Bradford assay, Bio-Rad, Hercules, USA)

SOD activity assay

To determine the activity of catalase in the myocardium. small tissue sections (>10mg) were immediately snap frozen in liquid nitrogen and stored at -80 °C. SOD activity assay was conducted according to the manufacturer's instruction (MyBioSource Inc., CA, USA). The reaction was carried out in a 96-well plate, and the samples were read in a fluorescence microplate reader (BioTek Instruments, Inc. Vermont, USA) at 450 nm. The values were corrected for the amount of protein (Bradford assay, Bio-Rad, Hercules, USA)

Chapter Five: Overall discussion and future directions

5.1 Overall discussion

The constant shortage of available organs is a significant obstacle and limiting factor in heart transplantation. The preclinical research could help us develop clinical approaches to donor heart preservation, allowing for prolonged storage and permitting remote procurement.

Maintenance of normal mechanical function of the myocardium relies on the high efficiency of aerobic metabolism. Unlike the cold storage method, the goals of normothermic perfusion are to preserve cellular ATP and supply adequate oxygen delivery and nutrients to maintain oxidative phosphorylation.[196] In the normal myocardium, the production of ATP is strictly coupled to myocardial oxygen consumption. Therefore, the level of cardiac functional recovery depends mainly on the integrity of the energy production apparatus, as 80% of oxygen utilization is devoted to the contractile work of the heart.[264] There is a close correlation of myocardial oxygen consumption with coronary blood flow. The metabolic factors (as compared to mechanical factors) control the regulation of vasomotor tone of the coronary vessels.[265] An efficient coronary circulation that meets the oxygen demands of the myocardium and must be able to adapt to the changing metabolic requirement.[266] Normal cardiac function is highly dependent on myocardial perfusion. So coronary arteries' function should be well evaluated and preserved as well.

In this dissertation, we evaluated the coronary artery function during ESHP. A large body of evidence indicates that the *ex situ* perfused heart gradually loses the coronary vasomotor tone.[104, 142, 253, 258] We have shown here that the *ex situ* perfused heart increases coronary blood to a supraphysiological level, decreases coronary vascular resistance

(CVR). Also, there is an uncoupling between the coronary blood flow and cardiac systolic function. We evaluated several mechanisms that may potentially play a part in decreased CVR during ESHP. These include vasoactive mediators, inflammatory mediators, and oxidative stress mechanisms.

5.1.1 Whole blood in regulating coronary vascular tone

In the second chapter of this thesis, we compared the effect of whole blood and diluted whole blood on coronary vascular tone regulation during ESHP. Tchouta and colleague[151] showed that the CVR is relatively higher in the setting of continuous plasma exchange ESHP than in the control heart. The higher coronary vasomotor tone in the plasma exchange hearts suggested preserved endothelial integrity. Mcleod[142] and Church[253], respectively, reported that in the setting of cross plasma circulation from a live, awake paracorporeal animal, the CVR was significantly higher than the heart perfused alone. It was indicated in these studies that there is something in plasma that maintains vasomotor tone.

This evidence also leaves us to speculate that some mediators in the plasma may play a part in regulating coronary vascular tone. The change concentration or functional loss of these mediators may cause the loss of coronary vascular tone in ESHP. The coronary vasculature is influenced by physical (arterial pressure, extravascular compression, hematocrit), neural, and chemical factors. Although there is a considerable interplay among these various factors, the predominant one is chemical.[267] Various circulating substances are involved in coronary flow regulation. The diluted whole blood in our study contained 50% of normal plasma and 50% of the normal blood concentrations

of leukocytes and platelets. Also, the vasoactive mediators like 5-HT, bradykinin, and histamine were half the concentration in the whole blood group. The correlation studies between the concentration of these vasoactive mediators with coronary blood flow rate were conducted and suggested that the change of some mediators (like 5-HT) may likely play a role in the decrease of CVR.

Additionally, plasma protein may have a protective effect on endothelial function. For example, McDonagh *et al* reported earlier that adding albumin and washed red cells to Krebs-bicarbonate buffer helps preserve normal coronary microvascular function and permeability.[268] Maintaining endothelial function may also help protect endothelial-dependent mechanisms of blood flow regulation and blood-blood vessel interactions.[269] In another study he reported that coronary tone and autoregulation were better maintained in blood added perfusate than Krebs buffer alone.[270] The CVR and oxygen delivery in the blood perfused heart were more stable and similar to *in situ* values.[271] Significantly more oxygen can be carried in blood perfusate than in Krebs buffer alone.[270] While Krebs perfused working heart may be undersupplied with oxygen.[272-274] In the Krebs buffer alone perfused heart, the coronary artery was markedly vasodilated in an attempt to meet myocardial oxygen demand.[272, 273] In addition, the red blood cell is more than just an oxygen carrier. It can also function as an oxygen sensor, participating in regulating of vascular tone.[275, 276] The red blood cell is capable of sensing oxygen content decreasing and release ATP in response to a hypoxia challenge.[277] It was suggested that ATP could subserve a regulatory role in vascular control.[278, 279]

In our study, coronary flow increased significantly irrespective of whole blood perfusate or diluted whole blood perfusate, suggesting that other factors may also play a

role in the loss of CVR in addition to the change of vasoactive substances. We hypothesized that ROS might be one factor. In our study, both groups had significantly increased the ROS production in the perfusate and the coronary vasculature. However, the whole blood group had higher ROS than the diluted whole blood group. Red cells contain antioxidant enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase, which may help to protect the heart from oxygen radical injury. This is supported by the study of Hendriks *et al.*, who reported improved recovery from hypothermic ischemia when the hearts were perfused with a red cell containing perfusate rather than Krebs solution.[274] However, the whole blood group had higher cell-free hemoglobin due to hemolysis during perfusion, which may be one significant source of ROS. This would create a high redox state in the vasculature of the whole blood perfused heart. In this study, the different vasoactive mediator profiles of whole blood versus diluted whole blood perfusate would mask the ROS mechanism to cause vasodilation. Thus, we investigated the ROS mechanism in the third project.

The whole blood perfusion indicated some disadvantages. Firstly, long-term *ex situ* blood perfusion of the heart has proven unsatisfactory because of the mechanical degradation of blood components produced during recirculation and oxygenation.[280] In addition, progressive hemolysis of the red cells during the machine perfusion system is unavoidable.[82, 105] The release of cell-free hemoglobin is a potent oxidant that can lead to the generation of ROS.[252] The *ex situ* perfused heart may undergo more severe hemolysis-related redox and inflammatory alterations due to the absence of the organs involved in scavenging hemolysis products, such as liver and kidney. This manifested that further investigation is needed for the oxygen carrier substitute in machine perfusion.

5.1.2 Reduced flow during ESHP

In the third chapter of this thesis, we perfused the heart at a controlled coronary artery flow rate. The targeted coronary artery flow rate is 500 mL/min, which has been used in another isolated working heart perfusion study.[148] It is generally accepted that coronary flow results from the interplay of coronary perfusion pressure, ventricular contraction, and coronary vascular tone.[94] Among which, the perfusion pressure is the decisive factor. We reduced the coronary perfusion pressure to maintain a constant coronary artery flow rate. Many studies suggested that controlled perfusion utilizing a low coronary perfusion pressure would limit injury and preserve cardiac function.[148, 188-191] However, pressure above or below certain limits would compromise myocardial protection in isolated heart perfusion.

Although coronary perfusion pressure may affect the development of myocardial edema, the inadequate flow may compromise myocardial oxygen delivery, while excessive pressure may damage the endothelium.[192] Kobayashi and colleagues[258] showed that hyperperfusion of the myocardium is detrimental, potentially resulting in significant myocardial edema. Aupperle *et al*.[200] also reported that lowering the perfusion pressure leads to improved ultrastructural preservation of the explanted heart. But what is the optimal perfusion pressure? When blood is perfused through the vascular system of animal's extremity, no flow is produced until the pressure gradient from arteries to veins reaches 10mm Hg.[281] So the perfusion pressure must be enough to provide the coronary flow to meet the oxygen demand of the myocardium.

Determining the optimal coronary flow rate above which the heart's oxygen demand is met is of critical importance to prevent the state of myocardial hypoxia and for better

organ preservation. Quader and colleagues[82, 282] reported that the minimum coronary flow can be determined in a rat *ex situ* heart perfusion model. The minimal coronary flow that meets the myocardium oxygen demand is the value below which the tissue resorts to anaerobic metabolism and oxygen consumption is flow dependent. It was determined from the best fit lines of the correlation between myocardial oxygen consumption and coronary flow rate. However, their studies used asanguineous perfusate, which is different from our setting. Whether this methodology could be applied in blood perfusate may need further investigation.

5.1.3 Redox state in the vasculature

An increase in vascular production of ROS has been traditionally considered a pathological response that leads to impaired vasomotor function.[283] In the fourth chapter of this thesis, we attempted to use different oxygen-derived free radical scavengers to control the ROS during ESHP. We are interested in antioxidant therapy for the target of vascular endothelium. The endothelial lining of the luminal surface of vessels serves as an interface between blood and the underlying myocardium. Endothelium helps regulate levels of vasoactive agents and control blood flow, vascular permeability, coagulation, fibrinolysis, leukocyte adhesion, and migration into tissues. We tested the hypothesis that the redox state in the vasculature may play a part in the lost CVR during ESHP.

Superoxide anion is continually released from mitochondria at several points in the electron transport chain as a byproduct of oxidative metabolism.[284] Oxidative stress is a state in which excess ROS overwhelms endogenous antioxidant systems. ROS has a distinct functional impact on each cell type in the vasculature and can play physiological

and pathophysiological roles.[246] Additionally, oxidative stress to the endothelium can be initiated or propagated by oxidants generated in the cellular milieu (plasma, vascular cells, extracellular matrix, adjacent alveolar compartment, etc.), released by activated leukocytes or generated by activated endothelial cells under pathological conditions.[285] Exposure of the endothelium to potentially harmful pro-oxidative compounds circulating in the bloodstream and formed in the tissue, such as oxLDL and oxidants released from activated platelets, has been implicated in vascular injury in atherosclerosis and thrombosis.[286] In the first two projects, we reported that the *ex situ* perfused heart was exposed to an oxidant-rich environment, indicated by the perfusate oxLDL. Also, there was an oxidative modification of the lipid and protein on the myocardium and coronary vasculature. This suggested that an oxidative stress environment would propagate the vascular redox state, further affecting the endothelial function.

The presumed mechanism is through the ability of ROS to induce biochemical alterations in the macromolecules in the vasculature, such as carbohydrates, nucleic acids, lipids, and proteins.[246-248] Oxidative modification of proteins can have multiple adverse effects on cellular physiology. For example, oxidative modification of enzymes has been shown to affect their activities.[287, 288] Multiple enzymatic systems produce superoxide and its derivates in the vasculature, including NADPH oxidase, xanthine oxidase (XO), nitric oxide synthases (NOS), and MPO. Superoxide not only can exert effects on vascular function, but it is also pivotal in generating other reactive species. For example, MPO utilizes $NO^{\bullet}$ to generate RNS, increasing oxidative stress.[289, 290] In the second chapter, we reported an increase of MPO activity and nitrotyrosine in the coronary vasculature, suggesting the oxidatively modified protein in the vasculature. In addition,

ROS, including superoxide anion, H_2O_2, $NO^{\bullet}$, damage catalase, and GPx, thus decreasing their activity. When ROS generation exceeds endothelial antioxidant defense mechanisms, endothelial cells may undergo apoptotic or necrotic death, leading to vascular injury.[285] In the fourth chapter, we reported the decreased GPx activity in both the myocardium and coronary vasculature and reduced catalase activity in the myocardium. The decrease of antioxidant enzyme activity would make the endothelium prone to the direct attach from ROS in the perfusate. Furthermore, in the absence of adequate detoxification of H_2O_2, SOD may aggravate oxidative stress.[291] Our results showed that adding SOD causes more damage to the endothelial layer and glycocalyx shedding, suggesting ROS-induced vascular injury. And endothelial injury would compromise its function.

Furthermore, oxidative stress may affect endothelial function directly. For example, a redox state can regulate ion channels and transporters and induce vasodilation or vasoconstriction. H_2O_2 can serve as a critical endothelium-derived hyperpolarizing factor (EDHF). As an EDHF, H_2O_2 induces vasodilation through a mechanism involving smooth muscle Ca^{2+}-activated K^+ channel (K_{Ca}) activation and subsequent membrane hyperpolarization.[292] A large body of studies have also reported that H_2O_2 induces potent K_{Ca}-mediated smooth muscle hyperpolarization and relaxation in porcine coronary arteries.[293-295] The H_2O_2 could be generated in the myocardium or within endothelium during normal oxidative metabolism.[284] Liu *et al*.[283] demonstrated that H_2O_2 produced in response to flow was a byproduct of complex I and complex III of the endothelial mitochondrial electron transport chain. In our study, the decreased catalase activity may cause the accumulation of H_2O_2. And inadequate detoxification of H2O2 may play a part

in coronary vasodilation during ESHP. However, the detailed mechanism during ESHP may need further investigation.

5.1.4 Uncoupling between cardiac function and coronary blood flow during ESHP

Blood flow must be altered appropriately, increasing when supply is unable to meet demand and decreasing when supply exceeds demand.[276] It is generally agreed that coronary flow and cardiac function change in the same direction.[94] However, in our study, there is uncoupling between cardiac work and coronary blood flow during ESHP. The constant coronary flow perfusion strategy cannot solve the uncoupling between cardiac work and coronary blood flow. We believe multiple factors may play a role in the lost uncoupling. H_2O_2 may be produced as a diffusible EDHF in the myocardium or the coronary artery, which induces H_2O_2-mediated coronary vasodilation.[284] The activity of the regulatory mechanism may also be impaired by such factors as hemolysis, damage to the myocardium, and coronary endothelium. These factors may also play a role in the apparently close relationship between cardiac work and coronary flow during ESHP.

5.2 Future directions

Overall, our work has new implications for future research:

- Antioxidant enzymes could be potentially used in isolated organ perfusion. Effective protective vascular oxidative stress using the administration of exogenous catalase proteins remains to be achieved. In our study, supplementing native enzymes cannot completely afford endothelium protection, most likely due to inadequate delivery. Previous studies reported that adherent leukocytes produced ROS diffuse into endothelial

cells and thus become inaccessible to circulating antioxidants.[296] However, the enzyme modifications may permit effective targeting more attractive pharmacokinetic and therapeutic profiles. For example, in order to increase the specific affinity of catalase for endothelium, it can be conjugated with antibodies directed against endothelial surface antigens. In addition to more specific targeting to the endothelium, the use of internalized antibodies may facilitate intracellular uptake of the antioxidant enzymes.[297, 298] It is conceivable that the derivatives that are characterized by prolonged function, specific targeting of the endothelium have the highest probability of serving as feasible therapeutic modalities.

- The preserved CVR has been reported in the cross-circulation ESHP model. However, the mechanism is seldom investigated. Whether the *ex situ* perfused heart receives the antioxidants protection from the live animal or other metabolic support mechanisms remains explored. As planned, we would perfuse the heart in a cross-circulated setting with a live animal and evaluate tissue redox state during perfusion.

- Coronary blood flow is tightly adjusted to the oxygen requirement of the myocardium, so metabolism in the myocardium may play an important role in the regulation of coronary artery as a feedback control mechanism. The mechanism of metabolic regulation of coronary blood flow during ESHP needs to be further investigated. Myocardial perfusion and CVR are regulated by signaling metabolites released from the local myocardium that act either directly on the vascular smooth muscle cells or indirectly via stimulation of the endothelium.[299] These metabolites may include adenosine and ATP. The source of adenosine is myocardial ATP and possibly to some extent via cyclic adenosine monophosphate (cAMP). Adenosine formation and release is tightly coupled

to cardiac metabolic activity and can serve to produce rapid adjustment of CBF.[267] Small and immeasurable decreases in tissue ATP can result in a several-fold increment in adenosine when oxygen supply is inadequate for cardiac demands.[300] It has also been reported that ATP, which also is a potent vasodilator, is released from isolated perfused guinea pig heart.[301, 302] The metabolic profile of some metabolites during ESHP need further investigation.

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