METABOLIC AND INFLAMMATORY ASPECTS OF CARDIOTHORACIC SURGICAL INTERVENTIONS

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METABOLIC AND INFLAMMATORY ASPECTS OF CARDIOTHORACIC SURGICAL INTERVENTIONS

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Voor papa, wat had ik je dit graag laten zien
Voor mama, wat ben ik blij dat je dit kan zien

Chapter 1

General introduction and outline of the thesis

D.A. Schipper

PREFACE

Cardiovascular disease (CVD) is the primary cause of death worldwide. Although pathophysiology and pharmacological treatment have extensively been studied, the need for novel insights persists, with incidence and prevalence of CVD only increasing. Due to the progressive nature of CVD, macro- and microbiological changes accumulate over time, eventually causing irreversible dysfunction. It is important to diagnose and treat patients before they enter this end-stage of pathology, since cardiopulmonary transplant then lasts as the only viable solution. Metabolism, and mitochondria specifically, have increasingly claimed a role in pathophysiology of a broad range of diseases over the recent years. This results in the possibility of novel therapies with a focus on the bioenergetics of cells and tissue. The importance of pro- and anti-inflammatory mediators and agents have been acknowledged in the peri-operative setting for a long time. Improved understanding of the mechanisms involved and the development of creative strategies intended to regulate the immune response can vastly improve surgical outcomes. This thesis aims to elucidate the metabolic and inflammatory aspects of a range of common cardiothoracic surgical interventions in three consecutive parts. These parts are preceded by an introduction in which a brief overview of cardiovascular disease is given. Mechanisms leading from disrupted metabolism towards myocardial ischemia and heart failure are highlighted. Additionally, amniotic membrane patches are introduced as a potential immunosuppressor to be used in cardiothoracic surgery. The final section of the introduction provides some historical background on cardiopulmonary transplant and the development of preservation solutions.

There is a wide variety of conditions and mechanisms that can lead to dysfunction of the cardiovascular system. This thesis does not intend to cover the entire spectrum of heart failure etiologies but addresses a selection of conceptually linked interventions. Notwithstanding the broad range of pathophysiological factors, we have focused on the metabolic and inflammatory perturbations in advanced cardiac failure and its most common therapies.

1.1 INTRODUCTION

Epidemiology

According to the World Health Organization, cardiovascular disease is responsible for the death of approximately 16 million people each year and is the most prevalent cause of death worldwide.[1] Prevalence of heart failure in the United States is 1.7-2.0%, similar to the estimated 2.0% in Europe.[2,3] The Rotterdam population study suggests an incidence almost twice as high, at 3.9% in The Netherlands.[4] The incidence and prevalence of

cardiovascular disease continue to rise, due to increased life expectancy, current Western lifestyle and improved healthcare standards.[5] The growing character is illustrated by an estimated 25% increase in prevalence by the year 2030. Additionally, over 40% of patients diagnosed with heart failure die within 2.5 years, despite advances in treatment.[6] Without adequate treatment, the vast majority of heart failure patients will end up on the waiting list for cardiac transplant. The so-called transplant gap is widening as the waiting list of recipients expands and the pool of available organs stagnates.

Metabolism

Metabolism is defined as the set of chemical processes that occur within a living organism in order to maintain life.[7] Cells cannot survive without nutrients, which makes metabolism the very essence of life. Metabolism can be subdivided into an oxygen-consuming, or aerobic, pathway and one that forms energy in a less effective, but oxygen-free manner, known as the anaerobic pathway.[7,8] Both pathways lead to the same end-point of ATP formation through a series of biomolecular steps. Cells are generally capable of obtaining nutrients through the process of diffusion. However, delivery of metabolic substrates over larger distances within an organism requires a faster, active infrastructure, in a process known as convection. In humans, as in all mammals, blood is the medium in which oxygen (O2) and nutrients are carried to end-organs. After nutrients and oxygen are properly delivered to cells, various metabolic steps can create energy in the form of high energy phosphates, most notably adenosine-triphosphate (ATP).[9] In both the aerobic and anaerobic metabolism, glycolysis plays a central role. In the process of glycolysis, glucose is converted into pyruvate and NADH, which results in the generation of 2 ATP molecules. NADH (along with FADH2) can then be re-oxidized by pyruvate oxidation and the formation of lactate as a byproduct, or can be used to maximally create another 34 molecules of ATP.[10,11] This second, more efficient form of ATP production requires mitochondria.

Mitochondria are organelles inside the cytoplasm of most human cells and are often referred to as the energy factories of the cell.[7] Mitochondria consist of an outer membrane and a folded inner membrane that contains the electron transport chain (ETC).[9] The ETC is a series of 5 mitochondrial complexes (complex I-V) that are responsible for creating an electrochemical proton gradient over the inner membrane through consecutive redox reactions.[9,12] The main function of mitochondria is the production of energy in the form of ATP, through the process of oxidative phosphorylation (OXPHOS).[13] Which pathway is predominantly used to achieve ATP formation, is dependent on the cell type and organ system.[14] Cells that require rapid energy production, e.g. muscle cells, utilize highly efficient OXPHOS as the predominant energy source, whereas glycolysis is the more favorable pathway for cells such as stem cells.[15,16] Differences in mitochondrial content between various organs has been demonstrated in rats, showing that myocardial tissue

has the highest amount of mtDNA as well as the highest OXPHOS capacity.[17] Comparably, mitochondrial content in pig hearts was found to be higher than in other organs, with mitochondrial content adapting as the animals aged.[18] Cardiomyocyte metabolism relies for 95% on OXPHOS and is facilitated by high mitochondrial density, taking up one-third of the cell's volume, more than in any other human cell type.[19,20] HOweverm the cell-preferred ATP production pathway is not static, as varying circumstances may influence OXPHOS and glycolysis dependency.[21,22] This is seen in tumor cells for example, where the Warburg effect shifts the metabolic focus away from OXPHOS and towards glycolysis.[23,24] Nonetheless, OXPHOS capacity is not solely dependent on mitochondrial quantity. The amount of respiratory chain complex and intrinsic mitochondrial activity add to the dynamics of OXPHOS capacity and ATP formation.[25] Another important role of the mitochondria is in cellular apoptosis.[26] Once released from the mitochondria, electron carrier cytochrome c induces apoptosis through caspase activation.[27-29] Mitochondria play an essential role in cell homeostasis as well as programmed cell death.[30] Thus, given the need of oxygen for energy production by OXPHOS, disruption of adequate cardiovascular function can impede cellular metabolism and cause organs to fail.

During OXPHOS, reactive oxygen species (ROS) are formed, mostly as a byproduct through mitochondrial complexes I and III.[31] ROS play an important role in cell signaling, induction of growth and the inflammatory response.[32-34] An abundance of ROS, however, is at the same time potentially harmful.[35,36] ROS can cause direct and indirect DNA damage and thus has a negative influence on aging.[37-39] Additionally, ROS-induced damage has been associated with the development of diabetes, cancer, and cardiovascular disease.[40-42] ROS can be neutralized through scavenging by antioxidants.[43,44] The inevitable mitochondria-induced ROS formation during ATP production creates a paradox, which has been adequately described as a love-hate relationship, in which the production of energy can harm tissue.[45] While ROS levels vary among different cell types and under fluctuating circumstances, ROS homeostasis is obtained by corresponding activity of counteracting antioxidants.[46] Only when imbalance between formation and scavenging potential arises, the signaling capacity of ROS is altered.[47] Consequently, loss of antioxidant potential or overproduction of ROS can both unsettle the redox equilibrium and thus cause oxidant damage.[48]

1.2 CARDIOVASCULAR DISEASE

Ischemic Heart Disease

In order to maintain normal left ventricular (LV) function, the provision of O2 and nutrient-rich blood to the myocardium through coronary arteries is essential. The left and

right coronary arteries have their offspring immediately at the proximal ascending aorta, and branch off into larger and smaller coronary arteries, forming various anastomoses to provide blood to the entire heart. The coronary artery system is, however, prone to disturbances.

Atherosclerosis is observed in medium and large-sized arteries when lipids accumulate and fibrosis develops. This process occurs naturally over time and is irreversible.[49-51] Although once regarded as a cholesterol accumulation disease, atherosclerosis is nowadays rather considered to be a state of chronic inflammation.[52] Upon lipid accumulation in the arterial wall, pro-inflammatory cytokines promote the recruitment of leukocytes, including macrophages which in turn phagocyte the lipids and become fat-laden foam cells, typical for atherosclerosis.[53,54] The end result of this immunological response is plaque formation in the endothelial wall, narrowing the arterial lumen. Over time and under influence of additional pro-inflammatory response, plaques can rupture and obstruct blood flow to end-organs, known as stroke in the brain and myocardial infarction in the heart.[55,56] Pathogenesis of atherosclerotic plaques is considered to be multifactorial.[57] Despite its essential inevitability, there are many factors that contribute to the progression of atherosclerosis, including high blood cholesterol levels, smoking tobacco, diabetes, an unhealthy diet, and hypertension.[58] Among the most predominantly affected vessels by atherosclerosis are the coronary arteries.[59] Calcification is a subtype of atherosclerosis mostly seen in elderly, adding to the vessel stiffness and affecting cardiovascular hemodynamics, especially in the coronaries.[60-62] Progression of atherosclerosis and calcification can cause angina pectoris, a syndrome in which distorted blood flow through the coronaries impairs LV function.

Generally, when an artery is occluded for over 50%, symptoms occur during moments of higher cardiac workload, increased stress or sudden temperature change. The predictable nature classifies the disease as stable angina. In addition to relief of angina symptoms, rapid diagnosis is essential in the prevention of further arterial occlusion and thus minimizing the risk of myocardial infarction.[63,64] If symptoms are experienced during rest, and are caused by chronic myocardial hypoperfusion, it is labeled as hibernating myocardium. This is a state in which impaired blood flow to leads to – at least partially – reversible LV failure.[65] Restoration of blood flow to the affected area can be achieved in various ways, which will be discussed in more detail in this introduction. A typical factor in hibernating myocardium is the metabolic activity of the affected area, indicative of cellular viability upon revascularization.[66] Hibernating myocardium should be distinguished from stunned myocardium, a short-term (5-15 min) state in which coronary flow is obstructed (near) completely.[67,68] During this short period no cell death is induced, and once perfusion is restored, LV dysfunction of limited duration occurs, ranging from hours to days.[69,70] When a coronary artery becomes occluded for an extended period of time (> 20 min), as is seen

in plaque obstruction, irreversible myocardial damage occurs resulting in tissue necrosis and replacement fibrosis of the affected area.[71] Additionally, undertreated stable angina can further progress into unstable angina, which is typically seen in lumen occlusion of more than 70%. Along with myocardial infarction, unstable angina forms a spectrum of clinical presentations known as acute myocardial syndromes.[72] Thus, unstable angina is defined as ischemia of the myocardium with the absence of necrosis and is associated with myocardial stunning.[73,74]

Cardiac hypertrophy, remodeling, and failure

Following a myocardial infarction, but also during long-term exposure to volume or pressure overload, cardiac remodeling and hypertrophy can occur. Despite the short-term benefit of this remodeling and hypertrophy, in the long-term hypertrophy is an independent risk factor for the development of heart failure and arrhythmias.[75] Cardiac hypertrophy and remodeling can be diagnosed using various methods, such as ECG and echocardiography.[76-78] Advanced heart failure is a clinical syndrome with many different etiologies, including myocardial ischemia, cardiac valve stenosis or regurgitation, and metabolic disorders.[79] Heart failure can be divided into two separate forms. Heart failure with preserved ejection fraction is attributed to diastolic dysfunction of the heart, whereas heart failure with reduced ejection fraction is known as systolic dysfunction.[80] Notwithstanding the substantial differences between both groups of heart failure, this thesis scales both forms under the global term 'advanced heart failure'. The exact definition of advanced heart failure varies among countries and institutions, but can best be defined as New York Heart class III-IV and an ejection fraction of <30%.[81,82]

1.3 THERAPEUTIC ASPECTS OF CARDIOVASCULAR DISEASE

Since lifestyle, in particular Western lifestyle, is an important risk factor for the development of cardiovascular disease, changes in lifestyle are logically considered the first-choice preventive and therapeutic intervention.[83,84] This includes dietary changes, increased physical exercise and discontinuation of smoking[85,86], as the risk of coronary artery disease can be significantly reduced by lifestyle interventions.[87] While changes in lifestyle are a low-cost option with considerable effect, the majority of patients require pharmaceutical aid in order to reduce the risk of adverse effects or further progression. To reduce the workload of the heart, ACE-inhibitors and diuretics are commonly used in heart failure. ACE-inhibitors can lower blood pressure through vasodilatation, antagonizing the renin-angiotensin-aldosterone system, while diuretics increase water excretion in various parts of the kidneys. Lower blood pressure diminishes the workload of the heart, reducing the risk

of myocardial infarction.[79,88,89] Corticoid mineral receptor blockers such as spironolactone have a similar blood pressure-lowering effect and may be added in the pharmacological regimen without additional risk to the patient.[90] Additionally, beta-blockers are often administered. Blockade of beta-adrenergic receptors causes heart rate to slow down, reduces contractility and lowers LV wall stress, thus reducing myocardial O2 demand.[91]

Notwithstanding the prominent roles that lifestyle change and pharmacological therapy play in the management of heart failure patients, surgical intervention remains one of the cornerstones of treatment for atherosclerosis and its sequelae. Percutaneous coronary intervention (PCI) and coronary artery bypass grafting (CABG) are the two most common interventions. In general, PCI is performed in the acute setting of ST-elevated myocardial infarction and acute coronary syndrome.[92] During PCI, the flow in the completely occluded coronary artery is restored by angioplasty. PCI is the preferred option in such an acute and complete infarction where each minute of ischemia induces more myocardial necrosis, as it is minimally-invasive and quick.[88] Multi-vessel coronary artery atherosclerosis results in chronic ischemia of the myocardium, rather than an acute occlusion. In these cases, patients are often treated with CABG.[93] Despite being more invasive than percutaneous coronary intervention, the superior outcomes in long-term mortality and lower revascularization rates make CABG the preferred intervention in multi-vessel coronary disease.[94,95] Nonetheless, in parallel with evolving techniques and with regards to secondary outcomes, the discussion on benefits and disadvantages of both therapies is vivid and ongoing.[96,97] Cardiac surgery is, as all surgeries, associated with postoperative complications. Superficial surgical site infection can be coped with relatively easy following extensive prevention and management guidelines.[98-100] This is not the case for nosocomial infections, and in particular deep sternal infections, which are associated with increased cost of care and mortality rates of more than thirty percent.[101,102] Deep sternal infections, or mediastinitis, require a more rigorous approach of antimicrobial care and surgical debridement.[103] Blood loss forms another important possible adverse event in cardiac surgery and can be broken down into surgical bleeding, such as suture line bleeding, and coagulopathy.[104,105] Although of different etiologies, both forms of bleeding expose patients to increased mortality risk.[106] Extensive hemorrhage cases require re-exploration, further increasing the mortality rate by a two- to threefold.[107] Administration of blood products in perioperative bleeding, however, plays an ambiguous role: while hemoglobin levels and thus the O2-carrying capacity of the blood normalize, the transfusion itself is a risk factor for infections and associated increased morbidity, mortality and cost of care.[108-110] These negative effects are regarded as a necessary evil, as no difference in morbidity and costs of care is found between a restrictive and liberal transfusion threshold.[111,112] Arguably even more problematic of a complication in cardiac surgery is the risk of developing arrhythmias, most notably atrial fibrillation (AF).

AF is the most prevalent arrhythmia and has an estimated lifetime risk for development of more than 20%.[113,114] Similar to heart failure, AF incidence is ever increasing with expanding life-expectancy.[115-117] During AF the atria contract irregularly and rapidly, which can distort physiological blood flow through the atria.[118] This can lead to stasis of the blood and therefore promote the formation of blood clots. AF is a risk factor for death, can cause serious adverse events such as stroke and leads to higher healthcare cost.[119-122] Pathophysiology of AF is complex and multifactorial, and has not yet been fully understood.[123-125] Some of the factors suggested to play a role in AF development include genetic predisposition, inflammation and structural remodeling of the atrium.[126]

Post-operative atrial fibrillation (POAF) provides an even more conspicuous challenge, as their postoperative state makes these patients even more vulnerable to comorbidities. POAF occurs in approximately 30% of all cardiothoracic interventions and is associated with increased hospitalization times and readmission rates.[127,128] Lowering the incidence of POAF would significantly reduce the risk of adverse effects and lower healthcare cost.[129] Although drug treatment and rhythm control are currently clinically used with reasonable efficacy.[130-132] it seems undisputed that the preferable solution in reducing the adverse effects is to prevent POAF from developing altogether.[133] While many factors can contribute to the development of POAF and its exact pathophysiology remains up for debate, recent studies have proposed a pivotal role for inflammation and oxidative stress in the development of AF.[126,134-136] In an animal model, reduced incidence of POAF was observed when dogs were treated with anti-inflammatory agents.[137]

Over the past two decades, stem cells in the form of amniotic membrane patches (AMPs) have established a prominent role in eye surgery.[138,139] Justification for its use is based in amniotic stem cells' potential to fight inflammation and thus reduce fibrosis.[140] This anti-inflammatory capacity of amniotic stem cells is further observed in wound healing, where the placement of stem cell-rich AMPs improves the healing process.[141] The potential of AMP is emphasized by the fact amniotic cells are immunosensitized and will therefore not induce a rejection response by the recipient's body.[142] These characteristics make amniotic stem cells of ongoing interest in tissue regeneration.[142-144] Given the anti-inflammatory properties of amniotic stem cells,[145,146] reduction of POAF development in patients treated with AMP has been hypothesized.[147-149]

1.4 CARDIAC ASSIST DEVICES

A common therapy for end-stage heart failure is implantation of a cardiac assist device, generally left ventricular assist device (LVAD) and total artificial heart (TAH).[150] LVADs

intend to improve left ventricular output by continuous flow from the ventricle into the aorta.[151] In a vulnerable group such as end-stage heart failure patients, implantation of LVAD is an invasive intervention, associated with intraoperative and postoperative complications, including bleeding, infection and pump thrombosis.[152,153] Nonetheless this cardiac assist device has demonstrated to greatly improve quality of life during the so-called bridge-to-transplant (BTT) therapy.[154,155] Although developed as a temporary solution, LVADs are a very good option as destination therapy (DT) with the right patient selection, as in this group the highly invasive procedure of heart transplant can be avoided.[156,157] Strict selection of DT patients would also allow the scarcely available cardiac grafts to be assigned to those who benefit most.[158] Additionally, patients that – for various reasons – are not eligible for transplant for various reasons could potentially benefit from DT.[159] Furthermore, the use of LVAD as DT does not negatively influence outcome of the device use itself.[160] As per September 2017, the FDA has granted LVAD approval to be used as DT. TAH, on the other hand, differs from uni- or biventricular assist devices in the fact that it is an orthotopic solution: the native functions of both ventricles and all 4 valves are taken over by the TAH.[161] Survival rates in patients that receive TAH as BTT therapy are significantly higher than those who did not receive device implantation, both during BTT as well as 1-year after cardiac transplant.[162]

Paradoxically, the implantation of a cardiac assist device can itself reduce the chances of successful future transplantation due to unwanted activation of the immune response. During the transplant waiting period, patients' anti-HLA-II antibody production is routinely monitored by the panel reactive antibody (PRA) test. The PRA test is used as an indication of the circulating antibodies to a random panel of donor lymphocytes (usually T lymphocytes) and is expressed as a percentage ranging between 0 and 99. Fundamentally, this test identifies the part of the donor population against which a potential recipient has formed antibodies. Patients with PRA levels of ≥10% test positive and are considered sensitized. Increased humoral sensitization elevates the risk for acute rejection, increases primary graft dysfunction, and shortens survival in patients upon cardiac transplant.[163-166] Increased anti-HLA sensitivity is often seen in patients that undergo device placement, up to approximately 30% of BTT patients.[167-169] Prior to cardiac transplant, various PRA-lowering interventions are considered once the screens are rising.[170,171] Given their immunomodulatory potency, amniotic-derived mesenchymal stem cells could be a preventive agent for sensitization.[138,139] It could be hypothesized that injections of micronized AMP along with mesenchymal stem cells reduce inflammatory response in a manner similar to suggested POAF reduction after AMP placement, thus being a preventive agent for sensitization.[172,173] This could safeguard those patients that receive LVAD or TAH in anticipation of cardiac transplant, and ensure increased PRA levels do not make them unfit for transplant.

1.5 CARDIOPULMONARY TRANSPLANTATION

Despite the many advances in treatment of heart disease, a substantial group of patients will remain symptomatic under optimal medical therapy. There is great heterogeneity among the causes of suboptimal response to therapy as well as the pathophysiological background. In rare cases, cardiac transplantation is indicated in congenital disorders, but more often it is reserved for patients with suboptimal response to all other therapies. For those patients, organ transplantation remains as the only final remedy.

Transplantation and its limitation

The first successful heart transplantation was performed in 1967 by Dr. Barnard and was received with great appraisal by the medical community.[174] Much of the groundwork, however, was covered by Dr. Lillehei, who is considered the father of open-heart surgery.[175] Four years before that, the first lung transplant was performed. The surgery itself was considered to be a success, despite the patient dying within weeks due to nephrotoxicity.[176] While other organ transplant programs have seen enormous improvements over the past decades, cardiac transplantation remains amongst the most challenging. Perioperative and adjuvant therapies including cyclosporine administration and the introduction of monoclonal antibodies have led to more than acceptable transplant outcomes, with 1 and 10-year survival rates as high as 80% and 60%, respectively.[177-179] Nonetheless, the gap between the number of patients on the waiting list and the number of available organs has not been closed. Increasing life expectancy and improvements in the pharmacologic treatment of heart failure patients are responsible for a rise in incidence and prevalence of cardiovascular disease. Over the past years, inclusion criteria for cardiac transplant waiting list have become less strict. In the United States, the inclusion of (ex-)smokers and obese patients has more than doubled the number of candidates on the waiting list for cardiac transplant in the past decade.[180] The number of cardiac transplants performed, however, has barely increased since 1994.[181] Although these numbers clearly illustrate the absolute need for more donors, one other key limitation in heart transplant may have even bigger implications: the high vulnerability of cardiac grafts. Maximal *ex vivo* preservation time for the heart is remarkably shorter than other solid organs, which has led to only 32% of the offered cardiac grafts being transplanted in 2010.[182] The main cause for these shockingly low numbers, is a maximal ischemic time of 4-6 hours for hearts.[183] In the period between donation and transplantation, organs are flushed and submerged in cold preservation fluid. This process aims to suspend metabolic activity and decrease energy utilization, minimizing the accrual of toxic metabolites and damage to the graft.[184] For lung graft preservation, the concepts and limitations are highly comparable, with a maximal cold ischemic time of 8 hours.[185] Given the comparability in transplant outcomes, preservation standards and maximal extracorporeal storage time between hearts and lungs,

this thesis investigates the preservation of lung grafts alongside cardiac graft. Despite many advances in transplantation medicine over the past decades, organ preservation solutions have not changed much. This is particularly important in cardiopulmonary transplant, as prompt adequate graft performance following implantation is essential. Cardiopulmonary graft injury, however, is not completely observable during the preservation period since most damage occurs upon re-oxygenation of the tissue. Once the blood flow is restored, unintentional opening of the mitochondrial permeability transition pore, allowing water to cause mitochondrial swelling and rupture, ultimately causing cytochrome c and other mitochondrial apoptotic factors to induce cell death.[186] Additionally, ROS are formed as part of ischemia-reperfusion injury and can cause direct damage to the transplanted organs.[187,188] Jointly, this leads to the concept that graft preservation is the current main limiting factor in expanding the success rate of cardiopulmonary transplantation. Expanding the maximal ischemic time of hearts and lungs could potentially be a major step toward a global organ sharing program, in which compatibility between donor and recipient would be achieved at a much higher rate and an increased transplantation rate of offered grafts could be accomplished.

Preservation solution

Since their first use in the 1960s, many forms of organ preservation solutions have been developed. Ultimately, all these solutions are based on three common underlying concepts: (1) hypothermic arrest of metabolism, (2) maintaining viability of the tissue during a slowed metabolic state to abate cellular swelling, and (3) minimizing reperfusion injury caused by free radicals and the inflammatory response. In order to achieve these goals, the ionic ingredients included in preservation fluids reduce the transmembrane K+ gradient, rapidly depolarize the myocardial cellular membrane, and stop cardiac electrical activity.[189] The organ storage solutions that have been developed can be categorized into intracellular (Na+ concentration less than 70 mmol/L and K+ concentration ranging between 30 and 125 mmol/L) and extracellular (Na+ concentration greater than or equal to 70 mmol/L and K+ concentration between 5 and 30 mmol/L) organ preservation solutions. Despite the major differences in composition, the post-preservation graft quality is comparable between both solution categories.[190] Given these persistent limitations of current preservation solutions and the lack of significant improvement of *ex vivo* graft durability, it seems imperative to develop novel strategies.

In search for expansion of preservation time, there has been a paradigm shift over the recent years, with an increasing effort focused on preserving organ bioenergetics rather than postponing cellular damage. One rather straightforward development that is of particular interest, is the enhancement of preservation solutions. Expansion of preservation solution with metabolic components and antioxidants have great potential given their relative

ease to use and low cost compared to more advanced alternatives such as continuous organ perfusion. One experimental solution that is of interest is the Harvard-developed Somah (Somahlution Inc, Jupiter, FL). Somah aims to extend the *ex vivo* longevity of organs by supplying metabolic needs rather than minimizing energetic demand, while providing ROS scavenging agents and tissue protection. Studies using porcine hearts have demonstrated the potential benefit of Somah for cardiac transplantation in hypothermic and subnormothermic settings.[191,192] However, in order to fully evaluate a novel solution's mechanism and clinical potential, assessment of *in vitro* effects is required as well as metabolic and redox pathways. Furthermore, there appears to be a deficit in knowledge of the biochemical differences between various organ preservation solutions and their effectiveness in preserving cardiac cellular metabolic and mitochondrial function. Along the same lines, there is a newfound need to better comprehend the mechanisms of action of novel solutions and their potential benefits in an *ex vivo* setting. This is of particular interest given the enormous potential *ex vivo* lung perfusion has shown over the past years. Various trials are currently ongoing with *ex vivo* lung perfusion being mostly used as a rejuvenation tool for marginal lungs and to expand preservation time.[193,194] One hiatus in this research may be the lack of comprehension of metabolics and redox potential in the current clinical practice.

Another perpetual restriction in the availability of donor grafts is the strict circumstances under which organs are donated. There is a primary distinction of organs donated after brain death (DBD) and donated after cardiac death (DCD). DBD settings are more controlled as the body is perfused with oxygenated blood during surgical procurement of each of the transplantable organs until finally the heart and lungs are surgically removed. This in contrast to DCD organ donation, where cardiac arrest has yet been established before procurement of the organs for transplantation. Such circumstances inherently lead to lower organ quality as a result of the increment in ischemic time before proper organ preservation. As such, the Maastricht classification gives an insight into organ quality before procurement, with categories I and II representative of uncontrolled DCD settings and categories III and IV being the controlled DBD organs.[195] The transplant donor gap is expanding for all organs, forcing researchers and medical investigators to explore unconventional solutions. Egan et al. suggested the use of cadaver lungs as early as 1991, demonstrating that 1-hour warm ischemic time did not lead to post-transplantation impairment of gas exchange after 4 hours of cold static storage in canine lungs.[196] In other fields improved techniques and preservation approaches have led to DCD kidneys and livers becoming increasingly acceptable as alternatives for DBD organs.[197-199] Nonetheless, DCD organs are currently deemed unsuitable. This has led to the present situation in which less than 20% of donated lungs are accepted for transplantation, and 10-year survival barely exceeds 30% post-transplant. We aim to clarify the influence of the clinical

standard as well as a novel solution on lungs during preservation in order to further evaluate their limitations and potential, and explore the possibility of utilizing DCD grafts for transplantation.

OUTLINE OF THIS THESIS

Cardiovascular disease continuously underlines its importance as the world's largest epidemic, with both incidence and prevalence of the disease increasing. The severity of cardiovascular disease varies widely, from relatively easy to treat stable angina to end-stage heart failure, requiring organ transplant. While much of the ongoing research is dedicated to improving current therapies, some underlying mechanisms of treatments are underappreciated and poorly understood, specifically concerning cardiac surgical interventions. This thesis investigates the metabolic aspects of cardiovascular disease and end-stage heart failure and explores mitochondrial bioenergetics of heart and lung transplant. Additionally, (anti)inflammatory facets in cardiac surgery and its complications are studied.

Chapter 2 of this thesis investigates metabolic changes in isolated mitochondria of the hypo-perfused heart compared with non-ischemic patients' myocardial samples. Respiratory control rates and complex I of the electron transport chain appear to be diminished in atrial tissue of patients undergoing CABG. **Chapter 3** of this thesis demonstrates the influence of cardiac assist device implantation on the body's resting energy expenditure, comparing LVAD and TAH. The results in this chapter indicate an increase in patients' REE after TAH implantation, which puts these patients at further risk of peri-operative complications.

Chapter 4 reviews the difficulties that are faced in cardiac transplantation, exploring the role of the mitochondrial permeability transition pore in IRI after hypothermic organ preservation. Additionally, (dis)advantages of various traditional and novel preservation solutions are discussed. Based on this review, preservation capacity of various solutions is investigated on rat myoblast cells in **Chapter 5**, suggesting improved cytoprotection and safeguarded mitochondrial function in experimental Somah solution when compared to conventional preservation solutions. Translating this work into a pulmonary transplant setting, **Chapter 6** explores the differences in metabolomics of porcine lungs during cold static storage in standard solution and Somah. These experiments demonstrate that Somah creates a favorable milieu for cold static ex vivo preservation over an extended time period. This is found in both the preservation media as well as in tissue samples obtained throughout the experiment.

Next, in **Chapter 7** the anti-inflammatory potential of the amniotic membrane patch (AMP) in cardiothoracic surgery is investigated. In a clinical case of complicated constrictive pericarditis, it is suggested that the anti-inflammatory properties of AMP contribute to the full recovery. This aspect of AMP is further investigated in **Chapter 8** when we present a case-control series in which micronized amniotic membrane patches are injected into the patient's heart upon cardiac assist device placement. The patients in this series appear to be less prone to immunosensitization, improving their chances of being well-suited as an organ recipient. In **Chapter 9** a strong trend towards significant reduction in atrial fibrillation incidence after cardiac surgery is observed when multiple patches are placed on the epicardium during intervention. The results suggest that AMP is a preventive agent for postoperative AF in a vulnerable patient group.

A summary, general discussion and the future perspectives of the presented work are jointly described in **Chapter 10** of this thesis.

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Chapter 2

Chronic myocardial ischemia leads to loss of maximal oxygen consumption and complex I dysfunction

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ABSTRACT

Background

Cardiomyocytes rely heavily on mitochondrial energy production through oxidative phosphorylation. Chronic myocardial ischemia may cause mitochondrial dysfunction and affect ATP formation. Metabolic changes due to ischemia alters cardiac bioenergetics and hence myocardial function and overall bioenergetic state. Here we evaluate differences in functional status of respiratory complexes in mitochondrial isolates extracted from left atrial appendage tissue (LAA) from patients undergoing cardiac surgery, with and without chronic ischemia.

Methods

Mitochondrial isolates were extracted from LAA in ischemic CABG patients (n=8) and non-ischemic control patients (n=6) undergoing other cardiac surgery (valve repair/replacement). Coupling and electron transport chain assays were performed using Seahorse XF^e 96 analyzer. Oxygen consumption rates (OCR) were measured to calculate respiration states.

Results

Respiratory control rate (RCR) in ischemic patients was significantly lower than control patients (6.17±0.27 vs 7.11±0.31, respectively; $p<0.05$). This is the result of minimal, non-significant state 3_{ADP} and state 4_O changes in chronic ischemia. Complex I respiration is diminished in ischemic tissue (99.1±14.9 vs 257.8±65.2 in control; $p<0.01$). Maximal complex I/II respiration ratio was significantly lower in ischemic patients (58.9±5.5 vs 90.9±8.8 percent; $p<0.05$), a difference that was also seen in complex I/IV ratios ($p<0.05$). There was no significant difference in complex II/IV ratios between groups.

Conclusions

Ischemic patients have aberrant mitochondrial function, highlighted by a lowered RCR. All ratios involving complex I were affected, suggesting that the insufficient ATP formation is predominantly due to complex I dysfunction. Complex II and IV respiration may be impaired as well, but to a lesser extent.

INTRODUCTION

The heart relies heavily on oxidative phosphorylation (OXPHOS) as its main source of energy, as ventricular and atrial contraction require large amounts of ATP. When oxygen delivery to the myocardium is suboptimal, ATP production through OXPHOS is lowered from approximately 90% of total cardiac ATP production to an estimated 60% [1, 2]. It could be speculated that chronic cardiac hypoperfusion may result in dysfunctional, but viable myocardium that is eventually remodeled in response to chronic recurrent ischemic events [3]. Proteins and enzymes involved in OXPHOS and the mitochondrial electron transport chain (ETC) are down-regulated during chronic ischemia [4]. These changes create a 'safeguarded' environment for a hypoperfused heart, providing protection against necrosis upon ischemia and reperfusion, as seen in ischemic preconditioning [5, 6]. Protection is achieved by limiting respiration in complexes I, II and III; the primary sources of reactive oxygen species (ROS) production. Mitochondrial changes found in the chronic ischemic myocardium are often only partially reversible upon restoring oxygen delivery [7], which is reflected clinically by the substantial number of patients that experience incomplete recovery of contractility following coronary artery bypass grafting (CABG) [8]. Chronic ischemic myocardium is associated with increased cardiomyocyte apoptosis and formation of fibrotic tissue, elucidating the importance of early blood flow restoration [7, 9]. Additionally, bioenergetic alterations seen in chronic myocardial ischemia are characterized by loss of mitochondrial respiration. Furthermore, uncoupling of OXPHOS has been suggested due to up-regulation of mitochondrial uncoupling proteins and is reflected by a decrease in respiratory control ratio (RCR) [10]. RCR in isolated mitochondria is determined by the ratio of ADP-driven respiration (state 3_{ADP}) to proton leak (state 4_O) [11]. Lowered OXPHOS capacity in tissue that has been exposed to extended ischemic periods could partially be explained by mitochondrial complex content [4]. However, while changes in RCR are an excellent method of revealing OXPHOS defects, these findings are non-specific, as any complex can function as a bottleneck for the effects observed upstream. Thus, it is important to investigate mitochondrial respiration on a complex-to-complex basis. The present study analyzed mitochondrial respiration states and complex-based respiration in order to investigate the mitochondrial bioenergetics of human heart left atrial appendage (LAA). The mitochondrial respiration was determined in freshly isolated human heart mitochondria using a Seahorse XF^e 96 analyzer. Additionally, electron transport chain assays were performed to assess each individual mitochondrial complex. Using this approach, changes in cardiomyocyte mitochondrial function were assessed in patients with ischemic cardiomyopathy who underwent CABG surgery compared to patients without any history of ischemic heart disease who underwent cardiac surgery.

METHODS

Patient samples

The LAA was ligated with a 40mm AtriClip (AtriCure, Mason, OH) from patients undergoing open heart surgery. All patients in both groups had the LAA ligated prior to the administration of cardioplegia, within minutes after cardiopulmonary bypass was initiated. Samples were immediately placed in ice-cold PBS and processed further in the lab, ensuring minimal ischemic time. Samples that had not begun mitochondrial isolation within 30 minutes of surgical removal were excluded from the study. The experimental (ischemic) group (n=8) consisted of subjects receiving CABG. Control samples (n=6) were from patients without coronary artery disease and with preserved ejection fraction who underwent aortic valve replacement surgery. Only patients undergoing cardiac surgery with a clinical indication for LAA removal were included. The LAA is removed by the surgeon, and regarded as clinical discard. In accordance with U.S. Department of Health & Human Services 45 CFR 46.101(b)(4), this tissue is exempt from informed consent, as it has been completely de-identified to the researchers involved. Judgement for the removal of the LAA was made by the surgeon independent of the research.

Isolation buffers

The formulation of the three buffers used for isolation was adapted and adjusted from Palmer et al [12]. Buffer A consisted of 20 mM mannitol, 70 mM sucrose and 5 mM HEPES in 100 mL ddH_2O. Buffer B was buffer A enriched with 2 mM EGTA and 0.2% fatty acid-free BSA. Lastly, buffer C was composed of buffer A, supplemented with 0.05 mM EGTA. Each solution was pH adjusted to 7.2±0.05 using KOH. All chemicals were purchased from Sigma-Aldrich.

Mitochondrial analysis reagents

Seahorse XF^{e}96 analyzer and XF^e 96-well plates were purchased through Seahorse Bioscience (North Billerica, MA). The mitochondrial assay solution was prepared in ddH_2O. Mitochondrial assay solution used for the mitochondrial coupling assay was enriched with 10 mM succinate and 2 µM rotenone and adjusted to pH 7.2±0.05 using KOH. Assay solution used for the electron transport chain assay was supplemented with 2 mM malate, 4 µM FCCP and 10 mM pyruvate, pH adjusted to 7.2±0.05.

Mitochondrial coupling assay

Ports A-D of the Seahorse XF^e cartridge were loaded with BSA-free MAS containing drugs that were injected consequently: 40 µM ADP, 25 µG/mL oligomycin, 40 µM carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) and 40 µM antimycin

A. Oligomycin, FCCP and antimycin A were reconstituted in 95% EtOH and ADP was reconstituted in ddH_2O (pH 7.2). All stock solutions were stored at -20°C.

Mitochondrial electron transport chain assay

Ports A-D of the Seahorse XF^e cartridge were loaded with BSA-free MAS containing drugs that were injected consequently: 20 µM rotenone, 100 mM succinate, 40 µM antimycin A and a combination of 1 mM N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) and 100 mM ascorbate. Stock solutions of rotenone, antimycin and TMPD were prepared in 95% EtOH and stored at -20°C, ascorbate and succinate stock were prepared in ddH_2O (pH 7.2, -20°C).

Mitochondrial isolation

Mitochondria were isolated by differential centrifugation techniques as described by others [12, 13]. Isolation procedures were performed in a 4°C cold room. Adipose and connective tissue were excised from the sample, leaving 1.5-2 grams of pure LAA muscle. The tissue was then placed in a petri dish with 5mL of buffer A and minced with iris scissors for 5 minutes. The tissue was washed sequentially with three 5mL aliquots of buffer A.

The minced tissue and 5mL of buffer A were transferred to a potter-elvehjem homogenization tube. Five hand-driven homogenization passes were performed while submerged in buffer A using the Teflon pestle. Centrifugation followed at 700 x g at 4°C for 10 minutes. The supernatant was collected and centrifuged three times at 20,000 x g at 4°C for 10 minutes. After each centrifugation step, the supernatant was discarded and the pellet was re-suspended in 5mL of buffer B, buffer A, and buffer C respectively. Before the final re-suspension in buffer C, the outer lipid layer of the pellet was carefully removed and discarded. This washing step was performed with 200 µL of buffer A to remove any contaminants, paying close attention that the pellet remained intact. The washing solution was then discarded, and the pellet re-suspended in 150 µL of buffer C. The suspension was transferred to an Eppendorf tube and stored on ice.

Protein Quantification

First a 1:10 dilution from the mitochondrial stock was prepared in phospholipase c (PLC) buffer. Serial dilutions of 1:20 and 1:40 dilutions were prepared and all 4 aliquots were quantified for consistency. Concentrations of mitochondrial proteins were quantified using the BCA Protein Assay Kit (Pierce™, Thermo Scientific, Waltham, MA).

Seeding mitochondria

After protein quantification of the mitochondrial isolates, a 1:10 dilution was made in ice-cold 1X MAS in order to ensure homogenous mitochondrial distribution. Mitochondria

were seeded in a chilled Seahorse XF^e 96-well plate by placing 25 µL of mitochondrial suspension, containing either 0.04 µg/µl or 0.08 µg/µl. The 96-well plate was centrifuged at 2,000 x g for 20 minutes at a temperature of 4°C to ensure attachment of suspended isolates to the bottom of each well [14]. Following centrifugation of the 96-well plate, all wells were replenished with warm 1X MAS, up to a total volume of 180 µL per well. The plate was then placed in the XF^e 96 analyzer.

Assay conditions

Homogenous and gradual warming of the plate was ensured by waiting 8 minutes before the oxygen consumption rate (OCR) was measured throughout the mitochondrial assay in real-time, using the Seahorse XF^e 96 analyzer. In this machine, probes create a micro-chamber inside each of the 96 wells of an experimental plate and measure O_2 tension. The change in O_2 tension over time during a measurement is translated into OCR (pmoles O_2/minute). Following each measurement period, the probes withdraw from the micro-chamber and allow the solution in each well to re-oxygenate.

Between measurement periods, drugs were administered to the wells through injection ports built into the measuring probes. The drugs injected during the *coupling assay* give insight into the respiration states of the mitochondria. Sequentially, state 2, state 3_{ADP}, state $4_{O,}$ state $3_{U,}$ and non-mitochondrial respiration state were determined during this assay, normalized per gram of mitochondrial protein. These respiration states were used to calculate the RCR, which is calculated by the ratio of state 3_{ADP} (RCR_{ADP}) or state 3_U (RCR_U) to state 4_O. The *ETC assay* measures complex I respiration in the presence of FCCP, pyruvate and malate before blocking complex I by rotenone. Subsequently, complex II is measured by addition of succinate and then blocked by antimycin A. Lastly, complex IV OCR is measured by addition of TMPD and ascorbic acid. An examplary representative graph of both assays, used to determine mitochondrial function, is displayed as a reference (**Figure 1**).

Statistical analysis

Each experimental group consisted of a minimum of 6 wells per experiment. Statistical significance between groups was calculated using unpaired student's t-tests at a confidence interval of 95%. All values presented are averages ± SEM. Calculations were performed using GraphPad Prism 6 (La Jolla, CA).

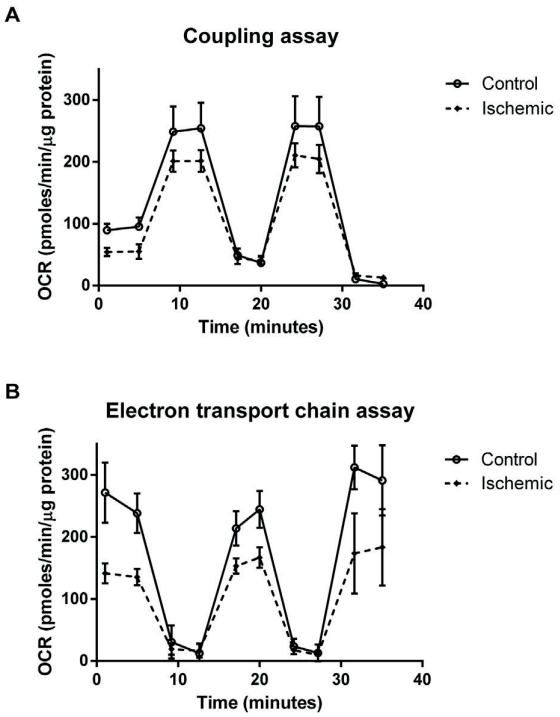

Fig 1. Representative Seahorse XF assay on isolated mitochondria. (A) Illustrative example of mitochondrial coupling assay performed on mitochondria isolated from chronic ischemic and control left atrial appendage (LAA), normalized per µG of mitochondrial protein. (B) Electron transport chain assay performed on the same isolates of chronic ischemic and control LAA. Each value shown has a minimum of 6 replicates, with error bars indicating SEM. (OCR = oxygen consumption rate.)

RESULTS

Mitochondrial respiration states

Mitochondrial respiration did not demonstrate significant differences between groups in State 2 (p=0.29), State 3_{ADP}, State 4_O, or State 3_U (p=0.21) (**Figure 2**). However, RCR_{ADP} measured in the ischemic group (6.17±0.27) was found to be significantly decreased compared to controls (7.30±0.32) ($p<0.05$; **Figure 3A**). The decline observed in RCR_{ADP} is a result of a combination of non-significantly lowered state 3_{ADP} respiration in ischemic samples (220.7±21.9) relative to the control group (245.6±17.9) (p=0.40; **Figure 2**) and increase in state 4_O respiration wihout statistical difference (p=0.40) between ischemic and control groups (36.56±4.25 and 31.87±3.10 respectively; **Figure 2**). RCR_U displayed similar results as seen in RCR_{ADP}, with significant decreased values in ischemic samples ($p<0.05$; **Figure 3B**). Lastly, the ratio between RCR_{ADP} and RCR_U was not found to be different between both groups (p=0.08; **Figure 3C**), indicating no change in coupling potential of ADP compared to uncoupled state.

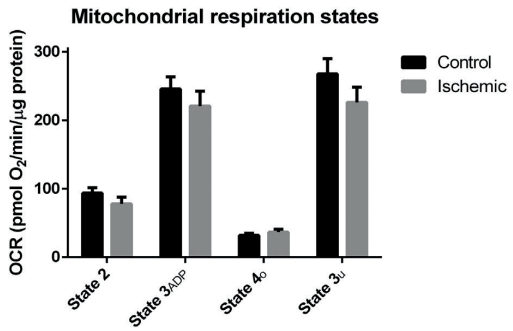

Fig 2. Mitochondrial respiration states in ischemic and control patients. Oxygen consumption rates of isolated mitochondria from chronic ischemic left atrial appendage (LAA) compared with isolated
mitochondria of control LAA, normalized per µG of mitochondrial protein. Mitochondrial assay media was enriched with rotenone and succinate to induce state 2. State 3_{ADP}, state 4_O, and state 3_U were achieved by the sequential addition of ADP, oligomycin, and FCCP, respectively. (OCR = oxygen consumption rate.)

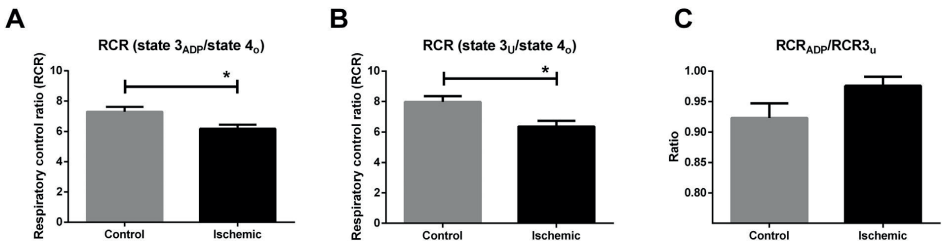

Fig 3. Respiratory control ratios in ischemic and control patients. (A) Respiratory control ratio (RCR) was determined as the ratio of state 3_{ADP} and state 4_O. (B) RCR was determined as state 3_{ADP} and state 4_O. (C) Both ratios were then compared. All values displayed are mean ± SEM, * indicates $p < 0.05$.

Electron transport chain assay

The ETC assay provided significantly altered uncoupled complex I respiration in ischemic patients (99.1±14.9), with lower values than controls (257.8±65.2) ($p<0.05$; **Figure 4A**). Ischemic tissue (162.4±16.11) demonstrated a decrease in succinate-driven complex II respiration compared to controls, bordering significance (258.5±47.6) ($p=0.05$; **Figure 4B**). Similarly, complex IV respiration in ischemic samples (172.6±20.2) showed a decrease on the verge of significance compared to controls (317±72.8) ($p=0.05$; **Figure 4C**) when mitochondria were stimulated with TMPD and ascorbic acid.

Additionally, in order to determine the relative change in OCR, respiratory complex ratios were calculated per complex from the absolute OCR. Ischemic tissue exhibited lower relative respiration (60.2±4.63 percent) in the complex I/complex II ratio than controls (96.9±7.0 percent) ($p<0.01$; **Figure 5A**). A similar decrease was seen in the complex I/complex IV ratio between groups ($p<0.05$; **Figure 5B**) There was no statistically significant difference between the groups in complex II/complex IV ratio, with 95.6±4.8 percent found in ischemic samples and 83.8±7.4 percent in controls ($p=0.20$; **Figure 5C**).

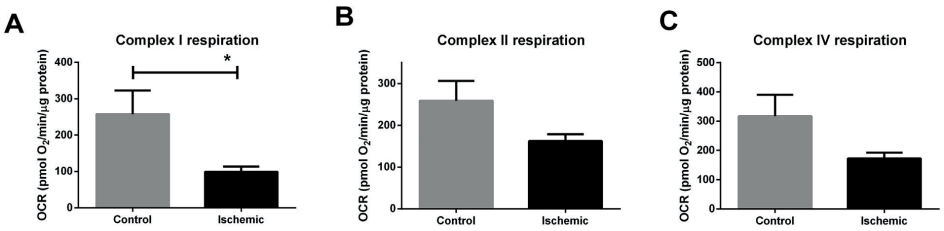

Fig 4. Mitochondrial complex driven respiration in ischemic and control patients. Oxygen consumption rates (OCR) of isolated mitochondrial from ischemic left atrial appendage (LAA) compared with isolated mitochondria of control LAA, normalized per µG of mitochondrial protein.

Mitochondrial assay media was enriched with malate, FCCP, and sodium pyruvate to induce complex I respiration. (A) Complex I respiration, measured as the resulting OCR after subtraction of rotenone driven OCR. (B) Complex II respiration, measured as the succinate driven respiration after subtraction of the antimycin A OCR. (C) Complex IV respiration, measured as the TMPD and ascorbate driven respiration after subtraction of antimycin A respiration. All values displayed are mean ± SEM, ** indicates $p < 0.01$, * indicates $p < 0.05$.

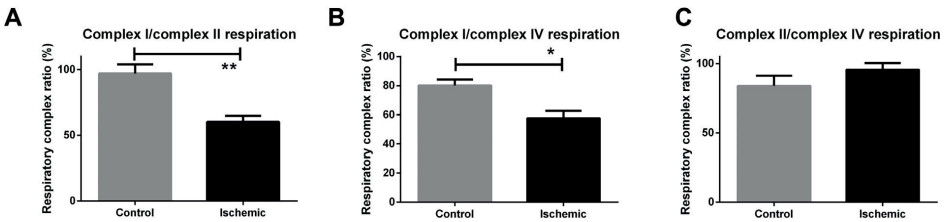

Fig 5. Mitochondrial complex respiration as ratios. Respiratory complex ratios (%) of isolated mitochondrial from ischemic left atrial appendage (LAA) compared with isolated mitochondria of control LAA. (A) Complex I respiration as a ratio of complex II respiration. (B) Complex I respiration as a ratio of complex IV respiration. (C) Complex II respiration as a ratio of complex IV respiration. All values displayed are mean ± SEM, * indicates $p < 0.05$.

COMMENT

Chronic myocardial ischemia is associated with a number of changes in mitochondrial morphology and function, which to date has been studied almost exclusively in animal models (**Table 1**). The present study intended to translate current animal models into a clinical human setting. The main findings in the present study demonstrated a decrease in RCR and complex I respiration in coronary artery disease patients with chronic ischemia.

Animal studies have shown a reduction in RCR by lowered state 3 respiration, indicating that mitochondrial ATP production through OXPHOS is suboptimal during myocardial ischemia [10, 15, 16]. Additionally, reduced mitochondrial complex protein expression was found in ischemic cardiac tissue [4]. Despite partial improvement in RCR after revascularization, mitochondrial protein expression showed no recovery [17]. However, in these studies it remains unclear what part of the ETC is responsible for the observed results. Moreover, despite the important role animal studies have in understanding isch-

TABLE 1
Mitochondrial Research Performed in Both Animal and Human Models of the Ischemic Myocardium

		Chronic Ischemia				
Author	**Species**	**Induction**	**Duration**	**Material used**	**Assay**	**Major findings**
Lim et al (1999)	Porcine	Fixed stenosis (1.5-2.0mm)	3 months	Cardiomyocytes	TUNEL assay	Increased apoptosis, regional and remote
Fallavollita et al (2002)	Porcine	Fixed stenosis (1.5 mm)	3-4 months	Blood samples	Blood gas analysis	Decreased OCR in rest
						Normal pH and lactate
McFalls et al (2006)	Porcine	Fixed stenosis (1.4 mm)	2-3 months	Isolated mitochondria	Clark electrode	Preserved state 3 respiration
						Reduced RCR (state3/state2)
						Increased UCP2 expression
Hu et al (2009)	Porcine	Fixed stenosis (1.5 mm)	3 months	Isolated mitochondria	Clark electrode	Reduced state 3 respiration LAD region
						Reduced RCR (state3/state2)
Kelly et al (2011)	Porcine	Fixed stenosis (2.0mm)	3 months	Isolated mitochondria	Clark electrode	Improved but still depressed RCR after CABG
						No improvement in complex I, II, III, IV or V proteins after CABG
Cabrera et al (2013)	Porcine	Fixed stenosis (1.5-2.0mm)	3 months	Isolated mitochondria	Clark electrode	No changes in state 2 or state 3 respiration
					Protein quantification	Reduced complex I, III, IV and V proteins
Page et al (2013)	Porcine	Fixed stenosis (1.5-2.0 mm)	3 months	Isolated mitochondria	Clark electrode	Reduced state 3 respiration LAD region
Stride et al (2013)	Human	Multi-vessel stenoses	Unknown	Isolated mitochondria	High resolution respirometer	Decreased combined complex I + II OCR
						Decreased complex II OCR
						Comparable bioenergetics in all 4 heart chambers
					Protein quantification	Reduced complex V presence
Schipper et al (2016)	Human	Multi-vessel stenoses	Unknown	Isolated mitochondria	Seahorse XF Analyzer	Reduced RCR (state3/state4)
						Decreased complex I, II-III and IV OCR
						Complex I decrease higher than other complexes

CABG = coronary artery bypass grafting; LAD = left anterior descending; OCR = oxygen consumption rate; RCR = respiratory control ratio; UCP2 = uncoupling protein 2.

emic myocardium, there is a critical or crucial necessity for research in human samples. An interesting study by Stride et al demonstrated lowered OXPHOS in the human heart in regions of chronic ischemia, without changes in RCR [6]. Ischemic samples in this study were compared to non-ischemic areas of the same subjects. This may be a notable limitation to the results, as Lim et al suggested apoptosis in remote areas of the chronic ischemic myocardium [18, 19]. Moreover, recent work presented by Matam et al demonstrated mitochondrial DNA mutations in right atrial appendage tissue of patients with coronary artery disease, highlighting the diffuse effect of ischemia on the heart [20]. Although not an ideal model, as those observations may not necessarily translate to the non-ischemic heart, the authors feel that the utilization of LAA tissue for human mitochondrial studies is an acceptable alternative for the left ventricular tissue. Therefore, in the present study we set out to investigate the individual role of the mitochondrial complexes in widespread RCR despression, using LAA tissue from patients undergoing CABG, compared with non-ischemic controls. The results of the present study demonstrate that a decrease in the RCR is a result of complex I dysfunction. With complex II and IV very closely reaching significant values, it is plausible these complexes may play a role in mitochondrial dysfunction as well. RCR was measured in order to attain a global understating of the mitochondrial respiration status. There was a significant decrease in ischemic patient samples for both ADP stimulated and uncoupled (FCCP) RCR with rotenone and succinate present in the assay media. The insignificant difference in ratio between RCR_{ADP} and RCR_{U} suggests that the changes seen in ischemic disease are attributable to mitochondrial dysfunction rather than incomplete substrate delivery. The significant changes seen in RCR are a result from an insignificant state 4_O increase and state 3 decrease. This points out that multiple angles are involved in the effects seen in chronic ischemia. Slightly altered ADP utilization as well as marginally altered proton leak, together lead to mitochondrial damage. The insignificant increase in state 4_O respiration in chronic ischemic tissue, which ultimately lead to RCR changes, could be a result of mitochondrial uncoupling, as suggested in the findings by McFalls et al [10], demonstrating increased uncoupling proteins. These findings are consistent with the known pathophysiology of chronic ischemic myocardium, and may contribute to a decrease in myocardial contraction. Conversely, these findings could also be interpreted as a protective aspect of chronic ischemia, where lowered OXPHOS and increased proton leak by uncoupling reduces ROS production and provides a stress-resistant state during repetitive ischemic events. In addition to the respiration states, the electron transport chain was investigated by assessing each individual mitochondrial complex. In ischemic samples we found that the OCR in complex I was significantly decreased. Maximal OCR loss in complex II and complex IV closely approach, but don't reach significant values. Subsequently, OCR ratios between complexes were calculated to interpret the magnitude of the oxygen consumption loss in each of the complexes. All ratios involving complex I were reduced in chronic ischemic samples, suggesting that

the principal impairment in respiration occurred at the level of complex I. The effects of chronic ischemia on state 3_{ADP} and state 4_O are small, but noticeable when put in the perspective of RCR values. However, the loss of maximal complex I respiration is very prominent.

While the use of human cardiac tissue represents a strength of the current study, there are some inherent limitations. Firstly, the amount of tissue obtained from the left atrial appendage ligation procedure is typically small, especially once the adipose tissue is trimmed. Consequently, the amount of tissue available for measurements was not sufficient to allow any further analyses such as protein gels, mitochondrial complex enzyme activity or citrate synthase assays. Such additional analyses would have provided important further information on mitochondrial function, and should be the topic of future studies. Secondly, all data in the present study were obtained in left atrial tissue, rather than left ventricular tissue. Although we recognize that the results obtained in left atrial tissue cannot be equated to results in left ventricular tissue, it is of interest to note that comparable mitochondrial bioenergetics were reported for the four cardiac chambers in patients with ischemic heart disease [21]. Moreover, microvascular changes were recently observed in the left atrial appendage of patients with ischemic heart disease, undergoing CABG surgery [22], that are similar to the microvascular changes found in chronically ischemic left ventricular myocardium [23]. However, while these findings suggest that metabolic and vascular responses to ischemic heart disease in atria and ventricles share similarities, we fully acknowledge that future studies are required to investigate mitochondrial function of left ventricular myocardium in patients with ischemic heart disease. Notwithstanding these limitations, the findings in the present study could be interpreted to suggest that the bioenergetics capacity of complex I may play a pivotal role during chronic ischemia of the human heart. Thus, the inability to consume oxygen at the same rate as non-ischemic tissue under ideal circumstances, in combination with lowered RCR values in ischemic samples, points to complex I as a major limiting factor.

In conclusion, the present study describes the effects of chronic myocardial ischemia on mitochondrial bioenergetics in the human heart. In ischemic hearts, RCR is reduced through lowered maximal oxygen consumption capacity and heightened proton leak, which may be a result of its cyto-protective state. This effect is seen primarily in complex I. Complex II and complex IV respiration are also affected, albeit to a lesser extent. Restoration of perfusion in ischemic myocardium is essential in patients with multi-vessel coronary artery disease. Failure to do so may lead to irreversible mitochondrial alterations and the formation of fibrotic tissue in chronically hypoperfused regions. Specific molecular targeting of mitochondrial bioenergetics could be used as a novel, adjunctive therapy to improve myocardial preservation and improve CABG surgery outcomes. Clini-

cal strategies to reduce mitochondrial damage could improve ATP production through OXPHOS, which would allow complete restoration of cardiac function and improve clinical outcomes.

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Chapter 3

Metabolic shift following mechanical circulatory device implantation in heart failure patients

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ABSTRACT

Background

End-stage heart failure patients, who are commonly energy depleted and at risk for cardiac cachexia, require surgical device placement as a bridge to cardiac transplant. However, this procedure can alter their energy requirements. Therefore, we compared their pre-operative metabolic requirements to their metabolic state after the implantation of a total artificial heart (TAH) or a ventricular assist device (VAD).

Methods

Before and after the implantation of cardiac assist devices, we measured the resting energy expenditure (REE) of chronic heart failure patients via metabolic carts, a non-invasive form of indirect calorimetry.

Results

Patients who underwent TAH placement (2632±247.9) demonstrated a significant increase in metabolic requirements compared to the pre-operative (PRE) group ($p<0.05$), but not in comparison to the PRE TAH group exclusively ($p=0.24$). When compared to the PRE group ($p=0.70$) and the pre-operative VAD group exclusively ($p=0.48$), there were no statistically significant differences in REE following VAD placement (1994±141.4).

Discussion

Our results indicate a device-dependent increase in REE after TAH placement. This may negatively contribute to the risk of cardiac cachexia among such vulnerable patients. However, adjuvant-targeted therapy may benefit those who are at a high risk of malnutrition and cardiac cachexia.

BACKGROUND

Cardiac transplantation is the current gold standard therapy for patients suffering from end-stage heart failure. As the gap between eligible donor hearts and patients awaiting transplant widens, however, the implantation of ventricular assist devices (VAD) is potentially a life-saving one. Likewise, the total artificial heart (TAH) offers those not eligible for VAD placement an alternative while waiting for cardiac transplantation.[1,2] These devices provide a bridge-to-transplant by offloading the heart while mechanically maintaining adequate blood flow in patients. Still, the type of device implanted into heart failure patients may alter energy demand. Placed in patients with left ventricular failure, VADs use a continuous flow pump to aid in perfusion. For patients who suffer from severe, biventricular failure, which is generally defined as a left ventricular ejection fraction of <25% and right ventricular ejection fraction of <20% , both ventricles are removed and replaced with a TAH that consists of two pulsatile pumps that drive both left and right ventricular blood flow.[2,3] Patients with severe chronic heart failure (CHF) have challenges with their nutritional status, including early satiety, anorexia, nausea, and delayed gastric emptying.[4] Additionally, they can suffer from cardiac cachexia, a low-grade systemic inflammatory response that is an independent risk factor for mortality.[5,6] Inflammatory and neurohumoral elements can lead to an imbalance between anabolic and catabolic pathways, which causes cardiac cachexia.[7] Development of cardiac cachexia is a complex process that is influenced by multiple factors, including malnutrition, dietary deficiencies, and intestinal dysfunction.[8,9] Given the poor prognosis of those who suffer from poor nutritional status, malnutrition, or cachexia, it is of vital importance to monitor and manage the anabolic and catabolic homeostasis of CHF patients. Prior to and after cardiac device placement, it is extremely important to assess the nutritional status of such patients in order to provide adequate medical nutrition therapy that can decrease morbidity and mortality.[4] Though individualized, such an assessment includes diet and weight history, body mass index (BMI), the evaluation of nutritional needs for other medical issues, and, if present, a diagnosis of malnutrition. It has been demonstrated that CHF patients have an average calorie deficit of -186.3 +/- 305 before the placement of a cardiac device, and that they are typically unable to meet their estimated nutrient needs compared to the average healthy adult patient.[10] BMI may also play a significant role in surgical outcomes because, following LVAD implantation, those with a low BMI are at an increased risk of mortality.[11-13] In patients with altered metabolic states and demands, nutrient requirements can vary widely[14] and cannot always be accurately assessed using predictive equations. In these situations, indirect calorimetry is the most accurate way to determine the resting energy expenditure (REE) and can provide a valuable tool to help ensure that patients are able to meet their calorie requirements pre and post operatively in order to improve and maintain nutritional status and overall outcomes[14]. Indeed, it has been found that those with CHF

have a higher REE, making it important to design medical nutrition therapy that accounts for this finding. However, little is known about the change in nutrient demand after the placement of a cardiac assist device.

For this study, we measured the REE of CHF patients via indirect calorimetry. We investigated the device-dependent effect on basal metabolic needs in patients following implantation. We hypothesized that the TAH restores circulation and leads to higher REE values, while the VAD only marginally influences REE.

METHODS

We measured the metabolic status of 18 CHF patients who were eligible for device placement (the PRE group) between January 2011 and August 2015 (**Table 1**). They were awaiting VAD (n=15) or TAH (n=3) placement. Following implantation, we again measured their metabolic states. Only 12 of 15 patients who were assigned a VAD had post-operative data, as three subjects passed away before post-operative results were obtained. One subject in the TAH group exclusively had available post-operative results because of the emergent nature of the intervention at the time. All surgeries were performed at the Banner University Medical Center, Tucson (AZ), and data collected under a University of Arizona approved IRB protocol. [IRB #1605601604].

To determine a patient's metabolic state, REE was quantified by indirect calorimetry, measuring oxygen consumption and carbon dioxide production. Every liter of oxygen used is equivalent to 5 kcal. The final value was calculated using the Weir equation[15]:

[3.94(VO2) + 1.11(VCO2)] 1.44 = 24 hr KCAL

REE was measured preoperatively within one week before surgery, and postoperatively within 14 days after device placement. We used Bonferroni multiple comparisons test to determine statistic outcomes. All results presented are mean ± standard error of the mean. Calculations were performed using GraphPad Prism 6.0.

RESULTS

Of all the preoperative patients, the overall REE was measured at 1934±81.9. We found that the change in REE following VAD placement (1994±141.4) was not significantly different from preoperative values (p=0.70) (**Figure 1A**). Patients who underwent TAH

placement (2632±247.9) demonstrated a significant increase in metabolic requirements compared to the PRE group ($p<0.05$) (**Figure 1A**). When comparing the post-operative VAD and TAH results with only the respective pre-operative VAD and TAH data, instead of the total PRE group, no significant differences were observed ($p=0.49$ and $p=0.24$, respectively) (**Figure 1B**).

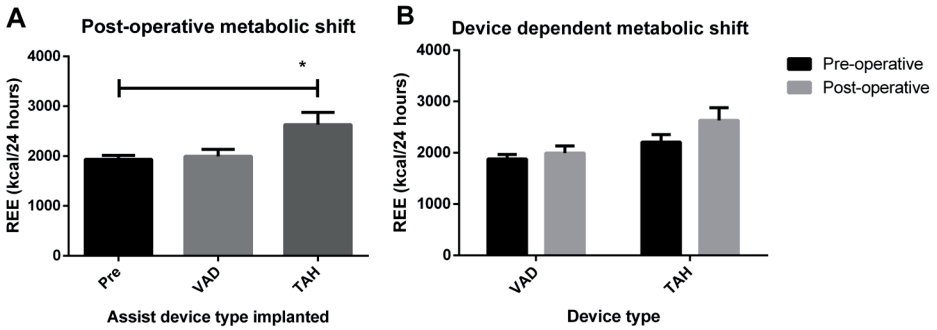

Fig. 1.
A Post-operative metabolic shift pre-operative patients' REE (n=18) compared to post-operative values of both VAD (n=12) and TAH (n=4) patients. All displayed values are mean ± SEM, with * indicating $p<0.05$.
B Device dependent metabolic shift change in REE per device. VAD patients' pre-operative REE are compared with values following VAD implant, and the same is done in TAH patients. All displayed values are mean ± SEM.

DISCUSSION

Patients suffering from heart failure often have increased metabolic demand and are at risk for cardiac cachexia, if their metabolic needs are not sufficiently met. Regarding the resting caloric demands in end-stage heart failure patients, this study confirms the findings demonstrated by others.[16-18] Additionally, the present study suggests that the REE is further increased in patients after TAH implantation, but not following VAD placement. This increase in metabolic need after TAH implantation was found in comparison to the entire PRE group, but not when compared exclusively to the pre-operative TAH values. Implantation of TAH or VAD is often performed in these patients as a way to lower myocardial workload and increase cardiac output. Our study investigates the effect of device implantation on the patients' basal metabolic states. This may be indicative of altered pre-surgical metabolic status in patients awaiting TAH placements. The TAH group exhibits increased REE pre-operatively and, although this observation is not statistically significant, it is plausible that the severity of heart failure in TAH patients would exacerbate the resting energy needs. Lack of a significant result in this group could very well be caused by the fact that only 4 patients underwent TAH placement during this study, and of those 4, only 3 were subjected to pre-operative measurements. Notwithstanding the fact that this group is underpowered, making it hard to draw conclusions, there appears

to be a clear trend towards increased REE. In addition to this limitation, another factor of influence is the surgical procedure performed on TAH patients, which is associated with increased surgical trauma. During TAH placement, both ventricles are excised from the patient's heart and replaced with pneumatic pumps, whereas VAD implantation is generally performed by apex cordis excision. Increased levels of REE are closely linked with degree of trauma or injury.[19-21] Thus, following TAH placement, an increase in REE due to surgical trauma is expected.

We conclude that TAH implantation alters metabolic needs in patients suffering from heart failure. Monitoring the shift in resting energy requirements in patients that receive a cardiac assist device would enable us to better understand the metabolic demands following surgery, and manage accordingly. In this patient group that is already at risk before surgery, avoiding malnutrition can be of enormous benefit. Moreover, it is essential to prevent cardiac cachexia in these patients. Lastly, as cardiovascular diseases are increasingly considered to be a pandemic along with the ever-increasing transplant-gap, cardiac assist devices are promoted as destination therapy rather than simply a method of bridging the time until cardiac transplant.[22-24] Adequate understanding of the metabolic needs of this relatively new group could be essential in the outcome of cardiac assist devices as destination therapy. We have seen a device-dependent increased metabolic demand in patients after cardiac assist device surgery. Increased energy requirement in patients could be counteracted using targeted nutritional therapy. Meeting the patients' nutritional requirements both pre- and postoperatively could lead to better surgical outcomes, fewer complications, and shorter hospital length-of-stay.

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Disclosures

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Chapter 4

The critical role of bioenergetics in cardiac graft preservation

Schipper DA, Marsh KM, Ferng AF, Duncker DJ, Laman JD and Khalpey Z

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ABSTRACT

The traditional philosophy of ex vivo organ preservation has been to limit metabolic activity by storing organs in hypothermic, static conditions. This methodology cannot provide longevity of hearts for more than 4-6 hours and is thereby insufficient to expand the amount of available organs. Albeit at lower rate, the breakdown of ATP still occurs during hypothermia. Furthermore, cold static preservation does not prevent the permanent damage that occurs upon reperfusion known as ischemia-reperfusion (IR) injury. This damage is caused by increased reactive oxygen species (ROS) production in combination with mitochondrial permeability transition pore (mPTP) opening, highlighting the importance of mitochondria in ischemic storage. There has recently been a major paradigm shift in the field, with emerging research supporting changes in traditional storage approaches. Novel research suggests achieving metabolic homeostasis instead of attempting to limit metabolic activity reduces IR injury and improves graft preservation. Maintaining high ATP levels and circumventing cold organ storage would be a much more sophisticated standard for organ storage, and should be the focus of future research in organ preservation. Given the link between mPTP, Ca2+, and ROS, managing Ca2+ influx into the mitochondria during conditioning might be the next critical step towards preventing irreversible IR injury.

INTRODUCTION

For patients with end-stage heart failure, heart transplant remains the preferred treatment and alternative options are limited. The increasing discrepancy between the number of hearts donated for transplant and the number of possible recipients on transplant lists is a global problem. Hypothermic *ex vivo* organ storage is used in the context of transplantation as an attempt to suspend metabolic activity and decrease energy usage, the accrual of toxic metabolites and damage to the graft (1). However, the limitations of *ex vivo* storage conditions including classic organ preservation solutions persist, most notably with ischemia-reperfusion injury (IR injury). Recently, there has been a major paradigm shift in the field, with emerging research supporting changes in traditional storage approaches. While inhibiting cellular processes in order to postpone energy loss is the classic approach to organ storage, novel research suggests achieving metabolic homeostasis instead reduces IR injury and improves graft preservation. This metabolic equilibrium is obtained by controlling the supply and demand of energy via both oxidative phosphorylation and glycolysis. The goal of this novel approach is to attain preservation of bioenergetics, defined here as stable mitochondrial membrane polarization and preserved mitochondrial functionality. During IR injury, pro-apoptotic factors such as cytochrome C and reactive oxygen species (ROS) are released into the cytosol as a result of mitochondrial disruption. Apoptosis can be induced this way, or by the mitochondria directly, either of which implicates mitochondria in organ graft dysfunction.

In this review we discuss the ideal milieu to maintain heart viability and the importance of controlling metabolic demand in order to retain mitochondrial integrity, which is essential in the inhibition of ROS and release of apoptotic factors into the cytosol upon reperfusion. We examine the role of bioenergetics in aspects of ex vivo donor heart preservation including organ storage solutions, storage time and mode, temperature norms, and IR injury. We suggest future directions accordingly. Lastly we consider the effects of circulating mitochondrial DNA (mtDNA) on the immune response and the possibility of mtDNA as a biomarker for IR injury.

Ischemia reperfusion injury

Ischemia reperfusion injury (IR injury) is one of the major problems in transplantation. Reperfusion injury occurs when blood supply returns to the tissue after a period of ischemia, and is encountered in numerous clinical cardiac scenarios including open-heart surgery, percutaneous coronary intervention and orthotopic heart transplantation. When myocardial blood flow is interrupted, acute myocardial damage can be limited by rapid restoration of blood flow and nutrient delivery. However, after prolonged ischemic time, reperfusion injury causes irreversible tissue damage due to increased reactive oxygen species (ROS) production in combination with mitochondrial permeability transition pore (mPTP) opening (2, 3).

ROS play an important role in both the cytosol and mitochondria of cells, and are necessary for normal mitochondrial function. Oxidative stress, which occurs due to an imbalance of ROS production and removal, causes macromolecular damage and is implicated in several disease states (4). Under certain pathological conditions, ROS overproduction and resultant tissue damage can be stimulated by Ca^{2+} overload, which is paradoxical since Ca^{2+} also has numerous positive affects in the mitochondria (2). Ca^{2+} overload caused by the loss of ATP, as in hypothermic *ex vivo* storage, is due to impaired function of the Na^{+}/Ca^{2+}-antiporter. In hypoxia, metabolism shifts to a glycolytic state, the tissue acidifies, and the Na^{+}/H^{+}-antiporter attempts to restore cellular pH. The corollary increased sodium gradient drives the Na^{+}/Ca^{2+}-antiporter, which is unsuccessfully activated due to the lack of ATP (figure 1). Na^{+}/Ca^{2+}-antiporter dysfunction leads to an increased intracellular and intramitochondrial Ca^{2+} concentration, ultimately causing ROS overproduction. In addition to the increase in ROS, Ca^{2+} is an important factor in propagating mitochondrial permeability transition pore (mPTP) opening.

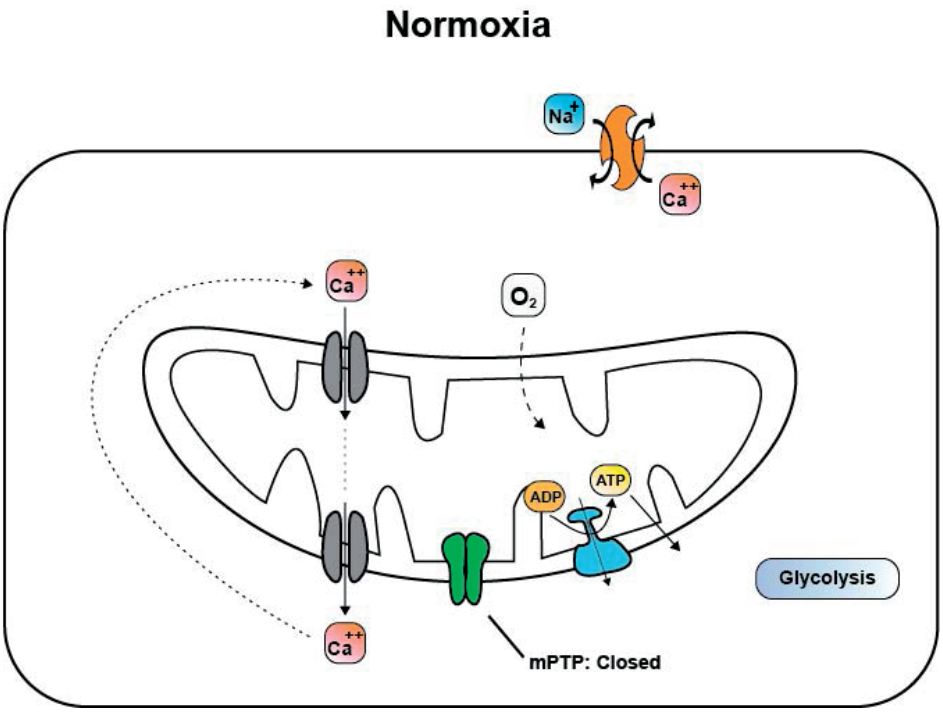

Fig. 1 Normoxic state metabolism: mitochondria produce ATP in the presence of oxygen. A calcium equilibrium is maintained and the mPTP remains closed.

Ca^{2+}-induced mitochondrial Ca^{2+} overload in combination with certain pathological conditions leads to the persistent opening of mPTP. Ischemia is an example of one such pathological circumstance, with a lowered AMP/ATP ratio and depletion of adenine nucleotides. The mPTP is nonselective and allows all molecules up to 1.5 kDa to move freely across the outer and inner mitochondrial membranes. Upon opening, the mitochondrial membrane potential is no longer maintained. This leads to the influx of water, mitochondrial swelling and rupture, and eventually the release of cytochrome C and other apoptotic factors from the inner mitochondrial membrane (2). The mitochondrial membrane potential is lost, resulting in an uncoupling effect. Persistent opening of the mPTP will not only impede the mitochondria from producing ATP through oxidative phosphorylation, but it will also actively breakdown the ATP that is produced via glycolysis in an attempt to restore the cellular pH levels and concentration gradient across the mitochondrial membrane. Using this reasoning, the low pH that occurs during prolonged ischemic periods thereby inhibits mPTP opening. Once reperfused and supplied with oxygen, the reverse occurs. The cell shifts to aerobic metabolism and pH increases, which opens the mPTP and further increases ROS levels. This mechanism accounts for the time delay observed in IR injury. Though acidic conditions can inhibit mPTP-opening, controlling pH alone cannot protect the tissue against mPTP-induced injury (5). However, well-maintained mitochondrial function and prevention of pathological circumstances inhibit mPTP opening and ultimately reduces IR injury (6). Lastly, during IR injury there is a release of mtDNA upon mitochondrial rupture, which becomes freely circulating. Given this mechanism, mtDNA has recently been considered to be a possible biomarker for myocardial infarction (7-9). In addition to the apoptosis induced by mitochondrial disruption, free mtDNA can add to the cardiomyocyte death rate (10, 11). In vitro studies have shown a dose-dependent increase in cell death upon liver IR injury caused by mitochondrial damage associated molecular pathways (DAMP) (12). Upon tissue trauma, Toll-like receptor 9 (TLR9) recognizes the mtDNA that is released in the circulation after mitochondrial disrupture as non-human material, as it strongly resembles bacterial DNA. As a response, the body initiates systemic inflammation (13). Interestingly, high circulating levels of mtDNA along with increased TLR9 expression has been used in recent clinical studies as a predictor of mortality in critically ill intensive care unit (ICU) patients (14). Furthermore, urinary mtDNA has been used as a biomarker of mitochondrial damage in acute kidney injury (15), and circulating mtDNA could play an important role in assessing cardiac graft damage from IR injury in a similar way. A recent study demonstrated that coronary artery bypass graft (CABG) surgery, a procedure in which cardiopulmonary bypass temporarily inhibits cardiac blood flow, leads to elevated free mtDNA levels in the blood (16). These findings strongly support the proposition of imposing free circulating mtDNA as a biomarker for IR injury in cardiac transplant.

Current transplant standards address mitochondrial function by resorting to hypothermic *ex vivo* storage and using ischemic pre-, post- and remote conditioning with various solutions as discussed below (17).

Organ storage solutions

Ex vivo storage of donor hearts is time limited, with effective allograft function highly dependent on using a storage solution. Historically, all *ex vivo* organ storage solutions have three common underlying concepts: 1) hypothermic arrest of metabolism, 2) maintaining viability of the tissue during a slowed metabolic state to abate cellular swelling and 3) minimizing reperfusion injury caused by free radicals and the inflammatory response. To attain these goals, the ionic ingredients included in preservation fluids reduce the transmembrane K+ gradient, rapidly depolarize the myocardial cellular membrane, and stop cardiac electrical activity (18). All existing organ storage solutions fall into one of two categories: intracellular (Na^+ concentration less than 70 mmol/L and K^+ concentration ranging between 30 and 125 mmol/L) and extracellular (Na^+ concentration greater than or equal to 70 mmol/L and K^+ concentration between 5 and 30 mmol/L) organ preservation solutions. Both types of solutions generally have comparable results on graft preservation, though this point has been debated (18, 19). For an overview and detailed composition of selected heart preservation solutions, see Table 1.

Developed in the late 1960's, Euro Collins was the first widely accepted preservation solution until F. Belzer introduced UW solution in the 1980's. UW solution remains the gold standard to which all new solutions continue to be compared still today, with Celsior as a notable contender (1, 20). Of the many novel components of UW solution, glutathione is metabolically most relevant. Glutathione can act as a reducing agent and antioxidant, limiting the formation of lipid peroxides and other cytotoxic end products of oxygen metabolism (20, 21). The basis of UW solution is lactobionatic acid combined with raffinose, instead of chloride as used in Collins solution (22). In other solutions, lactobionic acid was either replaced or used in combination with the potent buffer histidine, and the edema-preventing capacity observed in UW solution was conserved or even further improved (23, 24). Histidine is the first of three major components of another preservation solution, HTK solution, added to prevent acidosis and promote adenosine triphosphate (ATP) production during ischemia (25, 26). Ketoglutarate also acts to promote ATP production (27). Ketoglutarate, tryptophan and mannitol, all included in HTK solution and in some other solutions as well, possess antioxidant properties (Table 1) (20).

TABLE 1

Ingredient	Euro-Collins	UW-Solution	Celsior	Somah-solution
Na+	10,00	25,00	100,00	125,00
K+	115,00	125,00	15,00	7,00
NaCl	10,00	-	-	125,00
KCl	108,00	-	15,00	7,00
MgCl2	-	-	13,00	0,50
NaCO3H	10,00	-	-	5,00
NaOH	-	25,00	100,00	-
KOH	-	100,00	-	-
MgSO4	-	5,00	-	0,50
Glucose	180,00	-	-	11,00
Adenosine	-	5,00	-	2,00
Lactobionate	-	100,00	80,00	-
Glutamic Acid	-	-	20,00	-
Glutathion	-	3,00	3,00	1,50
Allopurinol	-	1,00	-	-
Mannitol	-	-	60,00	-
Raffinose	-	30,00	-	-
CaCl2	-	-	0,25	1,30
Phosphate	60,00	25,00	-	0,44
Insulin	-	40,00	-	1,00
Dexamethason	-	16,00	-	-
Pantafraction HES	-	50,00	-	-
Penicilin G	-	200.000,00	-	-
l-Arginine	-	-	-	5,00
l-Citrulline malate	-	-	-	1,00
Creatine orotate	-	-	-	0,50
Creatine monohydrate	-	-	-	2,00
l-Carnosine	-	-	-	10,00
l-Carnitine	-	-	-	10,00
Dichloroacetate	-	-	-	0,50
Ascorbic acid	-	-	-	1,00
Sodium phosphate	-	-	-	0,19
Histidine	-	-	30,00	-
pH	7,30	7,40	7,30	7,50

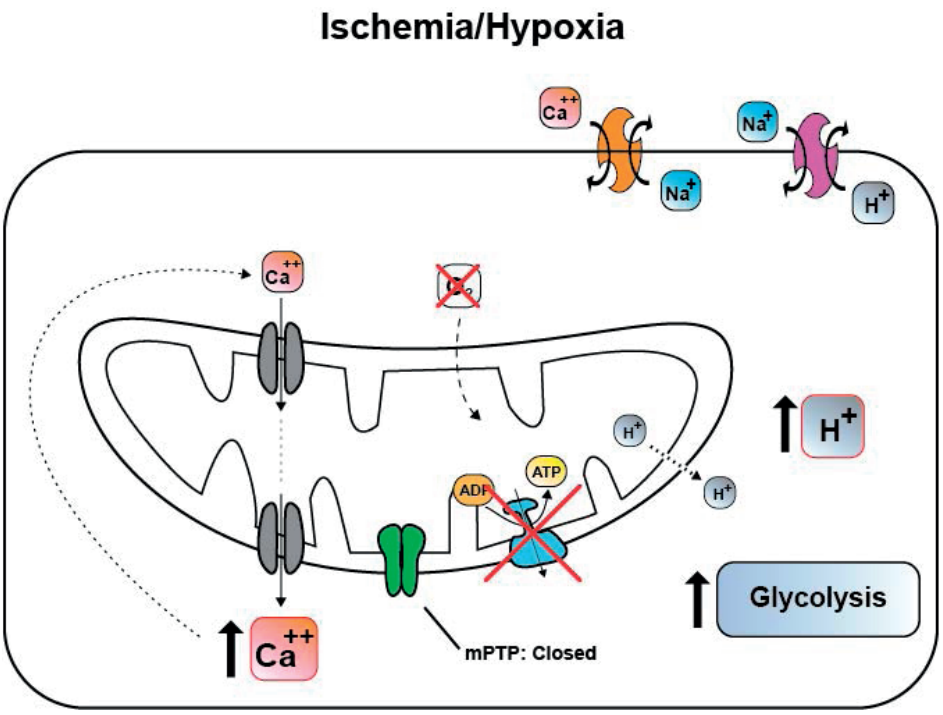

Fig. 2 Ischemic or hypoxic metabolism: lack of oxygen leads to an increase in glycolysis to maintain ATP levels and acidification of the cell occurs. The cascade of ionic changes to counteract cellular acidification generates calcium overflow in the cell and consequently in the mitochondria. At this stage, the mPTP is still closed, as the low pH is an inhibiting factor.

Though not included in HTK solution, another compound included in many organ preservation solutions (including UW solution) is adenosine. This endogenous purine nucleoside was originally added to replete ATP following ischemic storage via the purine salvage pathway, and was later found to have multiple important functions (28). Stimulation of A_{2A} receptors protects against the ischemia reperfusion-related inflammatory response by inhibiting rolling, adhesion and migration of inflammatory cells. A_{2A} stimulation also attenuates other inflammatory responses by inhibiting CD4+ cells, TNF-α, superoxides, and interleukin release (29). When organs are preconditioned with adenosine, activation of A_1 and A_3 adenosine receptors protects the heart against subsequent ischemia and reduces infarct size (30, 31). Adenosine-mediated preconditioning also reduces ischemic acidosis and enhances post-ischemic recovery of $[P_i]$, $[Mg^{2+}]$ and ΔG_{ATP} unrelated to cytosolic [ATP] (32).

All the discussed solutions and additives focus on maintaining viability of the tissue during a slowed metabolic state. A paradigm shift is emerging in the field, with a new focus on providing organs with necessary metabolic nutrients instead of slowing metabo-

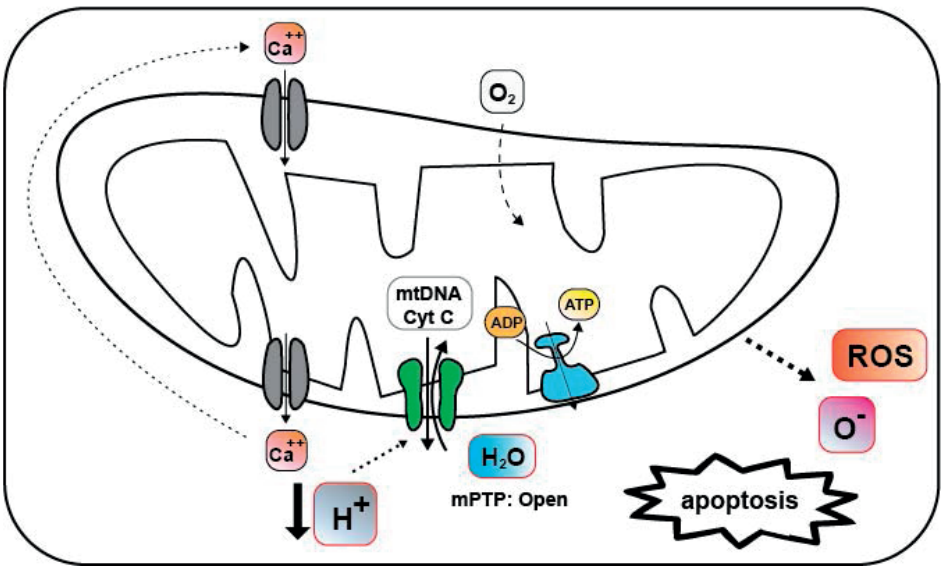

Fig. 3 Reperfusion metabolism: upon reperfusion, the mitochondrial ATP production restores, and ROS is generated. In addition, cellular environment returns to physiological pH. This sets an ideal circumstance for the mPTP to open, causing large molecules and water to enter the mitochondria. Once the mitochondrial inner membrane cannot swell bigger, the outer membrane ruptures and apoptotic factors are released into the cytosol.

lism. Somah solution attempts to meet the energy requirements of cardiomyocytes and coronary epithelium by preserving anaerobic metabolism and high-energy phosphates, while safeguarding the mitochondrial gradient (33). Interestingly, Somah solution is most effective at 21˚C (34), adding to the question whether conventional low *ex vivo* storage temperature is justified.

Ex vivo storage

Temperature

The heart normally relies heavily on oxidative phosphorylation and the Krebs' cycle for energy production. Once oxygen and other substrates are limited as during *ex vivo* storage, the cell shifts to anaerobic metabolism. This shift to glycolysis can have deleterious effect on stored organs due to the overall loss in ATP. Decreasing the temperature of an organ from 37˚C to 0˚C results in a 12-13 fold decrease in metabolic rate, analogous to van 't Hoff's rule (35, 36). This is the basis of the conventional cold storage preservation method that has been in use since the late 1960's – lowering organ temperature in order to slow cellular processes and accretion of mitochondrial by-products such as ROS.

Though slowing the unavoidable buildup of ROS may be beneficial, there are limits to cold preservation and acceptable *ex vivo* storage times are organ-dependent. Heart preservation is currently limited to 4-6 hours of cold ischemic time, with successful outcomes exponentially dropping as cold ischemic time increases past 6 hours (37). ATP production decreases dramatically during cold ischemic storage. This leads to loss of mitochondrial membrane potential, causes mitochondrial membrane permeabilization, and ultimately induces cell death (38). Furthermore, prolonged cold ischemia is an independent risk factor for organ dysfunction upon transplant. These major limitations of hypothermic storage have led to a paradigm shift in organ solutions exploring subnormothermic storage (34, 39). Recently, it has been shown that cardiac function can be preserved and myocardial injury reduced during long-term storage of swine hearts at subnormothermic temperatures (34, 40). Abandoning the hypothermic storage model has led to superior results, particularly with perfused and beating *ex vivo* hearts.

Static vs. perfused storage

Prior to transplant, organs have traditionally been stored under static conditions, without any semblance to the *in vivo* environment. This classic approach has been renovated with the advent of *ex vivo* mechanical perfusion. Mechanical perfusion simulates the *in vivo* environment with oxygen and substrate delivery, enabling organs to undergo continuous aerobic metabolism and washout of toxic metabolic byproducts (41). Hearts that are stored with machine perfusion exhibited lower lactate levels, increased adenosine monophosphate (AMP) / ATP ratio, and lower phosphocreatine levels compared to hearts in static cold storage (42, 43). Lastly, mechanical perfusion may also decrease IR injury (44). In a porcine model, perfused hearts show less mitochondrial injury after reperfusion on a Langendorff system (45). On high-risk transplant procedures, the use of an organ care system (OCS™) that provides continuous heart perfusion has had very promising results (46). Currently the OCS™, manufactured by TransMedics Inc., is the only commercially available device capable of ex vivo heart perfusion. Other limitations include the necessity to limit warm ischemic time. Failure to ensure rapid perfusion may exacerbate the Ca^{2+} overload caused by the loss of ATP. This loss of ATP will inevitably occur more quickly to an organ stored at warmer temperatures, further emphasizing the need for nutrient-rich storage solutions and/or blood. Lastly, as seen in the Langendorff model, continuous perfusion of hearts in the OCR™ has been associated with myocardial edema (47), a limitation that can be overcome by proper storage solution composition as well.

DISCUSSION

Despite continued improvements in organ transplantation, significant challenges remain. Many of the problems can be attributed to the lack of optimal *ex vivo* storage. The traditional approach with organ storage has been to slow cellular metabolism and all other cellular processes including cell death. Current standards call for *ex vivo* storage of hearts using existing organ storage solutions at hypothermic static conditions. While hypothermic conditions lower organ metabolism and therefore the need for energy, hypothermia itself can be damaging to the graft. Although this strategy may be successful for some organs, hearts require near-instant high ATP levels once transplanted. Thus the lack of ATP and shift to glycolytic metabolism during hypothermic storage is detrimental in hearts. Once reperfused and at baseline, beating hearts rely heavily on oxidative phosphorylation. Mitochondrial damage caused by persistent opening of the mPTP during reperfusion is therefore more damaging to the heart than to other organs. Given the link between the mPTP, Ca^{2+},ROS, managing Ca^{2+} influx into the mitochondria during conditioning might be the next critical step towards preventing irreversible IR injury.

Free circulating mtDNA has promising potential as a biomarker for IR injury. During cold static preservation it remains highly difficult to judge the prognosis of the stored organ, as most injury is induced during reperfusion. Increased levels of free circulating mtDNA can be correlated directly to mitochondrial damage and cell death. Though metabolic assessment of the allograft is not currently performed during static storage, implementation of *ex vivo* graft perfusion would allow real-time assessment of organ condition using mtDNA as a biomarker.

Considering the significant damage caused by IR injury following traditional hypothermic storage, it is surprising that organ preservation strategies have barely changed for decades. The lack of innovation in organ preservation solutions and other storage standards may be because all the solutions have the same aim discussed above. Instead of trying to minimize metabolic demand, the recent novel approach is to stabilize and replenish precursors for the bioenergetic supply. As long as substrates are readily available and metabolic waste can be removed, there would theoretically be no need for an organ to fail outside the body. Substrates can be provided by a storage solution or blood, and waste can be removed by utilizing *ex vivo* perfusion. *Ex vivo* perfusion systems at subnormothermia may emphasize normal mitochondrial ATP turnover and may have remarkably less ATP depletion and tissue necrosis and apoptosis than current storage methods, and should therefore be closely investigated. Maintaining high ATP levels and circumventing cold organ storage would be a much more sophisticated standard for organ storage, and should be the focus of future research in organ preservation.

CONCLUSION AND FUTURE DIRECTION

The classic approach of suspending cellular metabolism through hypothermia does not provide longevity of hearts for more than 4-6 hours. The breakdown of ATP, albeit at a lower rate, still occurs in hypothermic conditions. More importantly, the mitochondria undergo permanent changes that result in active ATP dissimilation and induce cell death. This limitation will continue to result in a large amount of discarded and underutilized organs for transplantation. Current organ preservation solutions aim to diminish the consequences of improper mitochondrial protection by including antioxidants and impermeants to prevent tissue damage caused by ROS and edema, respectively. These additives improve graft outcomes to some extent, but the main target to increase organ longevity and functional durability has been overlooked. Preserving the graft's bioenergetic homeostasis can be achieved in multiple ways and is an excellent target for future studies of *ex vivo* heart preservation. This is demonstrated by the promising results of marginal human grafts transplanted after continuous perfusion, as well as static porcine heart preservation in Somah at various temperatures. Overall there is a growing understanding of the consequences of permanent mPTP opening, the prevention of which is key to proper organ preservation until transplant. Along with the exploration of subnormothermic storage and use of *ex vivo* perfusion in order to optimize graft preservation, the role of mtDNA as a biomarker of metabolic organ integrity due to mitochondrial disruption is an exciting proposition.

The transplant gap for hearts is constantly increasing. It has become clear that classic cold static preservation approaches are insufficient to expand the amount of available organs. A combination of preservation solutions, techniques and technologies will aid the field of cardiac transplant in the search of increasing the pool of organs suitable for transplantation. Not only will an improvement in graft longevity increase the amount of viable organ being transplanted into patients directly, it also has the ability to open new paths to rejuvenating of marginal organs.

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The authors have no disclosures to report.

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Chapter 5

Novel vs clinical organ preservation solutions: improved cardiac mitochondrial protection.

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ABSTRACT

Background

Heart transplantation remains the gold standard for end-stage heart failure, with current ex vivo organ storage times limited to 4 to 6 h before critical tissue damage occurs. Many preservation solutions exist in an attempt to limit both ischemic and reperfusion damage. In order to compare the effects of various storage solutions, mitochondrial function can be used to provide a sensitive analysis of cellular metabolic function.

Methods

Experimental plates were seeded with cardiac myoblasts and kept in suspended animation for either 4 or 8 h at either 4° or 21°C, in Celsior®, Perfadex®, or Somah storage solutions. Cells were then reanimated for 1 h at 37°C to simulate a reperfusion or clinical transplant scenario. Cellular bioenergetics were measured immediately thereafter to examine biochemical differences between preservation solutions and their effectiveness on preserving metabolic function.

Results

The oxygen consumption rates of Somah solution were significantly higher than Celsior® and Perfadex® at 4°C, with the exception of Perfadex® at 4° for 4 h. This effect was sustained up to 8 h. At 21°C, oxygen consumption rates of Somah solution are significantly higher than Celsior® and Perfadex® at basal conditions after 4 h, but this effect is not sustained after 8 h.

Conclusions

The purpose of this experiment was to study the efficacy of various preservation solutions on a mitochondrial level. The significantly higher oxygen consumption rates of Somah at 4°C suggests that Somah solution may have the ability to protect cellular mitochondrial integrity, improve transplanted organ function by reducing ischemic-reperfusion injury, and thereby improve transplant outcomes. Given that Somah offers benefits over Celsior® and Perfadex® at 4°C, it should be a target in future organ preservation solution research.

BACKGROUND

The gold standard for patients suffering from end-stage heart failure remains heart transplantation. With the number of patients requiring transplants growing and the available organs remaining constant, there is a need for improved donor organ and graft preservation methods to extend the life of donor organs [1]. Conserving organ viability outside of an organism can be accomplished by warm or cold storage, with or without perfusion. Currently, the standard approach in heart preservation is cold static storage, often supplemented by the use of preservation solutions [2]. These approaches have allowed for an ex vivo storage time of about 4–6 h before critical tissue damage occurs [3]. Preservation of organs for longer than 4–6 h results in increased ROS levels, ATP depletion, Na+/K+ ATPase alterations, mitochondrial disturbances, accumulation of xanthine oxidase, and dysregulation of Ca2+ homeostasis that will negatively affect cellular viability [2, 4]. Under normal physiological conditions, the heart utilizes ATP as energy for the Na+/K+ ATPase, both of which are required for sustained myocardial contractility. During ischemia, ATP levels decrease while intracellular H+ increases due to the shift from aerobic to anaerobic respiration via glycolysis and lactate production. ATPase pumps that maintain homeostasis become dysfunctional as the synthesis of ATP slows. Disruption of these ATPase pumps in the mitochondria can be measured experimentally through examining extracellular flux and mitochondrial respiration. In an attempt to avoid the negative effects of decreased ATPase pump efficacy, both the cold storage approach and utilization of storage solutions exists.

Cellular metabolic demand decreases as much as twelve-fold under hypothermic conditions at 4°C [5], but metabolic processes persist and will continue to result in cellular damage. Though warm preservation might result in injury from non-controlled warm ischemic periods [2], it is less researched and could be a more effective and sophisticated storage method [6]. Regardless of storage temperature during perfused or static storage, preservation solutions such as Celsior®, Perfadex®, and Somah storage solutions are commonly used to decrease ischemic reperfusion injury (IRI) from reactive oxygen species (ROS), prevent intra- and extracellular swelling, and minimize energy usage by lowering metabolic demand. The conservation of mitochondrial bioenergetic measurements such as these is important for improving organ transplant outcomes during static storage. During storage and upon reperfusion, IRI can be attenuated by using storage solutions such as University of Wisconsin (UW) solution [7]. However, UW contains high molecular weight compounds such as hydroxyethyl starch that resulted in a highly viscous solution that is linked to organ dysfunction. Newer alternatives including Celsior®, Perfadex®, and Somah have since been created to allow for reliable organ preservation up to 6 h by providing immunosuppressant and antioxidant properties that minimize IRI [4, 7]. There are

numerous organ storage solutions, with many new solutions currently in development. Somah is a novel solution that acts to maintain membrane polarity by allowing higher levels of high-energy phosphates to be generated through the glycolytic pathway during preservation, and mitigates the consequences of IRI overall [8]. Somah was developed to meet the energy requirements of cardiomyocytes and coronary endothelium, in addition to priming the organ with substrates and metabolites during storage to facilitate resumption of biochemical, physiological, and mechanical work upon post-transplantation reperfusion [8]. The differences between Somah, Celsior®, and Perfadex® are summarized in Table 1. Properties of these three preservation solutions are fairly similar, however, Somah contains many additional protective and unique substrates, such as adenosine, insulin, and ascorbic acid, among others [8]. For these reasons, bioenergetic profiles were generated to further explore mitochondrial physiology and the effects of storage solutions on organ preservation.

Table 1 Comparison of organ preservation solutions

	Celsior[a]	Perfadex[a]	Somah[b]
IC/EX	EX	EX	EX
Na^+	100	138	125
K^+	15	6	7
Impermeant/Colloid	LactoB, mannitol	Dextran	LactoB, mannitol
Buffer	Histidine	Phos	Bicarb
Antioxidant	GSH, mannitol	-	GSH, mannitol
Osmolarity (mOsm/L)	320	292	305
Ca^{2+}	0.25		0.25
Mg^{2+}	13	0.8	13
Cl^-	-	142	-
Glucose	-	5	11
Others		SO_4^{2-}0.8 dextran 40 g/L	2 mmol/L adenosine insulin 10 mg/ml 1 mmol abscorbic acid

All units expressed in mmol/L unless otherwise indicated
Source [a]Reference [8]; [b]Reference [5]
Abbreviations: *IC* intracellular, *EX* extracellular, *Und* undetermined, *LactoB* lactobionate, *HES* hydroxyethyl starch, *Phos* phosphate, *Bicarb* bicarbonate, *GSH* glutathione

In the present study, we sought to compare the biochemical differences between organ preservation solutions and their effectiveness on preserving cellular metabolic and mitochondrial function. Our protocol involves putting cells in suspended animation for 4 to 8 h at 4° or 21°C, reanimating the cells over 1 h at 37°C with regular media, and then immediately recording the bioenergetics thereafter. This study evaluates mitochondrial function under clinically relatable conditions, investigates mitochondrial safeguarding, and examines the bioenergetic state of cells after exposure to Celsior®, Perfadex®, and Somah storage solutions.

METHODS

Microplate coating

Cell-Tak was purchased from Corning (Product #354240). Cell-Tak was prepared in a 2:1 ratio to 1 M NaOH. The wells of a 96-well Seahorse microplate (Seahorse Bioscience, North Billerica, MA) were coated with 40 µl Cell-Tak solution and the plate was allowed to dry at room temperature for 20 min. Following the drying period, wells were aspirated and washed twice with 1x DMEM. The coated microplates were stored at 4°C and used within 72 h.

Preservation solution treatments

Rat embryonic myoblasts, H9C2s (ATCC® CRL1446™), were maintained at standard cell culture conditions in DMEM medium enriched with 10% FBS (Seradigm, Product # 1400-500), 5% L-glutamine (Corning, Lot # 25005289), and 1% Antibiotic-Antimicotic Solution (Sigma Aldrich, catalog # A5955).

H9C2s were seeded in the previously described Cell-Tak coated 96-well Seahorse microplates at 25,000 cells/well. The seeded microplates were incubated for 48 h at 37°C before introducing experimental conditions to the cells. Each well was washed with 1X PBS (phosphate buffered saline) prior to the addition of 200 µl of experimental solution or control media. Experimental groups included Celsior® (Sanofi, Bridgewater, NJ), Perfadex® (XVIVO Scientific Animation, Wethersfield, CT), Somah (Somahlution®, Jupiter, FL) solutions, 1X PBS, and standard H9C2 media. The microplates were then exposed to either 4°C or 21°C for 4-h and 8-h time points. The 10 experimental groups were named 4CEL, 21CEL, 4PER, 21PER, 4SOM, 21SOM, 4PBS, 21PBS, 4MED and 21MED to indicate the temperature condition and storage solution of each group: CEL = Celsior®, PER = Perfadex®, SOM = Somah, and MED = media. Each treatment group consisted of 12 wells and each microplate included all of the experimental groups. Each experimental condition was repeated twice using freshly made solutions and a separately started H9C2 cell line. After each experiment, supernatant from each group was collected and snap-frozen for High Performance Liquid Chromatography (HPLC) analysis.

Mitochondrial stress test

Immediately after administering the experimental treatments, mitochondrial respiration of H9C2 cells was assessed using XFe96 Extracellular Flux Analyzer (Seahorse Biosciences, North Billerica, MA). Oxygen consumption rates (OCR) were measured in the presence of oxidative phosphorylation (OXPHOS) driving substrates. After 3 basal measurements, 3 measurements each were taken after the addition of oligomycin, FCCP, and rotenone/ antimycin A combination. These injected drugs block ATP synthase, uncouple

the oxygen consumption from ATP synthesis, and block mitochondrial complexes I and III, respectively. Results were then used to calculate the respiratory control ratio (RCR) and coupling efficiency (CE), which were the primary outcome measurements along with basal respiration. RCR was calculated by the following equation: proton leak ÷ maximal respiration; and CE by the following equation: [(basal respiration – proton leak) ÷ (basal respiration)] * 100.

Statistical analysis

The data were analyzed as a split-plot design. A plate was considered a "whole plot" with treatments: time, temperature, and the time-temperature interaction. Each solution was considered the split-plot treatment with individual wells used as the experimental unit for a solution. Where there was a statistically significant (alpha = 0.05) effect of the solution, Tukey's HSD was used to compare all pairs of solutions for differences in mean response. Where there was a statistically significant interaction effect involving a solution, a slice was conducted to test the significance of drug at each level of time, temperature, and/or time-temperature combination and all pairs of solutions were tested for differences in mean response at each level of the time and/or temperature, again, using Tukey's HSD multiple comparison procedure. Initial analysis suggested non-constant variance among treatment combinations. Therefore, a weighted analysis was conducted using the inverse variance for each solution treatment-plate combination as the weight. All analyses were conducted using SAS PROC MIXED.

RESULTS

An overall flow chart of the experimental setup and resultant data plotted under the 4°C and 21°C temperature conditions is shown in Fig. 1. Tukey's HSD was used for pairwise comparisons of solutions for differences in mean response under each respective time and temperature condition. Only organ preservation solution pairwise comparisons with at least a significance of alpha = 0.05 are mentioned in the figure legends for each experimental condition (Figs. 2, 3, 4 and 5).

4 Hour experiments

Comparisons of different solutions are presented in Figs. 2 and 3, showing experiments conducted at 4C and 21C, respectively. H9C2 cells in 4SOM and 4PER both showed a higher mean basal oxygen consumption rate (OCR) than cells treated with either 4CEL, without significant differences between 4SOM and 4PER. H9C2 cells in 21SOM solution showed a higher mean basal OCR than H9C2s in 21CEL or 21PER.

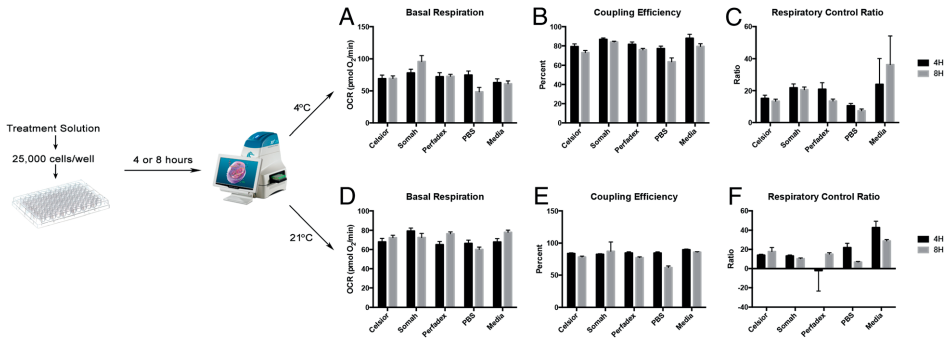

Fig. 1 Experimental Design Flow Chart. The conditions of the experiment were performed as shown, with data plotted for both 4 and 8 h time points under 4 °C in panels a-c and under 8 °C in panels d-f

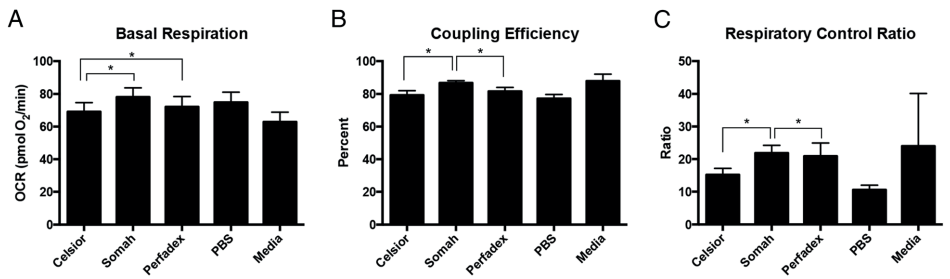

Fig. 2 Experimental Conditions at 4 h in 4 °C. For basal respiration (a), coupling efficiency (b) and respiratory control ratio (c), there is significance between the Somah-Celsior pairwise comparisons. For basal respiration (a), there is significance between Celsior-Perfadex pairwise comparisons. For coupling efficiency (b) and respiratory control ratio (c), there is significance between Somah-Perfadex pairwise comparisons. Error bars shown are SEM.

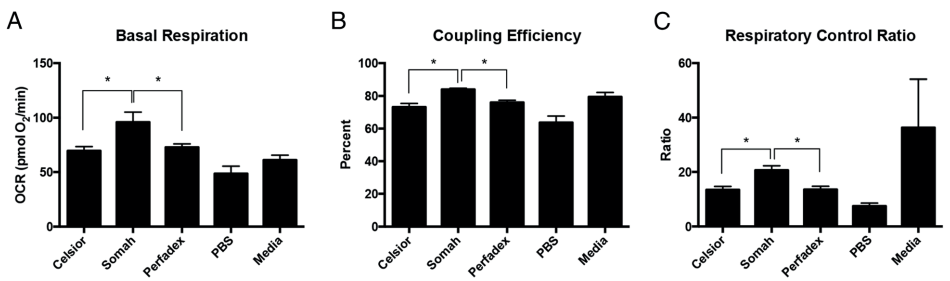

Fig. 3 Experimental Conditions at 4 h in 21 °C. For basal respiration (a), there is significance between the Somah-Celsior and Somah-Perfadex pairwise comparisons. For coupling efficiency (b), there is significance between the Somah-Perfadex® pairwise comparison. For respiratory control ratio (c), there is no significance between the pairwise comparison of the organ preservation solutions (Somah, Celsior and Perfadex). Error bars shown are SEM.

At the 4 h time point, 4SOM and 21SOM treated cells had a higher OCR than cells in 4MED and 21MED, respectively. 4SOM also had a higher coupling efficiency (CE) than 4CEL and 4PER, and 21SOM showed a higher CE than 21PER at 4 h. 4SOM showed a higher respiratory control rate (RCR) than both 4CEL and 4PER.

21MED showed decreased CE in comparison to 21SOM. 21MED had higher RCR values than all other solutions.

8 Hour experiments

Comparisons of different solutions are presented in Figs. 4 and 5, showing experiments conducted at 4°C and 21°C respectively. After 8 h, H9C2s in 4SOM had a higher basal OCR than cells in 4PER and 4CEL. The significant elevation in basal OCR seen in 21SOM during the 4 h experiment did not persist after 8 h.

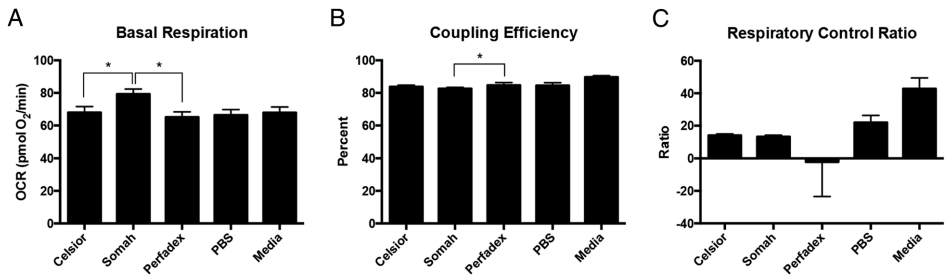

Fig. 4 Experimental Conditions at 8 h in 4 °C. For basal respiration (a) coupling efficiency (b) and respiratory control ratio (c), there is significance between the Somah-Celsior, and Somah-Perfadex pairwise comparisons. Error bars shown are SEM.

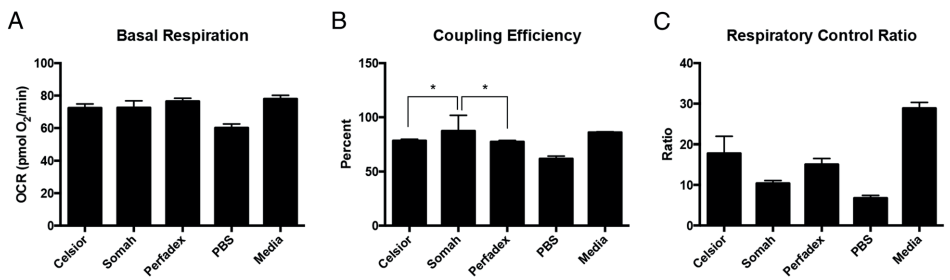

Fig. 5 Experimental Conditions at 8 h in 21 °C. For basal respiration (a) and respiratory control ratio (c), there is no significance between the pairwise comparisons of the organ preservation solutions. For coupling efficiency (b), there is significance between the Somah-Celsior, and Somah-Perfadex pairwise comparisons. Error bars shown are SEM.

4SOM had a higher CE than 4CEL; 21SOM had a higher CE than 21CEL and 21PER. 4SOM also showed this same RCR trend above 4CEL and 4PER. As seen in the 4 h experiments, 21MED had a lower CE than 21SOM. Finally, 21MED had a higher RCR than the other solutions, in accordance with the trend seen during 4 h experiments.

DISCUSSION

The goal of this study was to evaluate H9C2 cellular bioenergetics following a protocol designed to simulate cardiac transplantation by using clinically relatable temperatures and time points. To create this scenario, cells were submerged in Somah, Celsior®, or Perfadex® preservation solutions for 4 and 8 h either at room temperature (21°C) or "on ice" (4°C). Normal saline (phosphate buffered saline; PBS) without substrates was chosen as the negative control since the osmotic gradient of cells was expected to be preserved with cellular function steadily declining as endogenous energy stores were depleted. In contrast, normal H9C2 media acted as the positive control since this media is supplemented with the ideal growth substrates for this cell line at 37°C. While cultured primary cardiomyocytes are a valuable tool for studying the metabolic capacity of the heart, isolated cardiomyocytes can be fragile and difficult to maintain, especially under stress. As a result, the H9C2 cell line was chosen because these cells express multiple CYP genes comparable to the levels found in the human heart [9]. The role of these endogenous CYP metabolites has been shown to be important in the maintenance of cardiovascular health, and therefore offers an unique model for studying the metabolic activity of the heart [9].

It was hypothesized that the ideal preservation solution could create a state of suspended animation, in which cellular function and metabolism could be preserved for an extended period of time with the least amount of resultant metabolic dysfunction. Altered cellular metabolism is a more sensitive indicator of stress than some traditional outcome measures that may take time to accumulate and detect (e.g., DNA and protein analyses). The role of mitochondria in ischemic reperfusion injury during cardiac transplant is becoming increasingly evident [10]. Safeguarding mitochondrial bioenergetics could play a key role in improving transplant outcomes. Thus, to examine this endpoint, extracellular flux analyses were used to compare mitochondrial respiration of H9C2s after each treatment condition. Targeting various complexes and pathways in the mitochondria allowed us to identify any changes in substrate metabolism and mitochondrial (dys)function. Basal respiration was analyzed as a baseline bioenergetic measurement for each experimental condition. The basal respiration is often controlled by high ATP turnover, and in part dictated by substrate oxidation and proton leak [11]. This measure is therefore altered in response to ATP demand. Coupling efficiency and respiratory control ratios were analyzed since both parameters are ratios, and are therefore useful internal controls that are unaffected by differences in cell number. Additionally, changes in CE and RCR are good indicators of cellular and mitochondrial dysfunction.

Overall, Somah had a greater OCR than both treatment and control groups. This finding was statistically significant after 4 h at room temperature (21°C) compared to Celsior® and

Perfadex®. Compared to the control media, the OCR of Somah-treated H9C2s (4SOM and 21SOM) were increased compared to the control media (Figs. 2 and 3) at both 4o and 21°C after 4 h of incubation. By 8 h, the media and the preservation solution conditions become similar and the significant trend diminishes. 4SOM had an increased OCR value compared to 4PER and 4CEL experimental groups, as well as 4MED and 4PBS controls. Also at the 8-h time point, 21PBS had a significantly lower OCR than the other treatment groups, while 21SOM, 21CEL and 21PBS have similar OCR values. As expected, PBS was overall the least-ideal solution since it has the lowest OCR. At 4 h under both temperature conditions, the differences between all solutions, and notably PBS, were not obvious. This is likely due to the resiliency of the H9C2 cell line, reiterating why this cell line is a good model for investigating metabolic activity and drug metabolism in the heart [9]. An aim for a future study may include longer time points such as 12 or 24 h, though organs stored for extended periods of time are not considered clinically viable.

The basal respiratory OCR of Somah-treated cells was greatest and allowed cells to maintain metabolism at a more consistent OCR through the 4-h time point (Figs. 2 and 4). In comparison, base media, Celsior®, and Perfadex® took up to 8 h to return to a higher OCR at 21°C (Figs. 3 and 5). These same differences are even more pronounced at 4°C, where 4CEL is found to have a lower OCR than both 4PER and 4SOM.

Coupling efficiency (CE) is defined as the proportion of mitochondrial respiratory rate used to drive ATP synthesis (e.g., perfectly coupled OXPHOS has a coupling efficiency of 100%) [12]. CE is calculated as the change in basal respiration rate with the addition of oligomycin, and is thus presented as the fraction of basal mitochondrial oxygen consumption used for ATP synthesis. Since it takes the basal respiration into account, the CE also varies with ATP demand and is most sensitive to changes in proton conductance. Since CE is a ratio of two rates, it is an internally normalized value and can indicate mitochondrial dysfunction. Overall, both Somah and media treated cells have a high CE (90%), which shows that even though maximal respiration may be damaged (see RCR), the normal ATP turnover when compared to the leak of protons over the membrane has not changed. After both 4 and 8 h at 4°C, the CE of H9C2s treated with either Somah or media was significantly higher than all other solutions (Figs. 2 and 4) and Celsior® and Perfadex® were not significantly different at either time point. The shorter time point of 4 h led to a higher CE in Somah over the other solutions. 4SOM is comparable to the media control condition, while at 21SOM the CE was significantly lower than compared to Celsior® and Perfadex®, yet the CE of media treated cells remained significantly higher than the other groups. This might be due to an increased proton leak or a decrease in ATP production. If caused by a rise in proton leak, there might be an increased production of superoxide anions and other ROS that cause tissue or organ damage. In previous research, Somah has

shown to function best at 21°C [8]. This is an interesting link to our significantly lowered CE result and should be the subject of future research. However, given the similarities between Somah and media treatments in the hypothermic conditions of this experiment, Somah is considered preferred at 4°C, while media is favored at a warmer temperature.

The respiratory control ratio (RCR) is calculated as a ratio of the uncoupled rate of mitochondrial respiration to the rate with ATP synthase inhibited by oligomycin. The RCR is sensitive to changes in substrate oxidation and proton leak, which makes it sensitive overall to potential dysfunction [11]. One major benefit of the RCR as a measurement is that it is internally normalized, similar to CE. At 4 h, 4SOM results in a higher RCR than H9C2s treated with either 4CEL or 4PER, but has a similar RCR to media. Similar to the CE results, this change is attributable to either an increase in proton leak or a decrease in maximal ATP turnover, or a combination of both. Unlike the CE results, the RCR of Somah-treated cells remains significantly higher than all of the other solutions over the entire 8 h. 21MED also has a high RCR over both 4 and 8 h, with significance over all other treatment groups. As expected by 8 h, 21PBS has the lowest RCR of all groups. The experimental group most similar to the media-treated cells was the Somah-treated cells. While Somah best maintained a high RCR under hypothermic conditions, media maintained a high RCR at warmer room temperature conditions.

A notable limitation of the study is the limited number of storage solutions included in the comparison. As previously mentioned, there are numerous organ storage solutions currently available, and even more in development. Therefore, an aim in future studies includes incorporating more organ storage solutions into this experimental design, including UW solution, Custodiol, histidine tryptophan ketogluterate (HTK), and Collins storage solutions.

Another limitation was the use of non-human tissue for the measurement of bioenergetic values. While cardiomyocytes of multiple animal models closely resemble that of a human, it can still be difficult to draw solid conclusions regarding the clinical value of the findings presented. Even though this concern is valid, this study demonstrates a reliable and elegant method of measuring the bioenergetic profile of cardiac cells lines during storage in various solutions. Therefore, based on the methodology and experimental results, the results from this experiment can be extrapolated for translation to human tissue. It is important to establish non-human models since healthy human cardiac tissue is scarcely available. Despite these limitations as a clinical model, the results of this study suggest differences in metabolic preservation between the solutions, with Somah being the optimal preservation solution.

Additionally, the model explored in this study is limited by the fact that the cells are seeded in a monolayer, and not multi-layered as seen in an organ. The use of tissue biopsies has been very challenging, as the mitochondrial stress test performed in these assays is an extremely precise procedure, in which exact cell number are crucial when injecting different drugs in the wells of the assay plate.

Future studies to use multi-layer cardiac myoblast culture as well as creating micro-tissues by co-culture with fibroblasts and cardiac myocytes would provide key three dimensional bioenergetic data. Currently, these culture techniques are being developed with collaborators, as extracellular flux analyzers currently do not provide consistent and homogenous data that is reportable for these types of cultures. The benefit, on the other hand, of a single layered model is that these assays provide very accurate results, without any interference of other processes. Thus, these monolayer experiments serve as the first confirmatory step that protecting cellular mitochondrial integrity of transplantable organs may lead to improved organ function, reduced IRI and better transplant outcomes.

CONCLUSIONS

These results suggest Somah solution sustains a higher and more consistent OCR when compared to both Celsior® and Perfadex® at 4°C, and these effects can last from 4 h up to 8 h. These findings are additionally supported by current literature [8]. Given these results, Somah offers benefits over the solutions at 4°C and should be a target in future organ preservation solution research.

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Competing interests

Authors AF, DS, AC, KM, SK, and ZK declare that they have no conflicts of interest.

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Chapter 6

Improved metabolism and redox state with a novel preservation solution: implications for donor lungs after cardiac death (DCD)

Schipper DA, Louis AV, Dicken D, Johnson K, Smolenski R, Black S, Garcia JM and Khalpey Z

Pulmonary Circulation, 2017

ABSTRACT

Background

Lungs donated after cardiac death (DCD) are an underutilized resource for a dwindling donor lung transplant pool. Our study investigates the potential of a novel preservation solution, Somah, to better preserve statically stored DCD lungs, for an extended time period, when compared to low-potassium dextran solution (LPD). We hypothesize that Somah is a metabolically superior organ preservation solution for hypothermic statically stored porcine DCD lungs, possibily improving lung transplant outcomes.

Method

Porcine DCD lungs (n=3 per group) were flushed with and submerged in cold preservation solution. The lungs were stored up to 12 hours, and samples were taken from lung tissue and the preservation medium throughout. Metabolomic and redox potential were analyzed using HPLC, mass spectrometry and RedoxSYS®, comparing substrate and pathway utilization in both preservation solutions.

Results

Glutathione reduction was seen in Somah but not in LPD during preservation. Carnitine, carnosine and n-acetylcarnosine levels were elevated in the Somah medium compared to LPD throughout. Biopsies of Somah exposed lungs demonstrated similar trends after 2 hours, up to 12 hours. Adenosine gradually decreased in Somah medium over 12 hours, but not in LPD. An inversely proportional increase in inosine was found in Somah. Higher oxidative stress levels were measured in LPD.

Conclusion

Our study suggests suboptimal metabolic preservation in lungs stored in LPD. LPD had poor antioxidant potential, cytoprotection and an insufficient redox potential. These findings may have immediate clinical implications for human organs, however further investigation is needed to evaluate DCD lung preservation in Somah as a viable option for transplant.

INTRODUCTION

Lung transplantation is the only treatment for end-stage lung failure. Despite various attempts to increase graft availability, lung transplant suffers from a critical lack of suitable donor organs.[1] Fewer than 20% of post-mortem donor lungs are acceptable for transplantation and despite aggressive care, 10-year survival is only 29% to 31%.[2,3] While the majority of donor organs are donated after brain death (DBD), substantial evidence supports utilization of lungs donated after cardiac death (DCD), which would considerably increase the donor lung supply.[1,4] Common barriers to both DBD and DCD availability are ischemia-induced lung inflammation, vascular leakage and ischemia reperfusion (IR) injury occuring immediately after death and during the delay between recovery and transplant into the recipient.[5,6]

Over the past decades, promising prospectives have emerged in DCD lung utilization for transplant. The critical issue of warm ischemic times in a DCD setting has been addressed by continuous *ex vivo* lung perfusion (EVLP).[7] This approach has notable potential to expand the pool of available organs, both by assessment of graft quality as well as extending preservation time.[8-10] Nonetheless, in the past 15 years EVLP has not been a solution capable of diminishing the transplant gap. One important reason is that the lung evaluated in these experiments fall under Maastricht criteria III , with DCD occuring in a controlled hospital environment. Despite the impressive results demonstrated, the fraction of lungs addressed is only marginal, as the majority of possible DCD donations takes place after uncontrolled death, following Maastricht criteria I and II (**Table 1**). Expanding the DCD donor pool with lungs that fall under catergory I and II would clearly have enormous impact on the amount of available grafts. The use of Maastricht criteria III DCD lungs for transplant is generally considered as safe as DBD lung transplantation.[11,12] In addition to the relatively small number of DCD grafts that can be donated when solely focusing on Maastricht criteria III lungs, the limited number of medical institutions in possession of EVLP infrastructure stands in the way of this new approach from eliminating the donor lung scarcity. EVLP could be of enormous contribution in transplant, yet its biggest addition should be considered as a technique to improve and rejuvenate marginal lungs.[13-15]

TABLE 1
Maastricht criteria for DCD lungs.

Catergory	Desciption	Type
I	Dead on arrival (DoA)	Uncontrolled
II	Unsuccessful resuscitation	Uncontrolled
III	Awaiting cardiac arrest, treatment withdrawed	Controlled
IV	Cardiorespiratory arrest during/after diagnosis of brain death	Controlled

The study here presented therefore proposes a novel cold static storage solution to enhance current clinical procedures for DCD lungs donated for transplant.

A critical factor in transplant success is preserving the donor organ in optimal physiological condition between the time of collection and transplant. Current practice universally involves an *ex vivo* lung preservation technique based on cold static storage in a low-potassium dextran solution (LPD, Perfadex®) (**Table 2**). This solution provides protection against tissue edema, while the low temperature achieves a decrease in metabolic demand of the organ. Although LPD provides protection for up to 6 hours of storage[16] minimizing energy needs by hypothermia alone has not extended lung preservation time in clinical practice. Furthermore, IR injury occurs when blood flow is restored and oxygen is deliv-

TABLE 2
Composition of organ preservation solutions investigated, mmol/L.

Ingredient (mmol/L)	Somah	Perfadex
Na^+	125	138
K^+	7	6
Mg^{2+}	-	0.8
Cl^-	-	142
KCl	7	0.4
$MgCl_2$	0.5	-
$NaCO_3H$	5	-
$MgSO_4$	0.5	0.8
Glucose	11	5
Adenosine	2	-
Dextran 40	-	5%
Glutathione	1.5	-
$CaCl_2$	1.3	-
Phosphate	0.44	0.8
Insulin	1	-
l-Arginine	5	-
l-Citrulline malate	1	-
Creatine orotate	0.5	-
Creatine monohydrate	2	-
l-Carnosine	10	-
l-Carnitine	10	-
Dichloroacetate	0.5	-
Ascorbic acid	1	-
Sodium phosphate	0.19	-
pH (at 4°C)	7.5	7.4

ered to the lungs following implantation of a cold, statically stored organ. IR injury leads to apoptosis or necrosis of organ tissue triggered by mitochondrial disrupture.[17,18] In order to prevent irreversible damage to the organ it is critical to provide metabolic protection in addition to the traditional methods currently used for donor lung storage with LPD.

A novel solution, Somah (**Table 2**), aims to extend the *ex vivo* longevity of organs by supplying metabolic needs rather than minimizing energetic demand, while providing ROS scavenging agents and tissue protection. Studies using porcine hearts have demonstrated the potential benefit of Somah for cardiac transplantation in hypothermic and subnormothermic settings.[19,20] However, in order to fully evaluate a novel solution's mechanism and clinical potential, assessment metabolic and redox pathways are required. By investigating the metabolomics and redox aspects of this novel solution and comparison with the conventional organ storage solution, we aim to better comprehend the *modus operandi* of Somah and its potential benefits. In order to construct the optimal manner of pulmonary graft preservation, it is mandatory to have a broad understanding of tissue and solution interaction over time.

We evaluated the metabolomics and redox state of porcine Maastricht category I-like DCD lungs, statically stored in cold (4°C) LPD or Somah solution. Understanding the influence of different preservation solutions may provide an opportunity to expand the donor pool of lungs by safeguarding or modulating the metabolic state of DCD lungs prior to transplant.

METHODS

Storage Solutions

Somah was purchased from Somahlution (Jupiter, FL) and Perfadex® LPD solution was purchased from XVIVO Perfusion (Gothenborg, Sweden). Solutions were freshly prepared on the day of the experiment. Preservation solutions were randomly assigned to one lung per experiment and blinded to researchers handling the organs.

Organ procurement

Organs were procured from Yorkshire pigs (50-60kg) at the University of Arizona Food Product and Safety Laboratory, in accordance with United States Department of Agriculture and University of Arizona regulations. Pigs were sacrificed by electrocution and rapidly exsanguinated without anesthetics before organ harvest. Due to the traumatic nature of the injury, these circumstances mimic the Maastricht I-like criteria. The lungs were randomly assigned to either LPD or Somah and individually rinsed with 1 liter

of preservation solution by anterograde flushing through the pulmonary trunk, while ensuring exclusive flow through either the right or left pulmonary artery by temporarily sealing the contralateral vessel using Kelly forceps (**Figure 1**). Subsequently the lungs were surgically separated and submerged in their respective cold preservation solution. As a result of splitting the lung pairs into two separate lungs, biological variability was minimized. This way, each animal used in the study is represented in both the LPD and

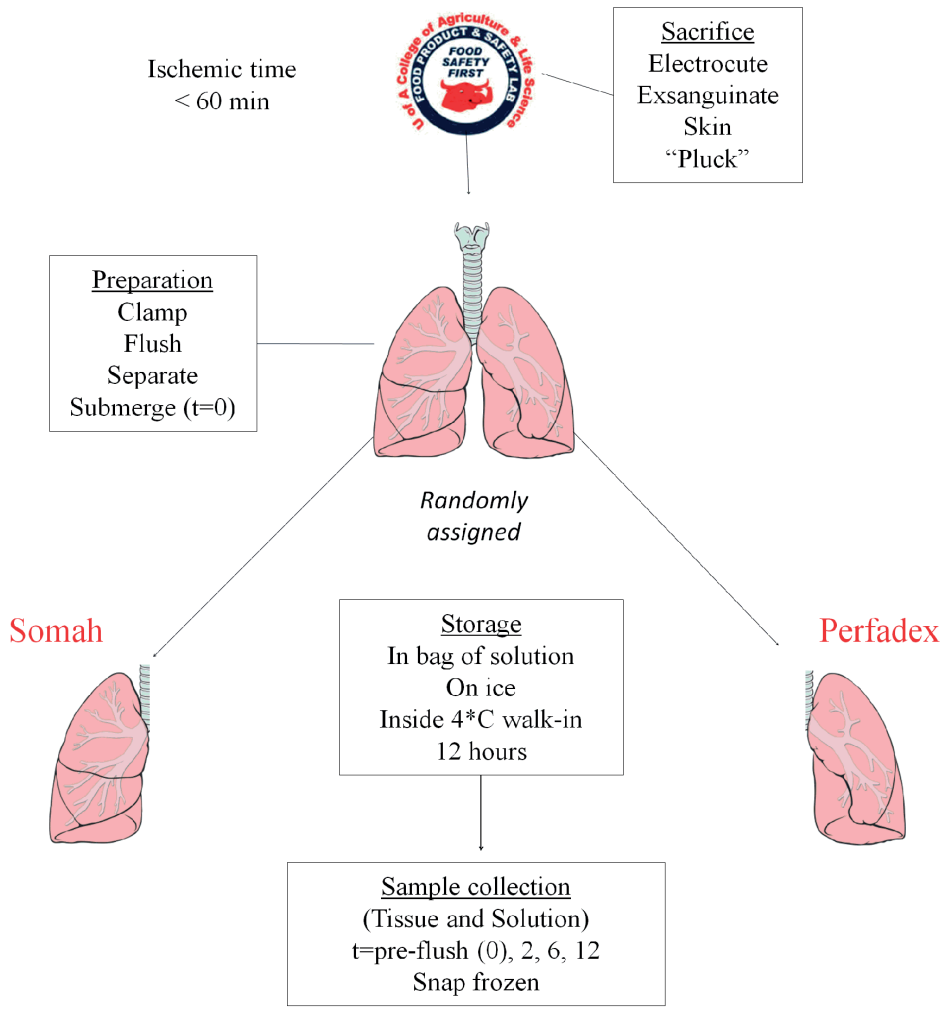

Fig. 1. Schematic overview of experimental set-up. Pigs were shocked and rapidly exsanguinated without anesthetics before organ harvest. The lungs were randomly assigned to either LPD or Somah and individually rinsed with 1 L of preservation solution by anterograde flushing through the pulmonary trunk. Subsequently the lungs were surgically separated and submerged in their respective preservation solution. Medium samples and lung biopsies were taken from the before introducing the preservation solution to the lungs (t=0) and at t=2 h, t=6 h, and at t=12 h. Tissue biopsies and solution samples were immediately snap frozen in liquid nitrogen and stored at -80°C.

the Somah group. Lungs that were not flushed, split and submerged within 60 minutes after circulatory death of the pig were excluded from our study.

Experimental procedure

Samples (5mL) were obtained from the preservation solution before submerging the lungs (t=0 hours) and then at t=2 hours, t=6 hours and t=12 hours post submersion (**Figure 1**). Similarly, tissue biopsies (~1gr each) were obtained from the apex of the inferior lobe of the lungs at the same time points. Tissue biopsies and solution samples were immediately snap frozen in liquid nitrogen and stored at -80° C until further processing for analyses.

Metabolic analysis

Metabolomic and statistical analyses were conducted at Metabolon, Inc. (Durham, NC) as described previously.[21] Briefly, tissue samples were homogenized (Covaris), then all samples were subjected to methanol extraction then split into aliquots for analysis by ultrahigh performance liquid chromatography/mass spectrometry (UHPLC/MS) in the positive (two methods, one optimized for hydrophilic, the other hydrophobic compounds), negative or polar ion mode. Metabolites were identified by automated comparison of ion features to a reference library of chemical standard followed by visual inspection for quality control.[22] For QA/QC, a pooled client matrix (or for plasma, an internal matrix) as well as several internal standards were assessed to determine instrument variability, with RSD = 3% (lung or medium) for internal standards and RSDs = 7% (lung) and 9% (medium) for endogenous biochemicals. For statistical analyses and data display, any missing values were assumed to be below the limits of detection; these values were imputed with the compound minimum (minimum value imputation). To determine statistical significance, a repeated-measures 2-way ANOVA was performed in ArrayStudio (Omicsoft) or "R" on log-transformed data to compare data between experimental groups; $P < 0.05$ was considered significant. An estimate of the false discovery rate (Q-value) was calculated to take into account the multiple comparisons that normally occur in metabolomic-based studies, with $Q<0.05$ used as an indication of high confidence in a result.

Redox potential analysis

Redox analysis was performed using RedoxSYS Diagnostics System (Aytu BioScience, Englewood, CO). The balance of all oxidants and reductants in each sample is measured as static oxidation reduction potential (sORP), which is an indicator for the oxidative stress a sample is subjected to. The driving current over the sample is measured in milivolts (mV). Lower values indicate less oxidative stress. Capacity oxidation reduction potential (cORP), also known as total antioxidant capacity (TAC), is measured in microcoulombs (μC) and expresses the antioxidant reserve in samples. Higher values indicate more ROS scavenging capacity.[23]

RESULTS

Glutathione metabolism

Somah demonstrated higher basal (t=0) concentration of reduced glutathione (GSH) in the medium than LPD ($p<0.05$; **Figure 2A**). GSH was rapidly depleted from the Somah medium, leading to comparable concentrations of GSH in both mediums after 2 hours. Oxidized glutathione (GSSG) remained stably elevated in Somah medium over LPD during the entire experiment ($p<0.05$; **Figure 2B**).

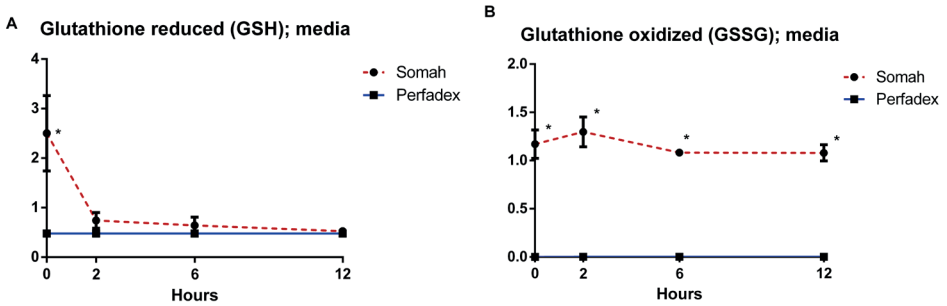

Fig. 2. (a) Reduced glutathione (GSH) in Somah and LPD medium over time. (b) Oxidized glutathione (GSH) in Somah and LPD medium over time. N=3 for each group, plotted values are mean ± SEM, * $P<0.05$.

Carnitine and carnosine metabolism

Both carnitine and carnosine levels in Somah medium were significantly higher during all time points ($p<0.05$; **Figure 3A-B**). In the tissue biopsies, significantly increased levels of carnitine were found in the Somah group from t=2 and were persistent during the entire experiment ($p<0.05$; **Figure 3C**). In addition to this finding, acetylcarnitine, a derivative from carnitine, and n-acetylcarnosine, an antioxidant intermediate in the histidine pathway, followed a similar trend in the biopsies. Though basal levels were not significantly different between groups, biopsies of lungs in Somah group contained statistically significant higher acetylcarnitine and n-acetylcarnosine values ($p<0.05$; **Figure 3D-E)**. The Somah group had higher values of n-acetylcarnosine in the medium throughout ($p<0.05$; **Figure 3F**).

Adenosine metabolism

Adenosine-enriched Somah solution demonstrated significantly higher adenosine concentration in the medium throughout the experiment, with adenosine gradually depleted during the entire 12 hours ($p<0.05$; **Figure 4A**). Tissue samples showed no differences in adenosine between groups (**Figure 4B**).

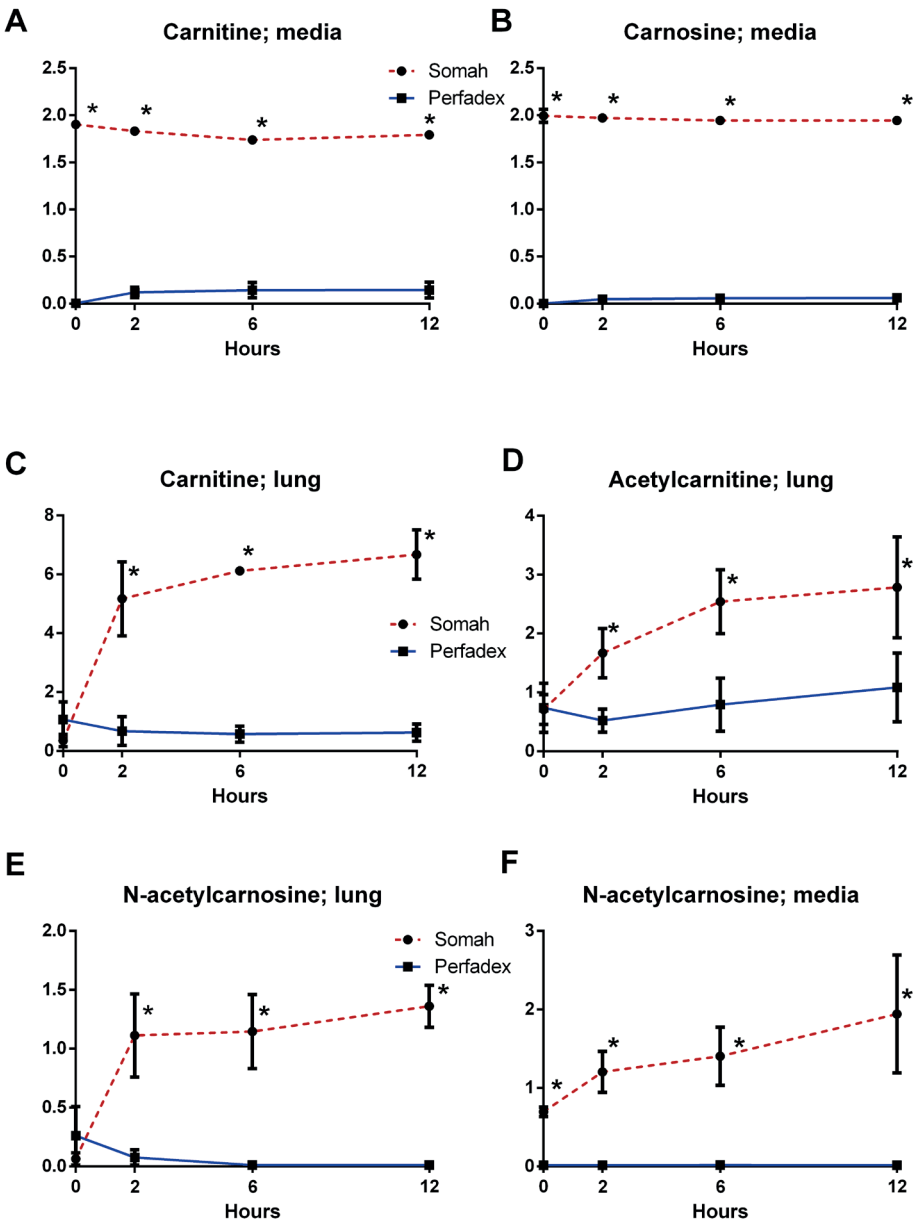

Fig. 3. (a) Carnitine in Somah and LPD medium over time. (b) Carnosine in Somah and LPD medium over time. (c) Carnitine in Somah and LPD lung biopsies over time. (d) Acetylcarnitine in Somah and LPD lung biopsies over time. (e) n-acetylcarnosine in Somah and LPD lung biopsies over time. (f) n-acetylcarnosine in Somah and LPD medium over time. N=3 for each group, plotted values are mean ± SEM, * P<0.05.

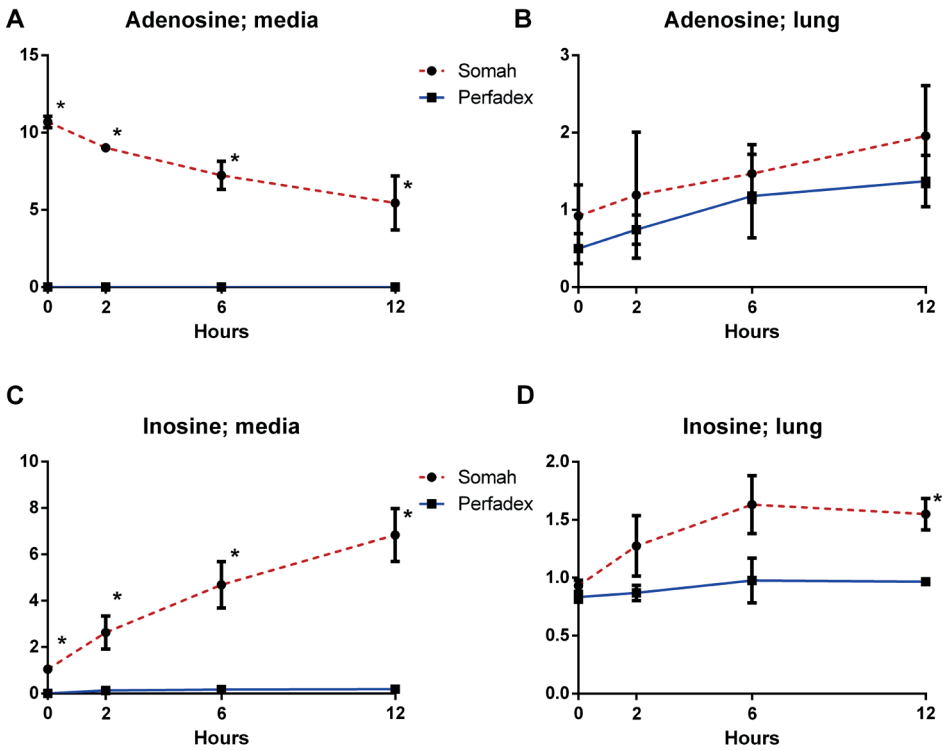

Fig. 4. (a) Adenosine in Somah and LPD medium over time. (b) Adenosine in Somah and LPD lung biopsies over time. (c) Inosine in Somah and LPD medium over time. (d) Inosine in Somah and LPD lung biopsies over time. N=3 for each group, plotted values are mean ± SEM, * P<0.05.

Along with adenosine catabolization, inosine in Somah medium increased gradually, a trend not found in LPD ($p<0.05$; **Figure 4C**). Inosine levels in Somah-preserved lung tissue were elevated at the beginning of the experiment and increased over time; the difference between groups did not reach statistical significance until t=12 ($p<0.05$; **Figure 4D**).

Carbohydrate metabolism

Somah contains higher basal levels of glucose, malate and fumerate, while fructose is higher in LPD ($p<0.05$; **Figure 5A-D**). Citric acid (TCA) cycle intermediates showed larger increments in Somah than in LPD across all time points ($p<0.05$; **Table 3**). In tissue samples, significantly higher mannitol/sorbitol was found when preserved in LPD after t=2 ($p<0.05$; **Figure 5E**). Malate in the tissue showed a trend of greater increase in Somah than LPD, but did not reach statistical significance (**Figure 5F**).

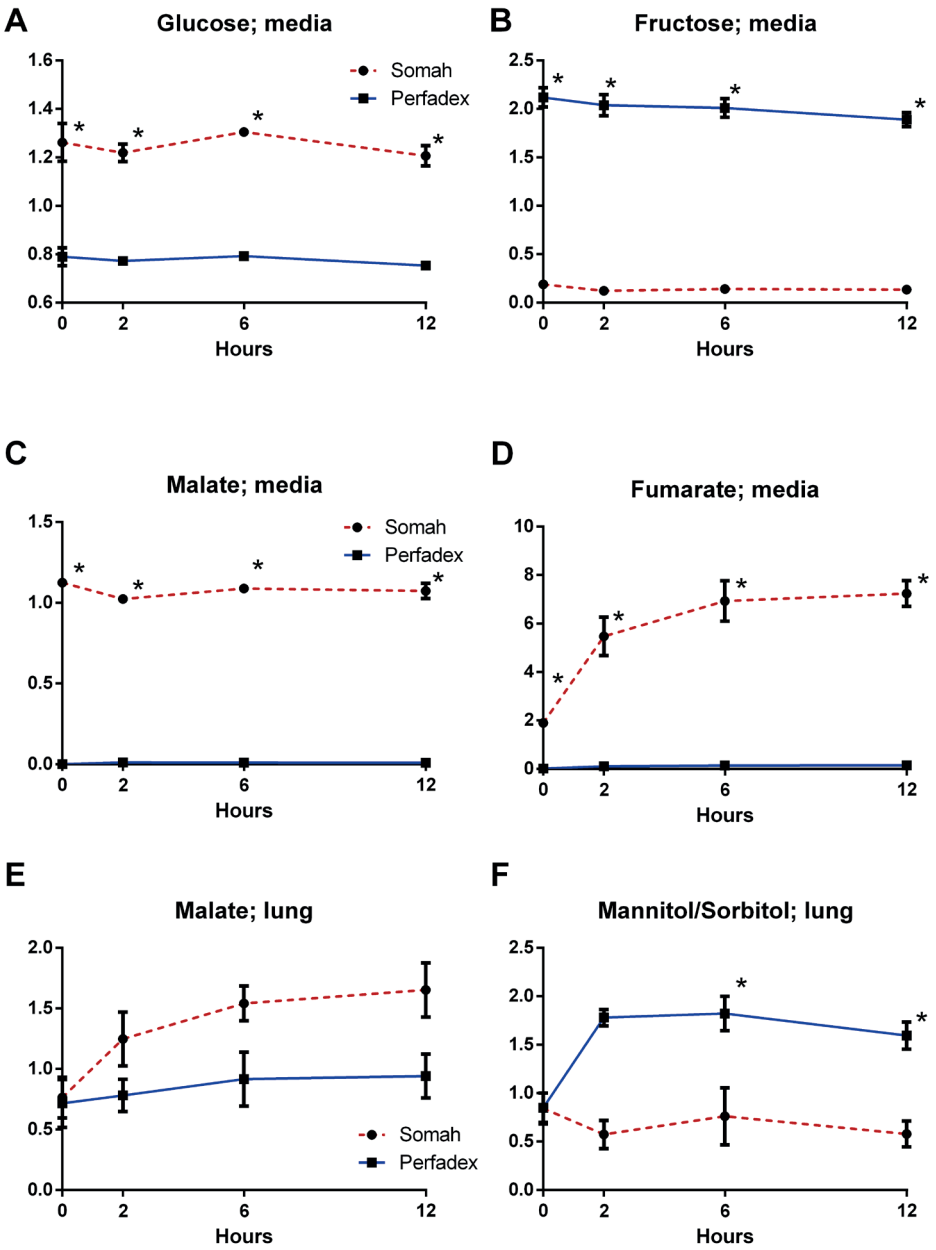

Fig. 5. (a) Glucose in Somah and LPD medium over time. (b) Fructose in Somah and LPD medium over time. (c) Malate in Somah and LPD medium over time. (d) Fumarate in Somah and LPD medium over time. (e) Malate in Somah and LPD lung biopsies over time. (f) Mannitol/sorbitol in Somah and LPD lung biopsies over time. N=3 for each group, plotted values are mean ± SEM, * P<0.05.

TABLE 3

TCA cycle components in preservation solutions over time; n=3 for each sample.

Media	Perfadex 0h	Perfadex 2h	Perfadex 6h	Perfadex 12h	Somah 2h	Somah 6h	Somah 12h	Perfadex 2h	Perfadex 6h	Perfadex 12h
Biochemical	Somah 0h	Somah 2h	Somah 6h	Somah 12h	Somah 0h	Somah 0h	Somah 0h	Perfadex 0h	Perfadex 0h	Perfadex 0h
citrate	0,6	**1,8**	1,62	1,28	**3,36**	**4,78**	**6,58**	**10,05**	**12,86**	**14,08**
alpha-ketoglutarate	1,35	0,99	0,82	0,6	**13,77**	**22,73**	**32,41**	**10,18**	**13,82**	**14,4**
succinate	**0,43**	**0,35**	**0,33**	**0,3**	**5,58**	**8,47**	**10,17**	**4,54**	**6,39**	**7,13**
fumarate	**0,01**	**0,02**	**0,02**	**0,02**	**2,88**	**3,65**	**3,81**	**7,21**	**9,8**	**9,99**
malate	**0**	**0,01**	**0,01**	**0,01**	0,91	0,97	0,95	**25,25**	**23**	**21,75**

0,55	**Green:** indicates significant difference ($p \leq 0.05$) between the groups shown, metabolite ratio of < 1.00
0,76	**Light Green:** narrowly missed statistical cutoff for significance $0.05 < p < 0.10$, metabolite ratio of < 1.00
1,71	**Red:** indicates significant difference ($p \leq 0.05$) between the groups shown; metabolite ratio of ≥ 1.00
1,32	**Light Red:** narrowly missed statistical cutoff for significance $0.05 < p < 0.10$, metabolite ratio of ≥ 1.00
1,20	**Non-colored text and cell:** mean values are not significantly different for that comparison

Redox state

Samples of LPD medium demonstrated elevated levels of sORP compared to Somah samples during the entire preservation ($p<0.05$; **Figure 6A**). Levels of cORP in Somah medium were significantly higher at each time point than LPD ($p<0.05$; **Figure 6B**).

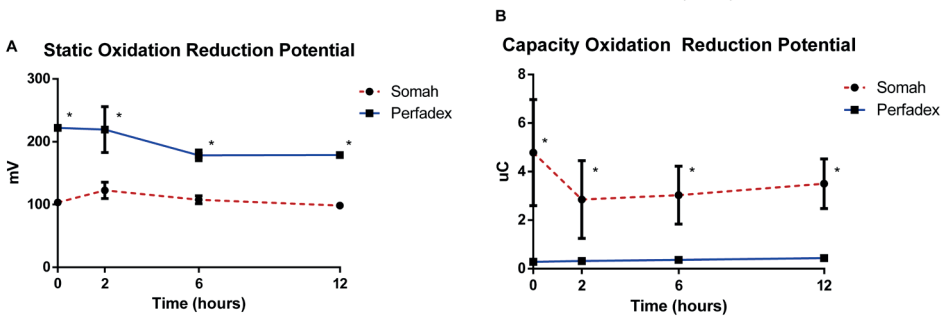

Fig. 6. (a) Static oxidation reduction (sORP) in millivolts (mV) of Somah and LPD medium over time. (b) Capacity oxidation reduction potential (cORP) in microcoulombs (mC) of Somah and LPD medium over time. N=3 for each group, plotted values are mean ± SEM, * P<0.05.

DISCUSSION

Limited *ex vivo* storage time of lungs has been one of the main restricting factors in the transplant success rate. Trials exploring the potential of EVLP as a preservation standard provide exciting prospects, but are increasingly aiming to rejuvenate marginal grafts rather than increasing preservation time. Moreover, EVLP has limitations that impede widespread usage of continuous perfused preservation. The need for improved cold static storage of donor lungs therefore remains unabated. Current static lung preservation protocols often lead to the transplantation of a marginal graft, and may lead to the organ being rejected. Extending *ex vivo* storage time with the right conditions would ensure better quality of organs transplanted. In addition to this enormous benefit, the organs' transplantable geographic radius would increase, providing the optimal HLA-match, and ideally facilitating a global organ donor program. However, little is known about the metabolomics of the lung allograft at hypothermia with the current golden standard lung transplant preservation solution, LPD. We evaluated the metabolomics pathways of the current practice organ preservation medium and compared the effects and implications of a novel solution on cellular and mitochondrial metabolism during cold static storage of porcine DCD lungs. Our study shows that LPD is unable to scavenge ROS effectively at hypothermia. ROS is a major cause of tissue apoptosis and necrosis in IR injury, down stream effects of which could be minimized. Both the lack of antioxidants and the redox status seen in the medium raise questions about our current use of LPD. Somah's ROS

scavenging ability could be an alternative substitute. Another important cytoprotective agent that is missing in LPD is adenosine, which is supplemented in Somah's formula. The reduction of adenosine along with the elevation in inosine, as observed in the Somah medium, are strong indicators of active adenosine catabolism during storage. LPD does not provide cellular protection or inflammatory modulation in a similar manner. Better characterization of the metabolomics in donor lung organ preservation solutions leads us to understand the foundations of limited longevity when lungs are stored in cold LPD. It also gives an insight on how Somah may better protect or even recondition DCD marginal lungs. This could theoretically prevent IR injury following lung transplantation and thus improve primary graft dysfunction and transplant outcomes.

Glutathione metabolism

Depletion of glutathione pathway intermediates in Somah medium after 2 hours suggests tissue utilization of these substrates. This reduction might be indicative of ROS scavenging and redox potential stabilization. GSH and GSSG can be used as a marker for oxidative stress.[24-26] The penetration of GSH appears to be suboptimal due to the static environment. It could be suggested that the tissue penetration would be better if the organ were continually perfused. Combining the benefits of continuous organ perfusion by EVLP and an optimal preservation solution that features higher glutathione metabolism has enormous potential in the search for increasing *ex vivo* graft storage time.

Carnitine and carnosine metabolism

Carnitine has an important role as a transporter of fatty acids over the mitochondrial membrane. The increase in carnitine in Somah lungs implies β-oxidation progression into the TCA-cycle and ATP production. More importantly, a deficit in carnitine during ischemic periods can cause a defect in metabolic processing and ATP production upon reperfusion.[27]

Furthermore, carnitine plays a role in gluconeogenesis through fatty acid oxidation as well as being a stabilizing agent in cell membranes,[28,29] providing protection against cytotoxic free fatty acids.

In addition to these findings, carnosine intermediates found in Somah-preserved lungs highlight a strong ROS scavenging role. This further supports the suggested antioxidant capacity of Somah. The accumulation of acetylcarnosine in lung tissue can play a vital, beneficial role against reactive oxygen species upon reperfusion of the lungs. Carnitine and carnosine levels are significantly higher after t=2 in Somah-preserved lung tissue and in Somah solution itself, implying the benefits of increased carnitine and carnosine uptake are seen in the Somah group and not the LPD group.

Adenosine metabolism

Adenosine in preservation solutions has notable influences on multiple levels. We have previously shown that adenosine has a cytoprotective role [30,31]; in lungs it is of great importance to protect cellular function during ischemic periods. Once the organ is transplanted, the need for adequate functioning (i.e. gas exchange) is vital.

Moreover adenosine possesses anti-inflammatory properties.[32,33] During ischemia, adenosine can play a pitoval role in preventing reperfusion injury.[34,35] The absence of adenosine in LPD raises concerns about the protective measures on the inflammatory response as well as IR injury.

Carbohydrate metabolism

Glucose levels, although being supplemented in both solutions, are higher in the basal formulation of Somah. Significant evidence of differerent carbohydrate metabolism is found in the basal variation in the medium, as Somah contains higher malate and fumerate levels and LPD demonstrates increased fructose levels. The effects of this contrast may be shown in the tissue biopsies. Lungs preserved in Somah demonstrate increased TCA cycle utilization, based on the increase in malate and succinate, whereas LPD lung tissue showed a muted response, although no statistical significance was found. This lack of significance might be due to the hypothermic storage conditions, effectively decelerating all cellular processes including TCA cycle reactions. Fructose metabolism in LPD lungs is demonstrated by the increments of sorbitol over time.

These finding suggest a better energetic profile for organs in Somah. This is supported by the accumulation of glycolytic metabolites such as pyruvate and lactate. Although this response is seen in both groups, the increase is greater in Somah medium. Altogether these results indicate the favorable impact of Somah on glycolytic circumstances, such as ischemic *ex vivo* storage.

Redox potential

The increased degree of sORP found in LPD samples are indicative of elevated oxidative stress, and the lower sORP levels in Somah highlight its ROS scavenging potential. Additionally the cORP results indicate greater antioxidant capacity in Somah throughout.[23] The measurement of sORP and cORP may provide some benefits over the more standard methods of ROS assessment. The application of traditional ROS assays have been up for debate as they are known to have various limitation. [36-38] By measuring the complete static and capacity ORP we aim to overcome the difficulties in ROS measurement, while demonstrating redox state, of which ROS is an integral part. Combining glutathione assays with redox potential provides a powerful evaluation of oxidative stress in the tissue.[26]

A limitation of our study is the absence of reperfusion after cold static storage. The investigators acknowledge the pivotal role of oxygen as a mediator of IR injury. As mentioned before, mitochondrial rupture upon reperfusion of the organ with oxygenated blood is a key mediator of tissue apoptosis. However, given the lack of understanding of what bioenergetic requirements are met during cold static storage as well as the protective means of current preservation solutions, a deeper understanding of the metabolic pathways was our focus. The present study aims to investigate differences in metabolic pathways, and methodology has been developed accordingly. As a result of the set-up, hoewever, it remains unclear if the effects are seen locally rather than throughout the entire pulmonary milieu. We also acknowledge that functional lung parameters were unable to be measured at hypothermia in this experimental setting and that further studies would be required following reperfusion of DCD lungs.

Lastly, biological variability between the donor pigs could be regarded as a limitation of our study. Our experimental set-up counteracted this issue by splitting the lungs before flushing with preservation solution, creating matched pairs. Thus, each animal was represented in both groups, one half of the lung serves as its own control, and the variation between animals was abated.

Conclusion

There has been little progress over the decade in lung transplantation preservation solutions. Altered cellular metabolism by hypothermia does decelerate energy depletion and tissue damage, but does not provide circumstances that exceed the 6-hour *ex vivo* time limit before critical damage occurs [39,40]. In pursuit of extending this time limit for cardiothoracic organs it is essential to minimize IR injury. The formulation of Somah solution could be a logical step towards maintenance of sufficient ATP levels, mitochondrial integrity and protection against IR injury. Although the tissue damage occurs upon reperfusion after transplant, the environment in which the graft is preserved plays a large role. Insufficient bioenergetic capacity or reserve and the loss in ATP associated with that is a major trigger for IR injury. Given the basal formulation of LPD, insufficient glycolytic capability and heightend fructose metabolism was expected. Consistent with this we observed an increase in fructose metabolites. The opposite was seen in Somah lungs, where the accumulation of TCA cycle components is representative of a favourable energetic profile upon transplant and glucose enrichment entails more glycolytic capacity, findings that were expected with Somah's basal formulation. Furthermore, DCD lungs remain underutilized as transplantable organs after cold static storage. The warm ischemic times often seen in DCD organ harvest as well as cytokine storm and inflammation upon cardiac arrest, further elucidate the need of preservation approaches that safeguard bioenergetic state and posses anti-inflammatory capacity. Our study illustrates the potential to enhance metabolic and redox

conditions in a way not seen in current practice with traditional LPD. Lung preservation solutions have hardly evolved over the past decade and this is underlined by insignificant extension of static *ex vivo* graft preservation time. Thus, in conclusion, to increase the lung donor pool, it is essential to find novel approaches that improve the metabolic state of the organ. Somah does so in a way that could introduce DCD lungs as an acceptable alternative resource pool for lung transplant with the potential of reducing primary graft dysfunction and its sequelae. Despite the highly encouraging results demonstrate in the current study, the effects of organ reperfusion after preservation remain unknown, as well as functional lung data. Studies investigating these factors and outcomes are needed in order to validate the suggested observations.

CONFLICT OF INTEREST

One author is an unpaid consultant for Somahlution, the manufacturer of Somah solution investigated in this study. All other authors declare no conflict of interest.

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Chapter 7

Anti-inflammatory properties of amniotic membrane patch following pericardiectomy for constrictive pericarditis

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ABSTRACT

Background

Since constrictive pericarditis is most often idiopathic and the pathophysiology remains largely unknown, both the diagnosis and the treatment can be challenging. However, by definition, inflammatory processes are central to this disease process. Amniotic membrane patches have been shown to possess anti-inflammatory properties and are believed to be immune privileged. Due to these properties, amniotic membrane patches were applied intraoperatively in a complicated patient presenting with constrictive pericarditis.

Case presentation

A patient with a history of multiple cardiac surgeries presented with marked fatigue, worsening dyspnea and sinus tachycardia. He was found to have constrictive physiology during cardiac catheterization, with cardiac MRI demonstrating hepatic vein dilatation, atrial enlargement and ventricular narrowing. After amniotic membrane patch treatment and pericardiectomy, post-operative cardiac MRI failed to demonstrate any appreciable pericardial effusion or inflammation, with no increased T2 signal that would suggest edema.

Conclusions

Given the positive results seen in this complex patient, we suggest continued research into the beneficial properties of amniotic membrane patches in cardiac surgery.

BACKGROUND

Constrictive pericarditis results in a thickened and less elastic pericardium, which can lead to incomplete diastolic filling and myocardial ischemia [1]. Since it is rare and the presenting symptoms are similar to those of several other disorders, the diagnosis can often be challenging. The diagnosis is usually made using cardiac catheterization or echocardiography as a part of the patient's initial clinical evaluation. Although there are multiple etiologies of constrictive pericarditis, in most cases, the pathophysiology is idiopathic or may occur following cardiac surgical procedures including orthotopic heart transplant [2]. Constrictive pericarditis is commonly treated with pericardiectomy; however even following surgical intervention, long-term survival decreases over time and further diminishes when patient history includes multiple cardiac re-operations [3].

It has recently been demonstrated that amniotic stem cell therapy consisting of either stem cells with extracellular matrix or extracellular matrix alone can decrease fibrosis and postoperative inflammation in humans [4, 5]. Specifically, extracellular matrix in the form of human amniotic membrane allograft has shown to significantly reduce post-ischemic cardiac dysfunction, improve ischemic heart repair, and increase blood flow recovery in rat and mouse models [6, 7]. This immunoprivileged tissue does not need to be donor-recipient matched to produce positive outcomes, further supporting its convenience of use and value [8]. In the context of these anti-inflammatory properties, and since inflammatory processes are central to the pathophysiology of pericarditis, amniotic membrane patches were applied intraoperatively in a patient presenting with constrictive pericarditis as outlined below.

CASE PRESENTATION

A 34-year-old male had a history of orthotopic heart transplant for hypertrophic obstructive cardiomyopathy and subsequent tricuspid repair for severe tricuspid regurgitation due to prolapse. The heart transplant was performed at a medical center in a neighboring state, with both prior surgeries performed by two different surgeons. He was doing well for over 3 years following these procedures, maintained with immunotherapy consisting of 2.5 mg Prograf® BID and 250 mg CellCept® daily as noted by the CT 7 Transplant Database. He then developed a one-week history of marked fatigue, worsening shortness of breath, and sinus tachycardia on electrocardiogram and was transferred to our facility from a neighboring state. Upon arrival, the patient's ejection fraction was significantly reduced at less than 20%, compared to his usual baseline of 60% last recorded 1.5 years prior to this presentation. Given the concern for acute transplant rejection, the patient

underwent endomyocardial biopsy via catheterization, which revealed evidence of Grade 1R mild acute cellular rejection without the presence of antibody-mediated rejection as evidenced by a pathologic antibody-mediated rejection (pAMR) of 0. Hemodynamic findings from right and left cardiac catheterization revealed equalization of diastolic filling pressures with discordance after volume loading, consistent with constrictive physiology. Additionally, Freidreich's sign was present with both steep x and y descent of the jugular venous pressure (JVP) tracing with increased left and right ventricular diastolic pressures with dip and plateau (square root sign). The patient then underwent a cardiac magnetic resonance imaging (MRI) study to further evaluate the constrictive physiology. Cardiac

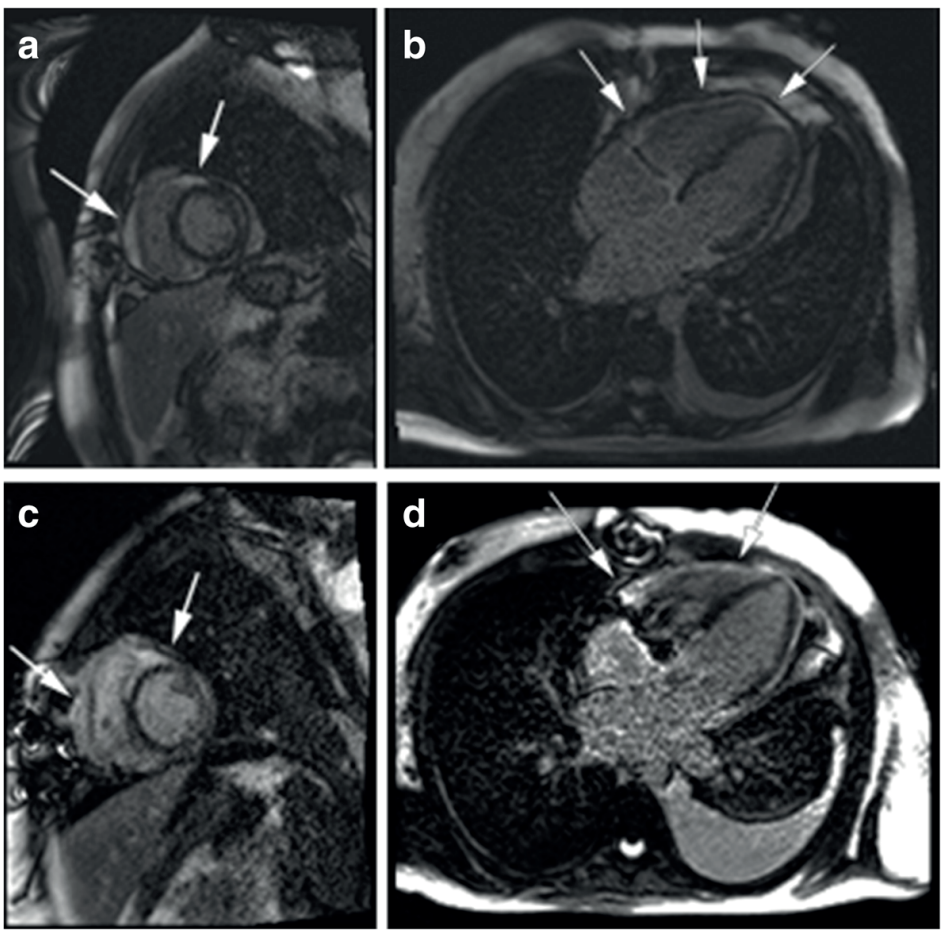

Fig. 1 Post-contrast T1-weighted cardiac MRI images. Preoperative MRI short-axis (a) and 2-, 3-, and 4-chamber long axis (b) delayed-enhanced inversion-recovery images demonstrate pericardial thickening and epicardial enhancement (arrows) with subtle periventricular septal flattening.

On dynamic cine imaging and anatomic imaging (not shown) there is septal bounce during Valsalva suggesting ventricular inter-dependance and biatrial enlargement with dilated IVC and hepatic veins. These findings are absent on postoperative cardiac MRI (c-d).

MRI revealed mild pericardial thickening, mild flattening and bounce of the interventricular septum, intermediate epicardial signal on pre-contrast T2weighted images and demonstrated intermediate delayed enhancement with post-contrast T1-weighted images consistent with fibrotic tissue (Fig. 1a, b). There was also moderate to moderate-severe tricuspid regurgitation. Given the hemodynamic values from cardiac catheterization and pericardial thickening, surgery was recommended to replace the tricuspid valve and remove the fibrotic epicardial material.

Using a redo sternotomy approach, complete phrenic to phrenic pericardiectomy, removal of Gore-Tex membrane from previous surgery and a tricuspid valve replacement were completed. Gross intraoperative findings of the pericardial space included thick, gelatinous material on the anterior surface of the heart, and the pericardium and Gore-Tex membrane were fused to the thickened pericardium on the anterior surface of the heart. (Fig. 2a) Prior to closure, four human allograft membranes were topically placed over the right atrium, right ventricle and left ventricle (Fig. 2b) for their anti-fibrotic and anti-inflammatory properties [7, 8]. The surgical pathology report later confirmed fibrosis of the explanted tricuspid valve, pericardial fibrosis, and chronic pericardial inflammation. Though constrictive physiology was still noted, post-contrast T1-weighted cardiac MRI images demonstrate significant improvement (Fig. 1c, d). Furthermore, a five-week postoperative fat-suppressed T2-weighted MRI revealed no appreciable postoperative inflammation and an absence of pericardial effusion (Fig. 3).

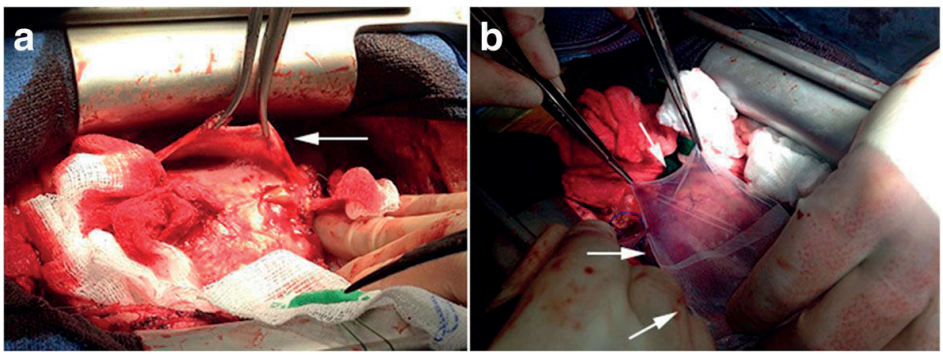

Fig. 2 Intraoperative images. Thick gelatinous material was found on the anterior surface of the heart, with the pericardium and Gore-Tex membrane fused to the thickened pericardium at the anterior surface (a). Four human allograft membranes were topically placed over the right atrium, right ventricle, and left ventricle (b) for their anti-fibrotic and anti-inflammatory properties.

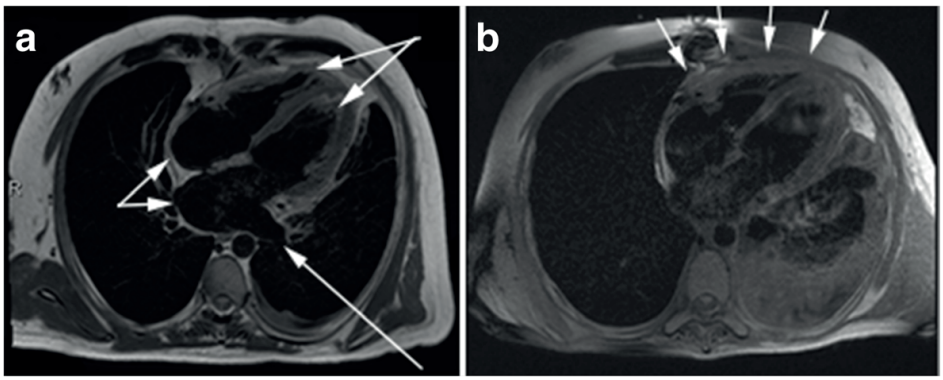

Fig. 3 T2 Fat Sat Horizontal long-axis fat-suppressed T-2 weighted images. Preoperative cardiac MRI (a) reveals hepatic vein dilatation, atrial enlargement and ventricular narrowing (arrows). Post- operative cardiac MRI (b) fails to demonstrate any appreciable pericardial effusion or inflammation, with no increased T2 signal that would suggest edema (*arrows*).

DISCUSSION

Constrictive pericarditis is difficult to diagnose given its rare occurrence and that the presenting symptoms are similar to those of several other disorders. In our patient, this diagnosis was further complicated by his history of orthotopic heart transplant. While the techniques continue to evolve, imaging patients in order to optimize diagnosis following cardiac transplant is limited [9]. Given our patient's surgical history and clinical presentation, the constrictive physiology from cardiac catheterization was considered to be a more reliable diagnostic measure than both the MRI and echocardiogram findings. The case discussed serves as a prime example for the need to develop alternate non-invasive imaging techniques or algorithms for improved diagnostic capacity in patients with prior orthotopic heart transplant.

Another confounding variable in diagnosing constrictive pericarditis in our patient was the steep y descent on JVP. Although Freidreich's sign is suggestive for constrictive pericarditis, our patient was also diagnosed with severe tricuspid regurgitation. However, despite these diagnostic difficulties, the patient was known to have prior Gore-Tex membrane placement during surgery. This iatrogenic component is another factor that may, in part, explain his constrictive physiology.

As discussed previously, pericardiectomy is often performed as a curative procedure for constrictive pericarditis. However there are instances, particularly for patients with advanced constrictive pericarditis or with radiation disease, in which pericardiectomy may not offer a cure or desired long-term result [1]. For these patients with higher risk

factors, it may be beneficial to explore additional or alternative treatment options. Current treatment of recurrent pericarditis has focused on targeting inflammation, and has shown overall positive outcomes [3]. Given our patient's extensive cardiac history of orthotopic heart transplant, reoperation for tricuspid valve repair, and Gore-Tex adhesions, extraordinary care was initiated in an attempt to resolve his constrictive pericarditis. As an emerging anti-inflammatory and anti-fibrotic treatment, the use of human allograft membrane has proven to be both safe and effective in humans thus far and continues to pique interest as an alternative therapy option [7, 8]. The anti-inflammatory properties of this treatment were exemplified, as our patient had no evidence of inflammation notable on T2weighted MRI five weeks postoperatively (Fig. 3b). Given the positive results, we suggest continued research into the beneficial properties of amniotic membrane patches in cardiac surgery.

CONCLUSION

Constrictive pericarditis is difficult to treat, and even a pericardiectomy may not offer a cure or desired long-term result. Given the inflammatory processes central to this disease process, amniotic membrane patches were used as anti-inflammatory treatment in a patient with a complicated history. With the amniotic membrane patch treatment and pericardiectomy, our patient had no evidence of inflammation five weeks postoperatively on T2weighted MRI, highlighting the importance of continued research in this area.

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Competing interests

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Chapter 8

Micronized human amniotic stem cell matrices improve panel reactive antibody (PRA) against HLA-I and II in patients undergoing left ventricular assist device placement (LAVD)

Schipper DA, Premyodhin N, Ashbeck JD, Johnson K, Kovacs KL, Skaria RS, Pilikian T, Laman JD and Khalpey Z

ABSTRACT

Background and Aim

Sensitization, the development of patient antibody against human leukocyte antigen (HLA) class I and II, is a major obstacle in heart transplantation. Mesenchymal stem cells as well as amniotic derived extracellular matrices have been associated with immunosuppression in various applications. This open label retrospective, observational study addresses whether local injection of human amniotic stem cells in liquid extracellular matrix is associated with reduced development of such anti-HLA antibodies, thus improving chances of long term transplant acceptance of a transplanted heart.

Methods

Ten patients at our institution undergoing implantation of LVAD received a concurrent myocardial administration of allogeneic human amnion-derived mesenchymal stem cells in liquid extracellular matrix to increase cardiac contractility. We retrospectively evaluated the effect of this therapy on anti-HLA class I and II antibody (panel reactive antibody, PRA) in comparison with control patients who did not receive intra-operative stem cell administration. The stem cell and control patients were stratified into ischemic and dilated cardiomyopathy groups. Baseline and postoperative class I and II antibody presence was evaluated where available using the Luminex single antigen bead PRA assay.

Results

Compared to baseline values, two patients with ischemic cardiomyopathy who received stem cell injection intra-operatively demonstrated reduced percentages of class I or class II antibody formation on post-operative evaluation two to four months later. The degree observed on the Luminex solid phase assay in these two patients was not increased compared to the controls, and a third case demonstrated reduction of anti-HLA antibody level. Results for patients with dilated cardiomyopathy varied.

Conclusions

Our results suggest that injections of allogeneic mesenchymal stem cells in liquid extracellular matrix suppress anti-HLA antibody formation through immunomodulatory means. This is a potentially important finding in the optimization of bridge-to-transplant approaches.

INTRODUCTION

Sensitization in the context of organ transplantation is defined as a recipient's state of having HLA-I and/or HLA-II antibodies circulating, or the susceptibility to formation of such antibodies upon transplantation.[1] Increased sensitization raises the risk of acute rejection and primary graft dysfunction, and it shortens survival in patients undergoing cardiac transplant.[2-5] Therefore it is of vital importance for potential organ recipients to remain de-sensitized while on the transplant waiting list. Sensitization can be measured by monitoring the presence of anti-HLA antibody via the Panel Reactive Antibody (PRA) test that evaluates which fraction of a random donor population the recipient has formed antibodies against, expressed as a percentage. Patients demonstrating PRA >10% are sensitized; although, some cardiothoracic surgeons argue that patients should be considered sensitized if they forms anti-HLA antibodies against more than 50% of the potential donor pool.[6] For patients on the waiting list for cardiac transplant, an additional hurdle arises in the case of left ventricular assist device (LVAD) placement as a bridge-to-transplant (BTT) therapy. Due to the markedly limited availability of organs, many patients are subjected to BTT therapy in order to improve quality of life or as a life-extending therapy until transplant. Compared with non-device patients undergoing medical management until transplantation, sensitization often increases following placement of an LVAD, with sensitization occurring in approximately 30% of BTT patients.[7-9] PRA levels are routinely monitored in patients awaiting transplant and, if levels are rising, various PRA-lowering interventions are considered, such as plasmapheresis, intravenous immunoglobulin and monoclonal antibody therapy.[10,11]

Anti-HLA antibody production is often measured with solid-phase assays, such as the commercially available Luminex single antigen bead assay. A solid phase bead is coated with denatured HLA antigens that can bind anti-HLA antibody. The presence of anti-HLA antibodies is then detected with a secondary antibody specific for the human IgG (subclasses G1 to G4) carrying fluorescent markers.

Because LVAD placement has the potential to promote inflammation and stimulate antibody production, limiting the inflammatory response in patients could possibly prevent sensitization after device implantation.[12,13] Amniotic membrane patches as well as amniotic-derived mesenchymal stem cells (MSC) have demonstrated anti-inflammatory potential in various medical applications, such as ophthalmology and dermatology, improving wound healing while limiting fibrosis.[14-16] While the number and viability of MSCs after cryopreservation prior to injection is debated,[17] growth factors and cytokines found in MSC mixtures and extracellular matrices are known to modulate the immune response.[18] Given their immunomodulatory effects, we hypothesized that micronized

matrices containing allogeneic MSCs are a preventive agent against sensitization.[19,20] This retrospective case series study presents the PRA results of patients who received myocardial MSC injection during LVAD implantation. It also explores changes in the observed degrees of mean signal interference (MFI) on the Luminex solid phase single antigen assay, as per standard of care in our institution. We hypothesized that PRA would not increase over time in these patients during their bridge-to-transplant period.

MATERIALS AND METHODS

From September 2011 to August 2017, sixteen patients with a formal diagnosis of dilated cardiomyopathy (DCM) or ischemic cardiomyopathy (ICM) were evaluated and approved for implantation of an LVAD device as BTT. During the transplant workup and prior to LVAD insertion, anti-HLA class I (-A, -B, -C) and class II (-DP, -DQ, -DR) antibodies were screened in patients with ischemic cardiomyopathy. Only anti-HLA class I screening data was available for patients with dilated cardiomyopathy. Radial flow LVADs (HeartWare International Inc, Framingham, MA) were implanted per standard of care. MSCs were obtained from amniotic fluid of multiple donors while micronized, liquid extracellular matrices (Amnio Technologies, LLC, Phoenix, AZ) were harvested from placental tissue. Following LVAD implantation, prior to chest closure, patients in the stem cell group received an administration of a human MSC mixture and extracellular matrix. This was injected with the intention of improving cardiac contractility after surgery. A mixture of 1.2 million MSCs in liquid matrix were injected into the anterior, inferior, and lateral walls of the left ventricular myocardium. Concurrently, 2.4 million MSCs and liquid matrix suspended in 5 ml normal saline was administered intravenously. Patients in the control group received routine chest closure without administration of MSC mixture per standard of care and did not receive intravenous MSCs in liquid matrix suspension. Peri- and postoperative blood product usage was monitored and recorded for each patient. There was no randomization of patients to each group.

The University of Arizona Institutional Review Board (IRB) (#1507990305A002) approved this analysis of human subjects. Consent was obtained from all patients. All procedures on the patient population followed relevant guidelines and regulations. Following the LVAD implants, while awaiting available donor hearts, patients were screened intermittently for class I and II PRAs (ischemic cardiomyopathy) or class I PRAs only (dilated cardiomyopathy) using the Luminex single antigen bead assay. Serum samples from patients were aliquoted, frozen, thawed, and centrifuged for 15 min at 15,000 rpm. HLA antibody screening and identification were performed according to manufacturer's recommendations using FlowPRA screening beads, LABScreen PRA Class I and II beads,

and LABScreen Class I and II Single Antigen beads (One Lambda®, Thermo Fisher Scientific, Inc. Waltham, MA). PRA was reported as a percentage determined by the current UNOS cPRA algorithm, representing the fraction of potential donor HLA-antigens to which an antibody response had been formed. When possible, antibody amount (but not titer) was determined by fluorescence, using a 1000 mean fluorescence intensity (MFI) cutoff point. In cases where background interference prevented a clean read of MFI, we subjectively used serial dilutions. Interference was qualitatively assessed using the MFI value of the internal negative control bead. MFI values less than 500 units were considered low interference, 500 to 999 units were classified moderate, and values 1000 and greater were high. The results of these tests were evaluated in this retrospective case series.

RESULTS

Ischemic cardiomyopathy

A total of six patients with a previous diagnosis of ischemic cardiomyopathy (ICM) received amniotic stem cell matrix injections during placement of LVAD device. Changes in their post-operative PRA levels from their pre-operative levels were compared to four control patients who received standard of care without amniotic stem cell injections. Stem cell case 2 demonstrated a decrease from 24% class I PRA pre-operatively to 7% class I PRA at the first assessment approximately four months (122 days) after implant. Stem cell case 1 demonstrated a decrease from 11% class II PRA pre-operatively to 0% at 62 days post-operatively despite intra-operatively receiving 675 ml of blood. One patient who received stem cells (case 3) demonstrated an increase from 0% class I PRA pre-operatively to 13% post-operatively and received 545ml of intra-op blood and 1 unit of blood with 1 unit of fresh frozen plasma in the immediate post-operative recovery period (**Table 1**). One control case showed an increase from 0% class I and class II PRA to 16% class I PRA detected on screening 47 days post-operatively with no intra-op or post-op blood or blood products administered.

Background fluorescence signal interference was observed in the Luminex single antigen assay for both class I and class II PRA values (**Table 2**). Of the four control cases of ischemic cardiomyopathy who did not receive amniotic stem cell matrix injections, analyzable interference data from our institution's HLA lab were available for two. In control cases, background signal interference qualitatively increased to moderate levels after one month post-LVAD placement, and it remained elevated at subsequent PRA checks until beginning to show a qualitative decrease between 243 and 273 days for control case 3. Interference data was available for four of the six cases that received stem cells prior to chest wall closure. A similar increase in both class I and class II interference was first

observed between two to four months after LVAD placement, but stem cell case 5 showed an earlier decrease in the degree of class I signal interference between 120 to 212 days. Stem cell case 4 demonstrated an unusual pattern of alternating low and high degrees of signal interference with no discernable trend. However, PRA levels were measured at 0% both pre- and post-operatively for this case. All other stem cell cases exhibited no increase in signal interference compared to control cases.

TABLE 1

Patient	Class I PRA pre-op (%)	Class I PRA post-op (%)	Class II PRA pre-op (%)	Class II PRA post-op (%)	Time from implant (days)	Closs clamp time (min)	Bypass time (min)	Intra-op blood volume given (mL)	Post-op blood given (units)	Oher blood products given (units)
Control 1	0	16	0	0	47	0	156	0	0	None
Control 2	0	0	0	0	122	0	0	0	0	2 FFP, 2 PLT
Control 3	0	0	0	0	181	0	91	0	0	None
Control 4	0	0	No data	No data	85	0	68	700	0	None
Stem cell 1	0	0	11	0	62	0	0	675	0	None
Stem cell 2	24	7	0	0	122	0	242	0	0	None
Stem cell 3	0	13	0	0	1135	0	107	545	1	1 FFP
Stem cell 4	0	0	0	0	30	0	0	450	0	Cell saver
Stem cell 5	0	0	0	0	120	0	0	0	1	None
Stem cell 6	0	0	0	0	563	0	0	0	0	None

Dilated cardiomyopathy

A total of four patients with a previous diagnosis of dilated cardiomyopathy (DCM) underwent LVAD placement with amniotic stem cell matrix injection prior to chest closure. Two control patients did not receive amniotic stem cell injections. Class I PRA was evaluated pre-operatively and twice post-operatively at various time points. Control case 1 demonstrated a pre-operative class I PRA of 0% that remained at 0% on both post-operative evaluations up to 487 days after LVAD placement (**Table 3**). Control case 2 exhibited a pre-operative class I PRA of 27% that remained elevated at 20% 212 days after LVAD implantation without amniotic stem cell injection. Three of the four patients that received stem cells demonstrated pre-operative class I PRA of 0%, which remained at 0% at both evaluations post-operatively up to 650 days after surgery. All three cases

TABLE 2

Control 2	LVAD date	Ab Screen	Days	Class I interference	Class II interference
	Aug-13	Sep-13	31	None	None
	Aug-13	Dec-13	122	Moderate	Moderate
	Aug-13	May-14	273	Moderate	Moderate
	Aug-13	Nov-14	457	Moderate	High
	Aug-13	Apr-15	608	Moderate	Moderate
	Aug-13	Jul-15	699	Moderate	Moderate
Control 3	**LVAD date**	**Ab Screen**	**Days**	**Class I interference**	**Class II interference**
	Jan-13	Feb-13	31	Low	None
	Jan-13	Jul-13	181	Moderate	Moderate
	Jan-13	Sep-13	243	Moderate	High
	Jan-13	Oct-13	273	Low	Low
	Jan-13	Dec-13	334	Low	None
	Jan-13	Jan-14	365	Low	None
	Jan-13	Apr-14	424	Moderate	None
Stem cell 1	**LVAD date**	**Ab Screen**	**Days**	**Class I interference**	**Class II interference**
	Dec-14	Feb-15	62	High	Moderate
	Dec-14	Apr-15	121	High	Moderate
	Dec-14	Jun-15	182	High	Moderate
	Dec-14	Aug-15	243	High	Moderate
	Dec-14	Dec-15	365	Low	Low
	Dec-14	Feb-16	427	Low	Low
	Dec-14	Apr-16	487	Low	Low
	Dec-14	Jun-16	548	Low	Low
	Dec-14	Sep-16	640	Low	Low
Stem cell 2	**LVAD date**	**Ab Screen**	**Days**	**Class I interference**	**Class II interference**
	Sep-14	Jan-15	122	High	Moderate
	Sep-14	Apr-15	212	High	Moderate
	Sep-14	May-15	242	High	Moderate
	Sep-14	Mar-17	912	Low	Low
Stem cell 4	**LVAD date**	**Ab Screen**	**Days**	**Class I interference**	**Class II interference**
	Sep-13	Oct-13	30	Low	Moderate
	Sep-13	Mar-15	546	Moderate	High
	Sep-13	Apr-15	577	low	Moderate
	Sep-13	May-15	607	High	High
	Sep-13	Jul-15	668	Moderate	Moderate
	Sep-13	Sep-15	730	Moderate	High
	Sep-13	Mar-16	912	None	None
Stem cell 5	**LVAD date**	**Ab Screen**	**Days**	**Class I interference**	**Class II interference**
	Jan-15	May-15	120	Moderate	Moderate
	Jan-15	Aug-15	212	Low	Moderate
	Jan-15	Nov-15	304	Moderate	Moderate
	Jan-15	May-16	486	Low	Low
	Jan-15	Nov-16	670	Low	Low

received either intra-operative blood or blood in the immediate post-operative recovery period. Stem cell case 4, which exhibited a pre-operative class I PRA of 0%, developed a positive, greater than 10% (the exact percentage was unavailable from the records), class I PRA detectable at the three-month post-operative follow up that declined to 0% upon re-evaluation at 153 days after LVAD placement. For this case, 1 unit of post-operative blood (350 ml) was given, but none was given in the post-operative recovery period.

TABLE 3

Patient	Class I PRA pre-op (%)	Class I PRA post-op #1 (%)	Time from implant (days)	Class I PRA post-op #2 (%)	Time from implant (days)	Closs clamp time (min)	Bypass time (min)	Intra-op blood volume given (mL)	Post-op blood given (units)	Oher blood products given (units)
Control 1	0	0	122	0	487	0	105	350	0	None
Control 2	27	25	61	20	212	0	58	0	1	None
Stem cell 1	0	0	672	0	731	0	0	375	0	None
Stem cell 2	0	0	120	0	243	0	108	0	3	None
Stem cell 3	0	0	274	0	458	0	109	350	0	None
Stem cell 4	0	>10	91	0	153	52	167	0	1	None

Fluorescence signal interference in the Luminex single antigen assay for both DCM control cases showed none to low degrees of signal interference for both class I and class II PRA post-LVAD placement, which remained at low levels on second evaluation up to 487 days later **(Table 4)**. Of the four DCM cases that received amniotic stem cell matrix injection prior to closure, two showed either no increase in background signal interference or very low levels. Stem cell cases 1 and 3 showed no appreciable signal interference post-LVAD and on the second evaluation 2 years later (around 730 days). However, stem cell case 2 showed a high degree of class I and class II signal interference at evaluation 90 days after LVAD placement. In this case, class I and class II interference remained at a moderate or higher degree of interference between 334 to 396 days until dropping to a low level beyond that point for both classes. Stem cell case 4 demonstrated a low degree of class I interference when the increase in PRA to > 10% was detected at around 91 days post-LVAD placement. Stem cell case 4 also developed a slight increase in class I signal interference 153 days after LVAD placement, but no appreciable increase in class II channel signal interference was noted. No further data points were available for this case.

TABLE 4

Control 1	**LVAD date**	**Ab Screen**	**Days**	**Class I interference**	**Class II interference**
	Sep-13	Jan-14	122	Low	None
	Sep-13	Jan-15	487	Low	Low
Control 2	**LVAD date**	**Ab Screen**	**Days**	**Class I interference**	**Class II interference**
	Oct-14	Dec-14	61	Low	Low
	Oct-14	May-15	212	Low	Low
	Oct-14	Jul-15	273	Low	low
Stem cell 1	**LVAD date**	**Ab Screen**	**Days**	**Class I interference**	**Class II interference**
	Apr-15	Feb-17	672	None	None
	Apr-15	Apr-17	731	None	None
Stem cell 2	**LVAD date**	**Ab Screen**	**Days**	**Class I interference**	**Class II interference**
	Jan-15	Apr-15	90	High	High
	Jan-15	May-15	120	High	High
	Jan-15	Sep-15	243	High	High
	Jan-15	Oct-15	273	High	High
	Jan-15	Nov-15	304	Moderate	High
	Jan-15	Dec-15	334	Moderate	Moderate
	Jan-15	Feb-16	396	Low	Moderate
	Jan-15	May-16	486	Low	Low
	Jan-15	Sep-16	609	Low	Low
Stem cell 3	**LVAD date**	**Ab Screen**	**Days**	**Class I interference**	**Class II interference**
	Jun-15	Mar-16	274	None	None
	Jun-15	Sep-16	458	None	None
	Jun-15	Mar-17	639	None	None
Stem cell 4	**LVAD date**	**Ab Screen**	**Days**	**Class I interference**	**Class II interference**
	Sep-14	Dec-14	91	Low	None
	Sep-14	Feb-15	153	Moderate	None

DISCUSSION

The results in this exploratory study involving a total of 16 subjects suggest that intra-operative treatment with MSCs reduces the risk of sensitization in patients that undergo LVAD implantation while awaiting cardiac transplant. Although this exploratory study was not powered for statistical significance, the data suggest a trend towards immuno-modulation by MSCs.

While improvements in cardiac function have been reported following targeted stem cell therapy, these findings have been overshadowed by retraction of a series of prominent studies [21] However, despite the current skepticism on cardiac stem cell therapies, improve-

ment of cardiac function through some stem cell therapies can be achieved albeit through different mechanisms than proposed previously.[22] In addition to improved contractility, a benefit may arise when using stem cells during BTT in terms of immunomodulation.[18] The latter mechanism of lowering sensitization by immunomodulation is key in the current study. The sixteen cases reviewed in this study all are patients at risk of humoral sensitization following LVAD implantation and/or administration of blood products. Patients in the stem cell intervention group received myocardial injections of amniotic MSC during VAD placement in order to aid cardiac function. In this study we evaluated immunomodulatory effects of MSC injections. Promising results were observed with stem cell injection during LVAD therapy for diagnosed ischemic cardiomyopathy, with two cases showing a mild decrease in either class I or class II PRA in patients who were sensitized prior to LVAD placement. One case demonstrated an increase in sensitization after LVAD therapy, but this patient developed a history of chronic driveline infections. These eventually required explant and re-implantation with a new device, and administration of intra- and post-operative blood as well as fresh-frozen plasma in a clinically complicated recovery period, thereby increasing the risk for sensitization **(Table 1)**. Mean fluorescence intensity, as noted qualitatively in the Luminex single antigen solid phase assay, showed a slightly earlier decrease in cases that received amniotic stem cell matrix injection compared to those that did not (212 days, down from 243 for control cases; **Table 2**). This may suggest a mild immunomodulatory effect imparted by the amniotic mesenchymal stem cell matrix for the ICM cases.

For the DCM cases, 3 of 4 patients who received amniotic mesenchymal stem cell injection maintained 0% PRA sensitization at both evaluations after LVAD placement, with stem cell case 4 developing a significant degree of sensitization three months (91 days) after LVAD placement. As one of the control cases also maintained 0% PRA sensitization after LVAD placement and the signal interference results were mixed, the impact of the amniotic mesenchymal stem cell matrix on the DCM cases is less clear. However, given that three of the DCM cases exhibited a low degree of signal interference and no appreciable increase in PRA, amniotic stem cell injection is unlikely to be responsible for the increase in the post-op class I PRA seen in stem cell case 4 for DCM (**Table 3**). Interestingly, the corresponding degree of signal interference at this clinical evaluation was low despite an appreciable increase in PRA. This confirms that these phenomena do not share a causal relationship and are worth evaluating separately as high degrees of interference can confound the interpretation of PRA results. While these findings suggest that differences in the underlying pathophysiologic changes in DCM versus ICM may have an impact on the immunomodulatory effects imparted by the amniotic mesenchymal stem cell matrix, the small sample size of patients reviewed in this case series limits our ability to definitively reach that conclusion.

It is well-established that numerous types of exposures can affect the development of antibodies to HLA antigens, with pregnancy and history of repeated blood transfusion being the most common. Previous studies have demonstrated that transfusion with leukocyte-reduced, irradiated, and ABO-matched blood products reduces the risk of developing significant allosensitization regardless of the volume of blood transfused.[23] All patients participating in this study at our institution received leukocyte-reduced, irradiated, and ABO-matched blood products per institutional protocol. In our study, no obvious relationship was observed between sensitization and volumes of intra- or post-operative blood or blood products administered.

As LVAD development has progressed through the years, various design and flow regimens have been associated with greater risks for sensitization. The third generation HeartWare devices implanted in patients in this study utilize a radial design with centrifugal flow and are likely to have a lower risk of sensitization, although minimal data on the development of anti-HLA antibodies is available for these specific devices.[6] Improved device design, including material optimization, novel flow regimens and blood-device contact area reductions that minimize platelet activation, have lowered the risk of sensitization. However, high risk patient populations with prior exposure from pregnancy, transplant, or routine blood transfusion with non-leukoreduced, irradiated, or ABO-matched blood may benefit from novel immunologic approaches to reducing PRA, thus improving their waiting list status.

Overall, these results suggest a beneficial role of amniotic stem cells for prevention against humoral sensitization. Mesenchymal stem cells are capable of repressing the T-cell response in a dose dependent manner.[24] Administration of MSCs leads to the inhibition of both naive and memory T-cells.[25] Furthermore, there is sufficient evidence of immunoglobulin impairment and B-cell chemotaxis deterioration, which suggests that inhibition of B-cell activation could result from HLA antigen exposure.[26,27] No clinically convincing pattern of elevation in PRA or the intensity of signal interference was associated with stem cell use in either DCM or ICM. Minimizing anti-HLA sensitization in transplant-eligible LVAD patients may broaden the potential donor pool, reduce the risk of transplant graft rejection, and lead to better outcomes and survival.

Amniotic-derived stem cells are well-established as an immunomodulatory and as anti-inflammatory agents in multiple fields. Our study illustrates the promising and exciting potential for improved outcomes in BTT patients. Additional research employing this intervention for patients at high risk of sensitization is needed to further investigate the potential value of this intervention.

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Chapter 9

Reduced post-operative atrial fibrillation following amniotic membrane patch placement

Schipper DA, Hallmark B, Marsh KM, Johnson K, Duncker DJ and Khalpey Z

ABSTRACT

Introduction

Atrial fibrillation after cardiac surgery occurs in approximately one third of patients and is associated with increased morbidity and mortality. Inflammation and oxidative stress may play a key role in the pathophysiology of new onset post-operative atrial fibrillation (POAF). Acellular human amniotic matrix is known to be anti-inflammatory, and therefore may have anti-arrhythmic effects in patients undergoing cardiac surgery.

Methods

During a three-year period, AMPs were placed in patients undergoing cardiac surgery (CABG, Valve Replacement, CABG+Valve Replacement). Patches were topically placed onto the anterior epicardium prior to partial pericardial and chest closure. We identified controls from the Society of Thoracic Surgeons (STS) database at the University of Arizona. Propensity score matching was performed based on 18 risk variables. POAF was defined using the STS criteria.

Results

After exclusions, 51 AMP patients and 798 controls were analyzed to include 45 AMP patients and 172 propensity matched controls. 4.4% of the AMP patients developed POAF compared to 21.5% in the propensity matched control group (odds ratio: 0.26, 95% CI:0.18-0.38) (odds ratio: 0.18, 95% CI:0.03-0.61). Of those undergoing CABG surgery, none of the 29 AMP patients developed POAF, compared to 17% of the matched controls.

Conclusions

Human amniotic membrane patch placement during cardiac surgery demonstrates a trend towards lower rates of POAF. This may lead to a reduction in thromboembolic complications, prolonged hospitalizations and repeat admissions. These preliminary results should initiate a multi-center double-blinded randomized controlled trial to further delineate the benefit of AMP placement in the prevention of POAF and the downstream impact on clinical outcomes.

INTRODUCTION

The development of postoperative atrial fibrillation (POAF) following open-heart surgery is a significant clinical and economic problem. New onset POAF is noted in approximately 27-40 percent of patients undergoing open heart surgical procedures.[1,2] In contrast, new onset atrial fibrillation is extremely rare after percutaneous cardiac interventions, accentuating surgical trauma and corresponding inflammation as key mediators in POAF pathophysiology.[3]

A number of significant clinical problems are associated with the development of POAF.[1,2] These include embolic phenomena, hemodynamic abnormalities, and ventricular arrhythmias as well as potentially a higher mortality rate.[2] Iatrogenic problems have also been noted with pharmacological and procedural-based therapies employed in the treatment of atrial fibrillation. The use of anti-arrhythmic medications is occasionally associated with problematic side effects and a potential increased mortality due to pro-arrhythmia.[4] The use of anticoagulant therapy can result in clinically significant bleeding. Furthermore, patient satisfaction is usually significantly diminished in the setting of postoperative atrial fibrillation.

Substantial potential economic detriments also derive from the presence of postoperative atrial fibrillation. As the length of hospital stay increases by an average of 2 - 5 days for patients with POAF, associated hospital charges increase as well. Approximately an additional $16000 in hospitalization fees are charged to each patient with NOPAF.[5] Assuming an event rate of approximately 20 percent, the annual cost for postoperative atrial fibrillation, based on an increased length of stay in both the intensive care and telemetry units and the occasional need for additional procedures, is estimated in the range of $ 1.0 to 2.5 billion per annum in the U.S. alone.

Amongst the risk factors that have been identified as associated with the development of POAF is age, which may produce fibrosis resulting in ultra-structural changes and secondary conduction abnormalities.[6] A prior history of atrial fibrillation has also been associated with an increased risk.[2] Various other medical co-morbidities appear to be associated with POAF including the presence of COPD, chronic renal failure, diabetes, and obesity.[2] The type of open heart surgery undertaken is associated with varying risks. The risk appears higher in those patients undergoing repeat surgery, valvular surgery, or concomitant surgery (e.g., valve surgery in conjunction with bypass surgery).

Pathophysiology of Atrial Fibrillation Occurring After Open-Heart Surgery

A number of pathophysiologic processes have been suggested as operative in the development of postoperative atrial fibrillation. Pathophysiology of POAF is complex and multifactorial, and to date no clear-cut single mechanism has been defined.[7,8] Those mechanisms that are thought to have a role include an inflammatory component from the surgical trauma and secondary reactions to such, excessive adrenergic stimulation, autonomic imbalance, neuro-humeral abnormalities, postoperative volume shifts, a genetic predisposition, potential underlying atrial fibrosis and conduction changes occurring with age or disease, stretch induced phenomenon, as well as the type of surgical procedure and the manner in which that procedure is performed.[9]

Recent studies have proposed a pivotal role for inflammation and oxidative stress in the development of AF.[10-13] Various inflammatory markers and mediators have been linked with the presence or the outcome of AF and may confer a prothrombotic state by promoting endothelial damage/dysfunction and platelet activation in patients with AF, thus linking inflammation and thrombosis.[14]

Management of POAF

A number of interventions have been undertaken in an attempt to reduce the frequency and the burden of postoperative atrial fibrillation. These include beta blockers, for which the most data exists. Significant variations in the success rate of beta blockers has been reported with rates ranging from absolute reductions of 5 to 15 percent and relative reductions of 30 to 65 percent. A number of anti-arrhythmics have also been employed in an attempt to reduce the frequency of postoperative atrial fibrillation. The relative reduction has been again in the range of 30 to 50 percent. Overall relatively positive data supporting the use of amiodarone have been reported. In general, β-Blockers, sotalol, and amiodarone all generally reduce risk of postoperative AF with no marked difference between them. There is evidence that use of these drugs will reduce length of hospital stay. Although drug treatment for rate/rhythm control are currently clinically used with reasonable effectiveness,[15-18] it could be suggested that the preferable solution in reducing the adverse effects would be to prevent POAF from developing.[19] Various studies have reported on prophylaxis of POAF both intraoperatively and prior to surgery, amongst which corticosteroid administration and specific intrathoracic surgical approaches.[20] In an animal model, reduced incidence of POAF was observed when subjects were treated with anti-inflammatory agents.[21] While human data is largely inconclusive, some benefit is observed in these approaches, especially with corticosteroid immunosuppression.[22] Beyond the systemic immunosuppression provided by steroids, local acting anti-inflammatory agents are of interest as they act on a specific target while lacking the notorious side effects often seen in systemic immunoregulatory drugs, such as electrolyte imbalances and hyperglycemia.[23] Extrapolating these

principles on inflammation and its impact on POAF pathophysiology, novel approaches of immunosuppression could be explored in cardiac surgery patients.

Amniotic Membrane Patch placement on epicardial surface

Placing an amniotic membrane patch (AMP) on the epicardial surface is intended for the reconstruction and repair of the epicardium following open-heart surgery.[24] Amniotic membrane patching has demonstrated regenerative properties due to the immunomodulatory features and stem cell potential.[7] This anti-inflammatory capacity of patching is widely acknowledged in wound management, where placement of AMP ameliorates the healing process.[25] In hearts, The immunomodulatory force of AMP has been observed in treatment of constrictive pericarditis.[26] Furthermore, patching with amniotic membrane tissue has demonstrated the ability to significantly reduce postischemic cardiac dysfunction, ischemic heart repair, and blood flow recovery in rat and mouse models.[8,9,10] Extracellular matrix has been shown to modulate inflammatory responses in myocardial infarction.[27] Placing an amniotic membrane patch on the epicardial surface provides an intact scaffold for cells to remodel various tissues, given the native matrix is damaged from surgery.

The amniotic membrane patch utilized in the present study is a minimally manipulated allograft derived from donated human birth tissue, consisting of both cells and extracellular matrix.

Providing this matrix to patients perioperatively is expected to decrease inflammation, and in turn decrease postoperative atrial fibrillation. In this study we retrospectively investigate the incidence of POAF in patients that received AMP and compared that with an equivalent patient group that did not receive AMP placement following open-heart surgery.

MATERIALS AND METHODS

Patient Selection and Statistical Analyses

Patients that had previously undergone cardiac surgery were excluded form our study, as well as patients with a history of atrial fibrillation or who were on other anti-arrhythmic drugs than β-blockers, e.g. amiodarone. After exclusion, a cohort of patients was established that had undergone CABG, valve replacement or repair, or a concurrent combination of these two interventions, performed by dr. Khalpey, a cardiac surgeon at Banner University Medical Center (Tucson, AZ), between 2013-06-04 and 2016-09-01. Of these 88 subjects, 49 received intraoperative amniotic membrane patch placement. Patients were not randomly assigned to either one of the groups, but were assigned based on the time of surgery, dividing into various time windows (Figure 1). In our analysis the patients

were subdivided in 3 different groups based on the type of the surgical procedure they were subjected to: CABG, valve surgery, or a combination of both. To determine whether the patch reduces post-operative atrial fibrillation or alters secondary outcomes, we first extracted a set of potential controls from the STS surgery database who had CABG and valve (or both) surgeries. Patients taking amiodarone, with arrhythmia, prior cardiac surgeries, or prior atrial fibrillation procedures were excluded. We then used propensity score matching to improve balance between the AMP and control group by choosing a subset of STS patients that are very similar to the patients who received a patch.[28] Specifically, we used the linear propensity score and estimated the balance and variance ratio statistics for a range of values for the number of matched controls per treatment case and the number of subclasses over which to balance observations, finding similar values for several combinations (Table 1 and Figure 2). After matching, a total of 45 AMP patients and 172 control patients were included. Five patients who received AMP could not be matched and were excluded from the study. In addition to incidence of POAF, postoperative stroke incidence and postoperative length-of-stay (LOS) were analyzed.

TABLE 1
propensity matching criteria

Matching criteria	Covariate	AMP	Control
Demographics	Age (yrs)	59.98	62.50
	Gender (male, %)	71%	71%
	Race (caucasian, %)	73%	73%
	BMI	28.44	28.59
Medical history	Heart Failure (%)	64%	59%
	Previous myocardial infarction (%)	42%	44%
	Valve disease (AV/MV/TV, %)	82%	80%
Medications	ACE inhibitor or ARB (%)	76%	75%
	Beta-blocker (%)	84%	83%
	Calcium-channel blocker (%)	16%	13%
	Long-acting nitrates (%)	0%	2%
Surgical time	Median bypass time (mins)	141.11	143.15
	Median cross-clamp time (mins)	97.78	100.27

We retrospectively compared the AMP and control groups both by aggregating all surgeries together, as well as separating by surgery type (CABG or valve). Due to the limited number of patients having both CABG and valve procedures, this category was dismissed from additional comparisons. Logistic regression was used to compute odds ratios and confidence intervals. In addition, we examined several secondary outcomes: stroke, discharge mortality status, readmission, and post-operative length of stay. All computations were performed in R, and the MatchIt R package was used for propensity matching.[29,30]

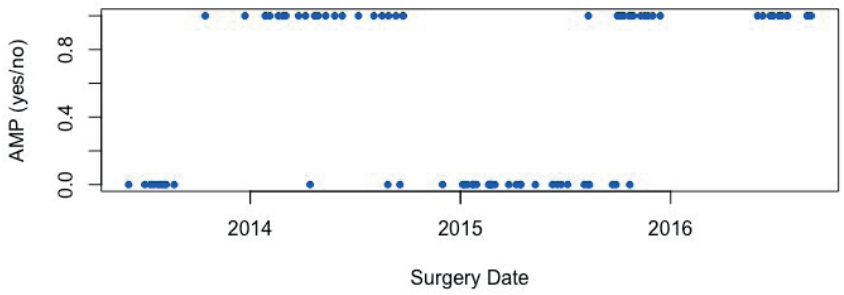

Figure 1

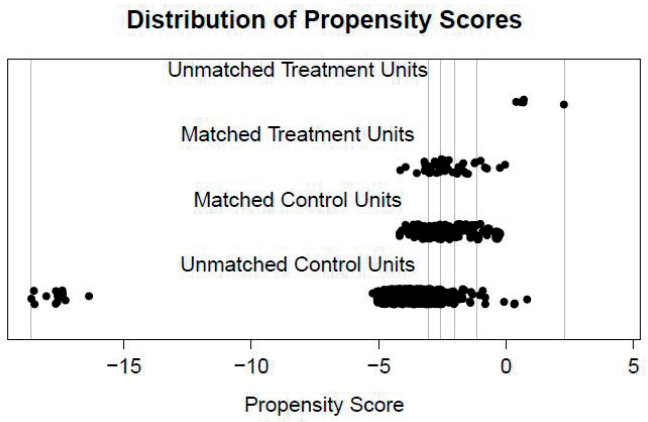

Figure 2

Amniotic Membrane Patch

PalinGen® Membranes (Amnio Technology, LLC, Phoenix, AZ), or AMP, are human allograft membranes obtained through the processing of placental tissue. Various growth factors can be found on AMP, including FGF, EGF, PDGF A & B and VEGF. Additionally, flow cytometry demonstrated the presence of surface antigens CD73+, CD90, and CD105+. These positive markers are in consistency with mesenchymal stem cell lineage.[31] Patients that were assigned to AMP placement had 3 patches placed on the anterior epicardium prior to partial pericardial closure, but after the surgical procedure was completed.

RESULTS

Atrial fibrillation

Out of the 45 patients that received AMP placement during surgery, 2 developed POAF. In the propensity matched control group, POAF occurred in 37 out of 172 patients (Table 2, Figure 3). However, despite this impressive reduction, results were not considered to be statistically significant as a result of low overall incidence of events. Patients that were subjected to CABG surgery demonstrated the most impressive benefit of AMP placement, as none of the 29 patients developed POAF, compared to 17% of the patients in the matched controls (n=84) (Table 2). Although not included in the results, it is noteworthy that none of the 5 unmatched and therefore excluded patients developed post-op atrial fibrillation after AMP placement.

TABLE 2

		Control			AMP		
	Surgery type	Number of cases	Incidence of POAF	Odds ratio (95% CI)	Number of cases	Incidence of POAF	Odds ratio (95% CI)
Propensity matched	All	172	21%	0.26 (0.18 - 0.38)	45	4%	0.18 (0.03 - 0.61)
	CABG	89	17%		29	0%	
	CABG + Valve	24	42%		3	33%	
	Valve	59	19%		13	8%	
All cases	All	794	26%	0.34 (0.29 - 0.40)	50	4%	0.12 (0.02 - 0.40)
	CABG	480	21%		31	0%	
	CABG + Valve	77	47%		5	20%	
	Valve	237	28%		14	7%	

The control group consisted of patients from other cardiac surgeons at the same institution. While the incidence of post-operative AF was homogenous among the majority of surgeons, it was noted that the incidence of POAF in surgeries performed by dr. Khalpey was generally lower than the overall incidence. To minimize the intra-operator variances, we compared the surgeries by only dr. Khalpey with and without patch placement. Overall, we see that Khalpey's post-op AF rate is lower than average, but also that the post-op Afib rate is lower for AMP patients. However, due to the small sample sizes, this effect was not statistically significant (p=0.23; Table 3). Similarly, the data after partitioning the results by surgery type, we observed trends towards a reduction in the number of post-op AF events, but without statistical significance.

TABLE 3

	Control			AMP			
Surgery type	Number of cases	POAF incidence	Percentage	Number of cases	POAF incidence	Percentage	p-value
All	34	5	13%	47	2	4%	0.23
CABG	13	2	13%	30	0	0%	0.11
Valve	17	3	15%	13	1	7%	0.63

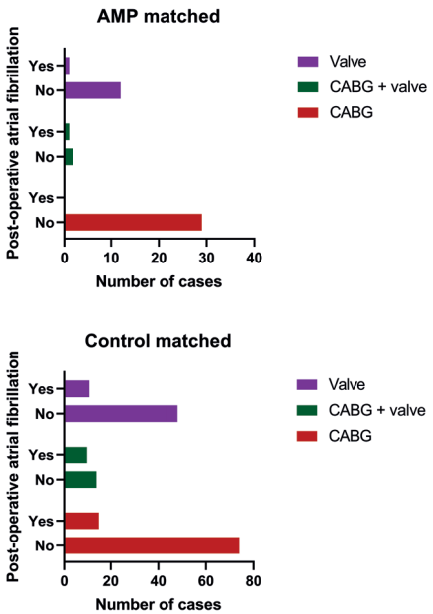

Figure 3

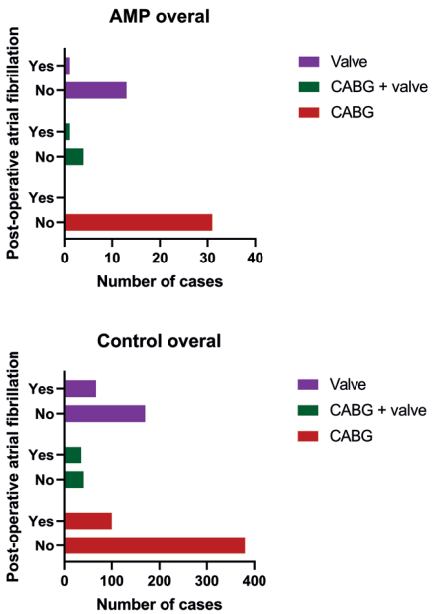

Figure 4

Lastly, in order to validate the propensity match scoring criteria, unmatched control and AMP groups were analyzed. Here, 2 out of 50 patients developed POAF after AMP placement, compared to 203 out of 794 amongst control patients, leading to POAF incidence of 4.0% and 25.6%, respectively (Figure 4).

Secondary outcomes

Various secondary outcomes were measured, firstly incidence in stroke. We do not see a significant effect of the AMP on stroke, though stroke is quite rare with only 3 events total, of which 2 were in the control (1.2%) and 1 in the treatment group (2.2%)(p=0.59; Table 4). We did not see a significant effect of the AMP on discharge mortality status. Overall, the AMP group had fewer patients die (2% vs 6%), but the counts are small, and the difference does not reach statistical significance(p=0.35; Table 4). Although the

TABLE 4

Surgery type	Control			AMP		
	Number of cases	Counts of stroke	Deaths	Number of cases	Counts of stroke	Deaths
All	172	2 (1%)	10 (6%)	45	1 (2%)	1 (2%)
CABG	84	0 (0%)	3 (4%)	29	0 (0%)	0 (0%)
CABG + valve	27	1 (4%)	3 (11%)	3	0 (0%)	0 (0%)
Valve	61	1 (2%)	4 (7%)	13	1 (8%)	1 (8%)

outcomes are slightly different between groups, the small number of cases limits inference at this time. Additionally, postoperative LOS was monitored and analyzed. Mean LOS did not demonstrate significant difference between both groups (p=0.40), while the median was equal (7)(Figure 5). Patients that were hospitalized for over 40 days were considered outliers and not included in the results.

Lastly, as documented before by peers, we found increasing age to be associated with an elevated risk for POAF development.

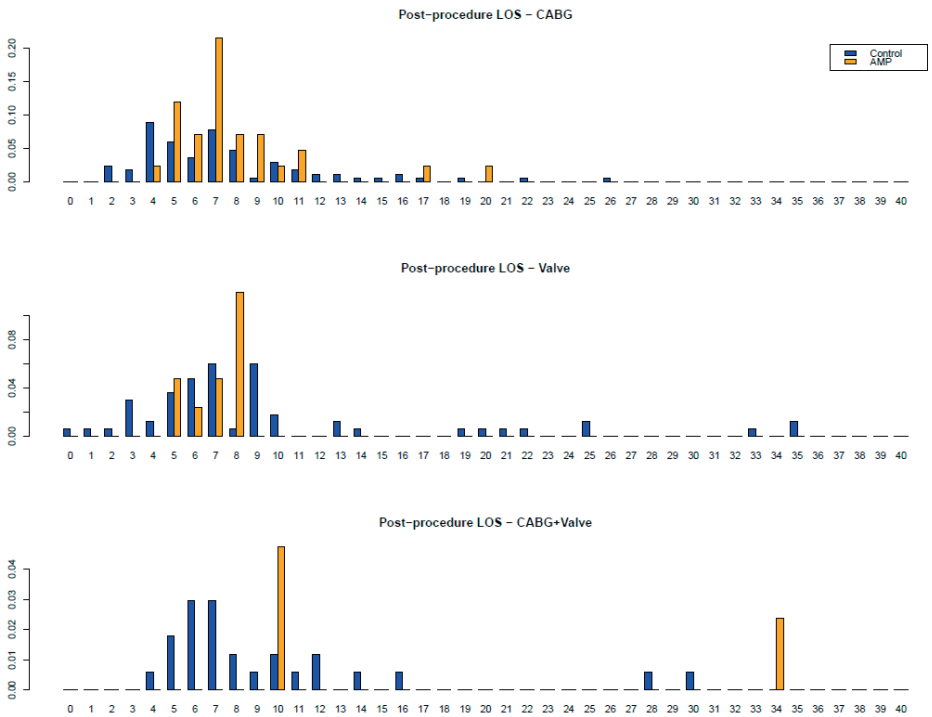

Figure 5

DISCUSSION

The results presented in this study suggest a strong reduction on the incidence of POAF by perioperative AMP placement, up to 84%. This effect is observed in comparison to a propensity matched control group that is closely resembling the AMP intervention group. Due to small sample size of the intervention group, statistical significance is absent, but the trend is impressive and noteworthy nonetheless. To increase statistical power, we did further analysis on the non-propensity-matched cohort, expanding the AMP group and control group to a total of 50 and 794 patients, respectively. POAF reduction remained present, potentially leading to 67% lower incidence after AMP placement. Secondary outcomes in our study do not demonstrate differences between AMP and control group, although the overall number of events is small in general.

As the amniotic membranes used in this study are immune-privileged, or immune evasive,[32,33] risk of adverse side effects are minimal. This study does not aim to conclusively identify the underlying mechanisms in general AF pathophysiology, but to evaluate local immunosuppression as a preventive agent for AF in a well-defined group of patients.

In the general population, the risk of AF development in are based on a combination of anatomical variances such as atrial size[34-36], age and other comorbidities. The combination of predisposing risk factors along with oxidative and inflammatory stress can lead to pathological arrythmia. While general risk and etiological factors for AF may still be of consequence, in the particular case of POAF the pathophysiological emphasis has strongly shifted towards inflammation. Pre-, intra-, and post-cardiac surgery, the heart is under significant inflammatory stress.[13] This targets the immune response as the primary actor for preventive measures, as has been suggested in the past. One major pitfall in prior attempts to locally suppress the inflammatory response, is the rapid washout of active substances. Pericardial flushing intraoperatively does have a protective downregulatory effect in POAF development, however the effect is not sustainable. Patients are at risk for POAF up to 5 days after cardiac surgery, elucidating the need for prolonged prevention. Systemic delivery of immunomodulating substances imposes the risk of perioperative infection, including surgical site infection and mediastinitis, as well as glucose and electrolyte imbalance, making it an unattractive option for the majority of patients.

Lastly, our study highlights the role of inflammation in post-operative AF development and its prevention. The AF reducing capacity of AMP is attributed to the immunomodulatory and anti-inflammatory capacities of the patch. Pathogenesis of AF, however, has been discussed for many years and various studies have demonstrated the complexity in AF development. Although these observations should not be neglected, our findings suggest

a pivotal role of inflammation in the genesis of POAF in particular. Moreover, the specific surgical setting of our patient group, in which tissue damage and temporarily altered blood flow are a prominent factor, appears to contribute the proposition that local anti-inflammatory therapy is key in AF prevention.

Limitations

While the results suggest that AMP placement counteracts the development of AF after cardiac surgery, some limitations should be taken into consideration. Primarily, the study design is suboptimal as this study is retrospective and does not contain a cohort of patients who are randomized to either receive the patch or not. Ideally this study would have been set up as a randomized controlled trial, with balance in the covariates between the treatment and control groups. Here, the AMP and control groups were primarily not balanced across covariates. We have addressed this imbalance by propensity matching the groups. The use of these matching criteria have provided us with excellent controls. Nonetheless the study is underpowered for primary and secondary outcomes such as length-of-stay in the hospital and readmission rates as a result of small sample sizes. Despite not reaching significance on the mentioned outcomes, the results of this exploring study demonstrates a decreasing trend. This is not the case for stroke incidence. It is important to note, however, that in AMP and control group the rates are too low to draw any hard conclusions. Another limitation to be taken into consideration is that all AMP surgeries were performed by one surgeon, potentially leading to selection bias. To counteract this possible limitation as much as possible, the AMP implantations have been divided in 3 different periods, all segregated by periods were AMPs were not used. Additionally, it is of importance to note that the occurrence of POAF is a process in which placebo effect is non-existent, as AF is a completely objective outcome.

End-points of the study did not include evaluation of inflammatory markers in blood samples such as c-reactive protein and interleukins. The surgical procedures that patients undergoing CABG or valve repair/replacement are subjected cause inflammatory markers in peripheral blood samples to be elevated regardless of the individual's risk of developing POAF. These are always elevated after cardiac surgery, and do not help to determine anti-inflammatory effects. AMP has a local immunomodulating effect, and systemic effects are not expected to be observed.

Safety of use

One major concern regarding the use of amniotic membranes is its safety of use. Stem cells and their utilization in therapies inherently carry the potential of tumorigenesis, and should therefore be approached with considerable caution. This is no different in AMP, as these patches are not manipulated and could be a source of stem cells during placement.

Amniotic membrane patches have been thoroughly studies in ophthalmologic and wound healing settings. In these fields there have been no reports of neoplasm formation due to stem cell presence. Furthermore, stem cell delivery into the heart is deemed safe and feasible.[37-39] Concomitant injections of stems to CABG are associated with improved ejection fractions, and the attenuation of left ventricular remodeling. These effects can be observed up to 5 years post injection.[40-42] Host versus graft response after stem cell delivery poses an additional hazard to AMP use. However, amniotic membrane is considered to be immune privileged. The membranes used in this study are dehydrated and consist of amniotic stem cells and growth factors, all of which do not inflict an host immune response.

In conclusion, this study suggests a strong reduction in post-cardiac surgery AF incidence following intraoperative pericardial AMP placement. The prevention of this serious surgical complication is attributable to local anti-inflammation through immunomodulatory components founds on the membrane surface. The observed effect of AMP administration is trending towards significance. Secondary outcomes were measured throughout but largely inconclusive. This is due to the study being underpowered for these outcomes, as a result of small sample size and event incidence. Despite some limitations to the study design, the results reported have the potency to be of immense impact in the clinical prevention of POAF, one of the most prominent comorbidities of cardiac surgery. We propose a multi-institutional double-blinded randomized controlled trial to further evaluated the demonstrated benefit of AMP placement in the prevention of POAF.

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Chapter 10

General summary, discussion and future directions

D.A. Schipper

GENERAL SUMMARY

In this thesis the metabolic and inflammatory aspects of various surgical interventions of the cardiopulmonary system are studied in three parts. After a general introduction (Chapter 1), **part one** (Chapters 2 and 3) describes the changes in bioenergetics and metabolism in patients with chronic cardiovascular disease, both at the mitochondrial and systemic level. In Chapter 2 demonstrates that oxygen consumption is diminished in mitochondria isolated from left atrial appendage tissue of patients that undergo coronary artery bypass grafting (CABG) surgery for chronic myocardial ischemia, as compared to non-ischemic control patients that suffer from cardiac valve disease. The decrease observed in this study could primarily be attributed to a loss of function of complex I. Chapter 3 investigates the influence of cardiac assist device implantation on whole body metabolism in a small cohort of end-stage heart failure patients. Here, implantation of total artificial hearts (TAH), but not of left ventricular assist devices (LVAD), was associated with an increase in resting energy expenditure (REE). This could mainly be attributed to the difference in working mechanism of both devices and the degree of surgically induced trauma.

Part two (Chapters 4-6) addresses various challenges encountered in cardiopulmonary transplantation. Chapter 4 reviews the current standards in donor heart preservation, while underlining the importance of protecting bioenergetics during the *ex vivo* period. Multiple preservation solutions and methods are discussed, and suggestions for improving the current clinical preservation standards were made. In this review, there is a particular focus on the role of mitochondria and the mitochondrial permeability transition pore (mPTP), and their contribution to ischemia reperfusion injury (IRI). In Chapter 5 we demonstrate improved mitochondrial protection *in vitro* when using a novel preservation solution on primary rat cardiomyoblasts. In Chapter 6, this innovative preservation solution is found to provide a preferable *ex vivo* milieu for porcine lungs during cold static storage, based on metabolomics, substrate utilization and depletion of both tissue and preservation solution, and sustained redox potential.

In **part three** of this thesis (Chapters 7-9) the anti-inflammatory properties of an amniotic membrane patch (AMP) are investigated in a selection of cardiac surgical settings. First we present a clinical case of difficult-to-treat constrictive pericarditis in chapter 7. In this case report, AMP is suggested to contribute to the inhibition of the inflammatory response, thus acting as a therapeutic agent. A comparable immunomodulating effect is observed in Chapter 8, in which micronized AMP was injected into patients' myocardium following LVAD placement in a case series of 16 patients. While LVAD implantation often leads to increased humoral sensitization of anti-HLA II antibodies and thereby limits ones

suitability to be a graft recipient, the addition of micronized AMP results in suppression of the anti-HLA antibody formation in the majority of cases. Finally, in Chapter 9 we evaluate the anti-inflammatory potential of AMP in preventing development of post-operative atrial fibrillation (POAF), one of the most prevalent complications of cardiac surgery. After the surgical procedure, but before chest closure, AMPs were placed on the epicardium, causing a marked trend towards reduction in AF development. The observed effect could be attributed to local suppression of the immune response that plays a pivotal role in the development of POAF.

DISCUSSION

Metabolic aspects of myocardial ischemia and heart failure

Chapter 2 demonstrates lower respiratory control rates (RCR) in myocardium of patients that suffer from chronic ischemia (Figure 1). This was due to a combination of lower state 3 respiration and higher state 4 respiration. Additionally, a decrease in complex I functionality was observed. These findings suggest that mitochondria in the chronically ischemic myocardium consume oxygen and produce ATP less efficiently than healthy cardiomyocytes, in confirmation of prior findings in animal studies.[1-3] Despite this important animal-to-human translational aspect, some limitations should be acknowledged. The left atrial appendage used in this study is not ideal, since the left ventricle is the part predominantly affected by ischemia in coronary artery disease. For obvious reasons, ventricular tissue cannot be easily and safely obtained in living humans. However, it has been demonstrated that remote areas of the heart are affected by ischemia. Thus, left atrial appendage tissue can serve as a valid alternative for ventricular tissue in ischemic hearts.[4] Another factor that should be taken into consideration, is the lack of matching in patient characteristics. No additional patient demographic information was available to the investigators as the tissue used was regarded as clinical discard. However, this disadvantage is in part mitigated by the nature of the material used in this study. Mitochondria are the only organelles in the body that possess their own DNA, which allows them to replicate independently from other organelles. In contrast to human cellular DNA, mitochondrial DNA (mtDNA) is exclusively inherited from the maternal side.[5,6] No genetic recombination or switching takes place in mtDNA during replication, limiting the variances in mtDNA to spontaneous *de novo* mutations,[7] as well as fusion and fission, albeit to a marginal degree.[8] Thus, it could be argued that inter-personal biological differences are relatively small in the isolated mitochondria used in our study, and among humans in general.

Mitochondrial integrity could play an important part in the clinical outcome of CABG surgery. The patients in the chronic ischemia group in our study all underwent CABG

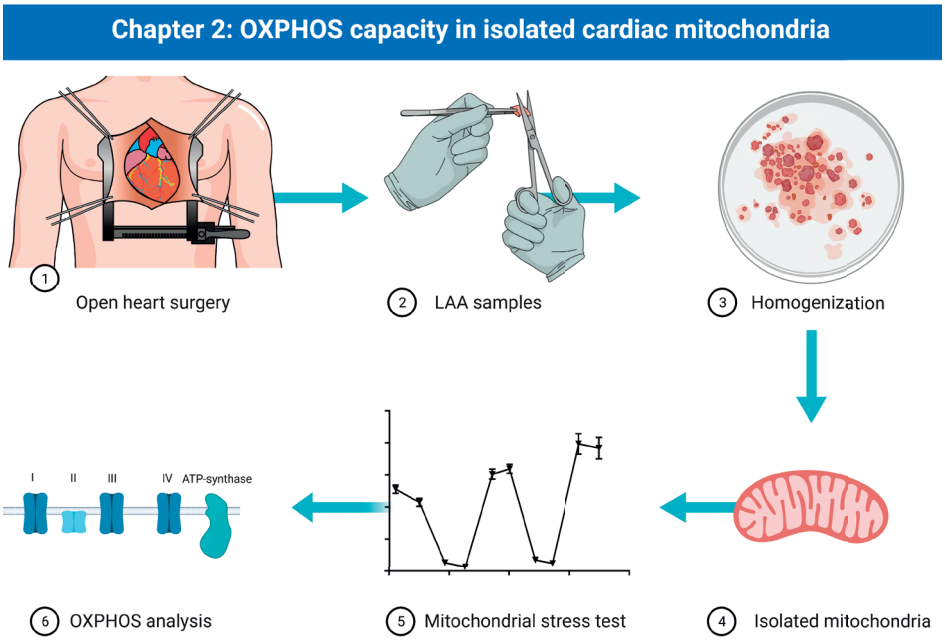

Fig. 1. Graphical abstract of Chapter 2. During cardiac surgery (1), tissue biopsies were taken from the left atrial appendage (2). This tissue was trimmed and homogonized (3), resulting in isolated mitochondria through a series of steps (4). Then, mitochondrial stress test was performed using Seahorse XF analyzers (5), allowing for thorough analysis of OXPHOS capacity (6).

surgery in order to restore myocardial perfusion, and thus resulting in improved ATP production through OXPHOS. Under circumstances of decreased substrate and oxygen availability, the myocardium is thought to remain in a hibernating state by switching to glucose as its primary energy source in order to remain viable.[9] This hibernating state provides a greater antioxidant scavenging profile, and is protective against ischemia induced injury.[10] It would be interesting, though not practically possible, to determine the effects of restored oxygen supply on mitochondrial function. It was demonstrated that restoration of blood flow in the hypo-perfused region can revert hibernating myocardium in an animal model.[10] This leads to improved, but less than optimal cardiac contractility. A comparable effect is seen in humans, where a substantial part of patients suffering from ischemic heart failure do not completely regain myocardial contractility upon revascularization.[11] This is mostly due to apoptosis and fibrosis due to ischemia. However, loss of mitochondrial protein expression is also observed during ischemia.[12] It could be speculated that inherent loss of mitochondrial functionality and protein expression limits the recovery of ejection fraction, even after revascularization of the myocardium. The typical symptoms and signs of ischemic heart failure result from disruption of homeostasis between energy demand and supply in the human myocardium.[13] There is a growing consensus defining acquired

heart failure as a disease of bioenergetics. In a recent study it was demonstrated that mtDNA alterations are present in the early stages of heart failure.[14] This finding is in addition to earlier observations of mitochondrial dysfunction in patients with heart failure.[15] Given the role mitochondria play in the development and maintenance of heart failure, even after coronary blood flow recovery, mitochondrial repair could be an interesting target of novel therapies.

Metabolic effects of cardiac assist devices

The observed shift in cellular metabolism in Chapter 2 as a result of chronic ischemia affects myocardial contraction, and therefore diminishes the ejection fraction. CABG surgery is intended to (partially) restore the perfusion of the entire heart in the case of coronary stenosis.[16] The subjects in **Chapter 3** of this thesis have been diagnosed with end-stage heart failure. In these patients, CABG is not considered a viable option anymore due to remodeling of the heart along with a severely depressed ejection fraction. These patients may be eligible for cardiac transplant as the solely remaining definitive treatment. However, given the scarcity in graft availability, bridging the patients with a temporary left ventricular assist device (LVAD) may be needed to improve quality of life, or even to survive. Additionally, with incidence continuously rising, cardiovascular disease is forecasted as the leading cause of death in the world for decades to come, allowing cardiac assist devices to be increasingly considered as destination therapy.[17-20] Given these circumstances, a surge in the implementation of cardiac assist devices is expected, and management of patients that undergo implantation is continuously optimized. If suffering from severe heart failure, the body's basal metabolism is affected by the ongoing state of continuous distress and due to a shift towards a more catabolic state. In addition, heart failure patients continuously combat challenges related to their nutritional status such as early satiety, anorexia, nausea and delayed gastric emptying,[21] putting them at risk of cardiac cachexia. In Chapter 3 we observed in a small group of patients that the REE is further elevated in patients that underwent total artificial heart (TAH) implantation, while LVAD placement had no significant influence on REE. The implications of these findings are mostly peri-operatively, when patients receiving TAH implantation rather than LVAD should be closely monitored for malnutrition. Interestingly, TAH and not LVAD implantation was associated with an increase REE, which may be due to by various factors. One notable distinction between TAH and LVAD is the effect on hemodynamics: TAH is a pulsatile flow device, whereas most LVADs are constructed to generate a continuous LV output. The benefits and pitfalls of both continuous and pulsatile blood flow have been the topic of debate. Pulsatile flow is associated with greater end-organ perfusion and improved coronary artery flow, thus increasing metabolic supply.[22,23] Despite these benefits, pulsatile flow in LVADs does not increase overall survival rate in patients, due to increased risk of thrombi formation and infection.[24] Nonetheless, novel techniques

and optimal flow settings are under evaluation, aiming to further tailor LVAD flow to physiological values while minimizing side-effects.[25-27] Another factor that may contribute to the differences in REE between devices, is the surgical procedure that is performed during implantation. Increased levels of REE are closely linked to degree of trauma or injury.[28-30] As TAH placement is generally more invasive than the implantation of an LVAD, an increase in REE is expected to be higher in the first. Additionally, following initial increased risk of malnutrition during the post-implantation period, the presence of a cardiac assist device can reverse cardiac cachexia over time as a result of restoration of flow.[31] Although the study in **Chapter 3** was limited by the small number of patients, it provides a useful insight in the shift in metabolic needs of patients that undergo device placement. In this group, stringent nutritional management may improve quality of life, minimize length-of-stay and thus reduce cost of care.

Part one of this thesis has focused on the role of metabolism in advanced heart failure. Disruption of metabolism on a cellular level is one of the key pathophysiological mechanisms in ischemic heart failure. Current therapies are rightly focused on restoring tissue oxygenation in order to provide sufficient energy. This thesis, however, describes alterations on a mitochondrial complex level, highlighting complex I as the main contributor to mitochondrial dysfunction leading to loss in cardiac contractility. Furthermore, we have elucidated the metabolic needs in heart failure patients that are subjected to assist device placement, along with the increased challenges that this vulnerable group is subjected to upon implantation. In conclusion, **part one** accentuates the perils of disrupted metabolism in the etiology and sustainment of heart failure. Macromolecular dysfunction in the ischemic human heart likely contributes to loss of cardiac contractility, and diminished ejection fraction while implantation of an assist device challenges these patients' metabolic needs.

Organ preservation and transplantation

End-stage heart failure is a chronic, progressive disease and the only definitive cure is organ transplantation, a therapy that continues to be limited mostly due to graft scarcity. **Part two** of this thesis investigates current challenges as well as novel developments in cardiopulmonary transplantation. With more and more patients developing end-stage heart failure and stagnating availability of donor organs, the transplant gap persists despite an increase in number of cardiac transplants (Figure 2A-C).[32] Less than half of all donated cardiac grafts actually end up being transplanted.[33] We discuss approaches to expand the pool of available cardiothoracic graft for transplantation.

Presumably, one of the most apparent methods to increase availability of organs would be to improve the organ storage conditions after harvest. Thorough understanding of the

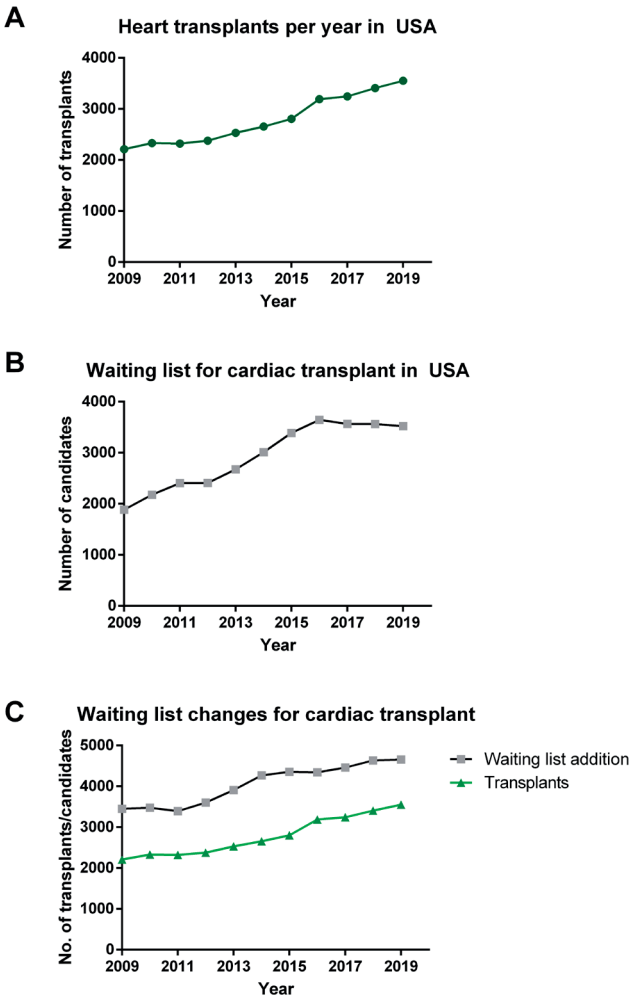

Fig. 2. A Number of hearts transplanted in the US per year over the past decade, showing a steady increase. B Number of cardiac transplant candidates actively on the waiting list, which has plateaued over the past 5 years. C Number of cardiac transplants performed in the US per year in comparison to the number of patients added tot he waiting lists for transplant.

limitations of current clinical practices is imperative in order to propose advancements. **Chapter 4** identifies the mPTP as a limiting factor in aiming to exceed *ex vivo* time past 4-6 hours before critical damage is initiated during cold static storage of cardiac grafts.[34] The circumstances that cause mPTP dysfunction are provoked by ischemia but not fully triggered until reperfusion, leading to graft dysfunction after transplantation.[35] We hypothesized that the mPTP plays a pivotal role in IRI of cardiopulmonary graft and its function should be protected in order for the *ex vivo* period to be extended (Figure 3).

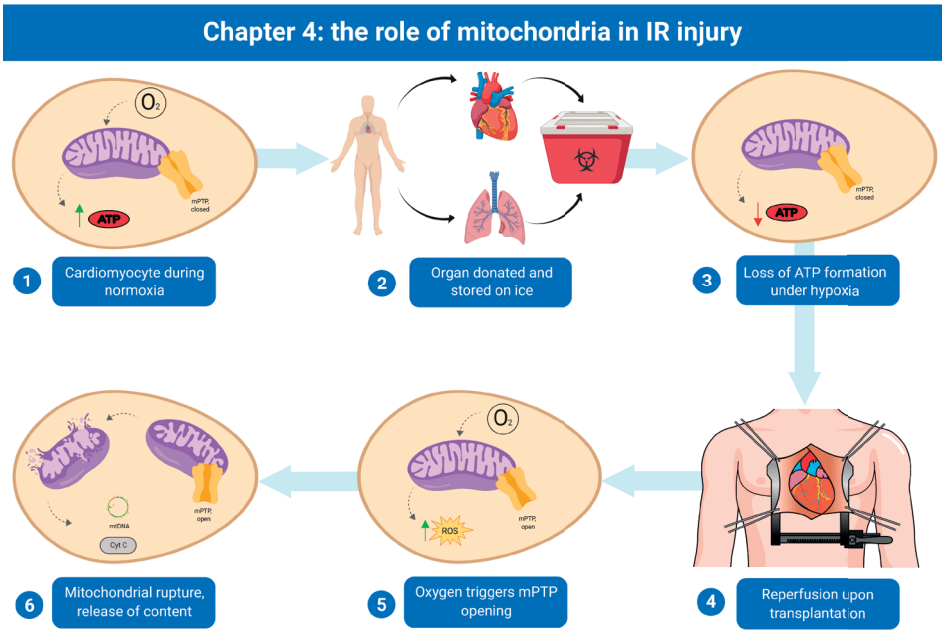

Fig. 3. Graphical abstract of Chapter 4. Normal oxygen consumption and closed mPTP during normoxia (1). Grafts are being stored on ice during *ex vivo* preservation, to minimize cellular metabolism (2). During this time of hypoxia, mitochondria are not capable of producing ATP through OXPHOS (3). Once transplanted into a recipient, the graft is reperfused and mitochondrial ATP production is restored (4). The re-introduction of oxygen opens the mPTP, triggering ROS formation and calcium influx (5). This results in mitochondrial swelling and disrupture, causing apoptotic factors to be released (6).

The significance of metabolic preservation has been recognized for many years, most notably in deep hypothermic circulatory arrest, where profound cooling of the body allows for prolonged cardioplegic periods with minimal brain damage in cardiothoracic surgery.[36] Similarly, it has been suggested that a state of so-called suspended animation, in which cellular metabolism is halted without damage occurring, can be achieved.[37] Suspended animation in cardioplegia by a combination of adenosine, lidocaine and magnesium (ALM) has been proposed by Dobson et al.[38] ALM is based on the combination of polarized cardioplegia, similar to what is observed in hibernating animals, and inotropic stimulation to resuscitate cardiac activity.[39,40] Through suspended animation, hearts preserved in ALM attained significantly higher levels of cardiac output after 8 hours of cold static storage than control grafts.[41] Alongside this concept of suspended animation to improve storage times, studies on preservation solutions offering metabolic stability are emerging as well.[42] One such a fluid has been assessed in Chapter 4, where "Somah" is described as a solution that is formulated to provide mitochondrial protection and improved bioenergetics. The metabolic substrates, free radical scavengers, and various ionic modulators allow Somah

to establish a favorable environment for preservation of cardiac tissue and endothelium.[43] Previously it has been demonstrated with porcine hearts that Somah achieves metabolic protection through maintenance of high-energy phosphates post-preservation and improved myocardial ejection fraction after reperfusion.[44] While the results of these studies are encouraging, the mechanistic pathways involved have not been well established. To evaluate the clinical feasibility of Somah solution, an *in vitro* model using cardiac myoblasts studied the mitochondrial effects of Somah preservation in **Chapter 5**. The most important findings in this study were that after preserving the cardiac myoblast for 8 hours at 4°C, the cells stored in Somah had significantly higher basal respiration, coupling efficiency and RCR than those stored in conventional storage solutions. The observed RCR decrease in the control groups can most likely be attributed to increased proton leak due to ROS formation as well as lowered maximal OCR due to mitochondrial complex V damage. Treatment with Somah solution prevented the mitochondrial membranes from becoming leaky and preserved physiological integrity of mitochondrial complexes. Coupling efficiency was preserved when using Somah, suggesting mitochondrial and metabolic protection as indicated by optimal maintenance of basal OCR and proton leak.

This study acts as a proof of concept for metabolic protection by Somah in rat cardiac myoblasts. Here, the metabolic preservation properties of various organ preservation solutions were investigated. We have demonstrated that Somah has superior protective capacity of cellular bioenergetics than Celsior and Perfadex, two clinically used preservation solutions. The findings, however, are limited by the fact that various cellular processes influence the mitochondrial function. These aspects have not been excluded, as would have been the case when experimenting on isolated mitochondria or permeabilized cells. Nonetheless, this study is a foundation for future research in animal models.

Building on the reviewed literature and *in vitro* experiments, **Chapter 6** focused on *ex vivo* organ preservation using Perfadex and Somah (Figure 4). This chapter is the only one in this thesis that made use of pulmonary rather than cardiac grafts in the experimental set-up. The general principles for heart and lung organ preservation are highly similar and preservation solutions are fundamentally identical, limiting the maximal *ex vivo* time to 4-6 hours for both of the cardiopulmonary organs. Also, in accordance with a recent attention shift towards the use of organs donated after circulatory death (DCD) as a new source for transplantable grafts, we used DCD lungs in our experiments.[45-47] The experimental end-point in this chapter was not to elucidate a difference in maximal *ex vivo* time between both solutions. Instead we studied the influence of preservation solution composition on organ metabolism during cold static storage.

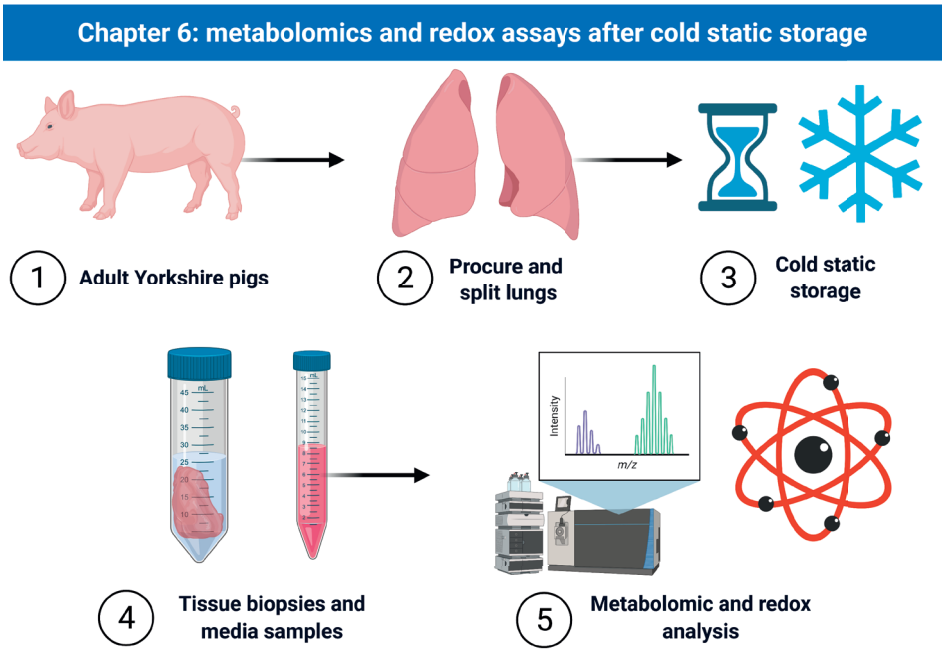

Fig. 4. Graphical abstract of Chapter 6. In this porcine study (1), DCD lungs were split and randomized into 2 groups (2). The lungs were preserved in experimental and control preservation solution, and were kept in cold static storage circumstances as per clinical standard (3). Tissue biopsies and media samples were taken throughout the experiments, and the lungs were preserved for extended periods of time (4). On these samples, metabolomics and redox potential was measured (5).

Here, lungs stored in Somah demonstrated superiority in metabolic pathway utilization and antioxidant scavenging during cold static storage. We found a larger glycolytic capacity in Somah solution than in Perfadex and this was reflected by the TCA cycle component accumulating over time. In Perfadex, on the other hand, the fructose pathway was used more in order to sustain metabolism. High adenosine levels found in Somah during preservation suggested conditions in which the organ was less prone to IRI injury. ROS scavenging capacity was found to be greater in Somah than Perfadex, as reflected by GSH and GSSG levels in the media. Lastly, we measured the oxidation reduction potential (ORP). Evaluation of ORP is a relatively unknown method in redox research. Measurement of redox potential and linked ROS and/or antioxidant capacity has been traditionally challenging. Absolute tissue ROS levels as a (by)product of biological processes or oxidative stress are not only difficult to monitor, but should be regarded in the right context as well, as absolute values can be misleading. Therefore a cautious approach is required when looking into redox potential. Over the recent years ORP has emerged as a useful parameter to assess oxidation reduction potential and antioxidant capacity in a quick and reliable manner.[48-50] This has been subdivided into static ORP and capacity

ORP, indicative of oxidative stress and antioxidant capacity, respectively. Both endpoints favor Somah over Perfadex in this study.

The analysis of metabolomics in **Chapter 6** has provided valuable understanding of regularly used Perfadex's impact on pulmonary graft during cold static storage. Expanded knowledge of the metabolic aspects of organ preservation solutions is imperative in proceeding and advancing *ex vivo* storage and improving transplant outcomes. Alongside this aspect of the study, we analyzed the metabolic consequences of preserving organs in Somah. We found that Perfadex, as expected given its formulation, does not provide metabolic protection, while Somah appears to have the potential to enhance metabolic and redox conditions. One major limitation of this study is the lack of post-preservation reperfusion. Our study does not address this issue, and thus it remains unclear if Somah's theoretical advantages in prevention of IRI persist after reperfusion. As previously described, oxygen and mitochondrial calcium overload are the driving currents in IRI and the lack of reperfusion in our model is limiting. Further research on reperfusion after preservation is needed in order to fully comprehend the role Somah – and other bioenergetics preserving solutions – may play in organ preservation. It seems inevitable that cardiopulmonary graft preservation will eventually shift towards *ex vivo* continuous perfusion, comparable with recent developments in preservation of other organs. Recent trials assessing continuous perfusion in human heart and lung preservation have confirmed non-inferiority compared to cold static preservation.[51,52] Clinical trials are currently ongoing to further establish the effectiveness in expansion of *ex vivo* storage time.[53-56] In spite of these developments, cold static storage will continue to be the standard procedure as a result of the current infrastructure in most medical centers. Increasing *ex vivo* time and expansion of the donor pool with DCD organs will provide benefits for the transplant waiting list.

In **Chapter 6** we demonstrated that Somah provides an excellent environment for pulmonary graft storage. This is reflected by the gradual depletion of substrates through metabolic pathways and is further elucidated by the metabolomic byproduct formation and by redox potential homeostasis. Although Somah has intentionally been developed for cardiac grafts, its role in preservation could be translated to lungs as a result of comparable storage criteria of both organs. We suggest additional research to be performed in order to further evaluate this exciting prospect.

Part two of this thesis addressed the challenges in preservation of cardiopulmonary grafts. Current restraints in *ex vivo* time are characterized by lack of innovation in the clinically used preservation solutions, and a hiatus in knowledge of the solutions' metabolic properties. We have addressed and illustrated this issue by reviewing the literature and proposing a revision in preservation solution composition. We suggested an improved outcome

through mitochondrial and metabolic protection, introducing Somah as an exemplary solution. Bioenergetic preservative properties of Somah were then explored on cultivated cell, suggesting an improvement compared to traditional solutions. This effect was further examined and demonstrated in **part two's** final chapter, where Somah appeared to provide a preferable environment for lung grafts in terms of metabolomics and redox safeguarding. The results in Chapters 5 and 6 establish Somah as a preservation solution with clinical potential. Ideally large, multi-center trials would be set up in order to determine what role Somah can fulfill in cold static storage of cardiopulmonary grafts.

Amniotic membrane patches and their role in inflammation

After discussing the metabolic aspects of heart failure and common therapies in the first two parts, **part three** of this thesis explored an experimental tool in cardiac surgery: the amniotic membrane patch (AMP). The AMP is obtained from human fetal tissue and consists of extracellular matrix, proteins, and cells.[57] AMP is mostly known for its use in wound healing and corneal transplant surgery.[58-60] Anti-inflammatory and anti-fibrotic effects are associated with AMP, attracting attention from various other fields.[61-63] As in any form of stem cell therapy, safety of use is an essential factor to be taken into account. Pluripotency inherently carries a serious risk of neoplasm formation. Amniotic membranes are deemed safe to use in the setting of patching and transplantation.[64-66] Here, we studied the potential of AMP in cardiac surgical interventions where anti-inflammatory agents are considered.

In **Chapter 7** of this thesis, AMP was introduced in a case report with a patient suffering from pericarditis. A long history of cardiac surgeries made this patient prone to complications, including a serious risk of death. Given the immunomodulation that is associated with AMP, the pericardium was patched to reduce the inflammatory state. This patient's pericarditis was cured without complication, as indicated by the absence of pericardial effusion and inflammation on MRI. Although this case report does not provide hard evidence for patching in common cases of pericarditis, it does raise interest in the use of local immunosuppression as a therapeutic tool in complex patients such as this one. More importantly, this case report triggered our interest in other areas of cardiac surgery where inflammation is the cornerstone of pathophysiology. The first was described in **Chapter 8**. In patients that are awaiting cardiac transplant as a remedy for end-stage heart failure, cardiac assist device implantation is often considered as a bridge-to-transplant (BTT). The implantation of an assist device can trigger the immune system to form antibodies, reducing the patient's chances of successfully accepting a donor heart upon transplantation.[67,68] In addition to immunosentization, patients during their BTT period are vulnerable to other immunological issues as well, such as the activation of coagulation factors, causing device failure and risk of death due to thrombi formation.[69-73] Anticoagulation is routinely

given to LVAD patients, but inherently carries risk of bleeding.[74,75] Additionally, systemic inflammation is often seen after implantation of cardiac assist devices.[76-78] Properly targeted anti-inflammatory therapies combined with anti-embolic and antibiotic treatment can prevent the issues associated with LVAD implantation, while improving the chances of successful graft acceptance.

As a tool to minimize the risk of antibody formation, AMP was micronized and then injected into the myocardium during surgical device implantation. We found that two patients who were previously considered to be sensitized (i.e. PRA>10%), were now at baseline PRA levels. Two patient experienced an increase in PRA, at this time marking them as immunosensitized, although one patient's case was complicated by chronic infection of the LVAD driveline. Three patients that received micronized AMP injections maintained PRA levels of 0% throughout. Control cases demonstrated an increase in PRA levels, although one patient returned to a non-sensitized state over time. This final observation somewhat blurs the otherwise encouraging results. Clearly, no conclusions can be drawn from the non-significant results in this study that was limited by small patient group, and more research is needed. Randomized control trials should be set up in order to validate our initial findings in this exploratory case series.

Finally, in **Chapter 9** AMP was used to locally suppress the intra-thoracic inflammatory response after cardiac surgery (Figure 5). Peri-operative epicardial AMP placement led to a very strong trend of reduction in POAF, the most common arrythmia after cardiac surgery. We found that secondary outcomes did not differ from the control group, although the study was largely underpowered for these outcomes. In general, the groups were relatively small, which is a limitation of the study. Propensity matching of control group and elimination of negative outliers among operators of control cases were executed to further validate the findings. Reduction of POAF incidence in cardiac surgery persisted in patients that received AMP.

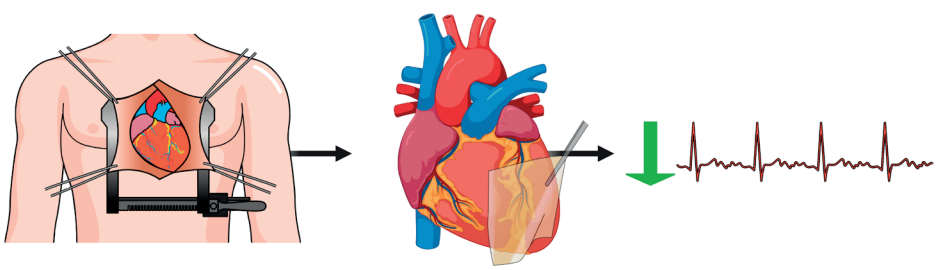

Fig. 5. Graphical abstract of Chapter 9. During cardiac surgery, amniotic membrane patches were placed over the epicardium, and the effects on incidence of post-operative atrial fibrillation were determined.

Prevention of POAF is one of the pillars of post-operative care following cardiac surgery.[79,80] Not only is POAF a risk factor for the serious comorbidities stroke and death, it also places a large economic burden on health care. The protracted hospitalization that is associated with POAF accounts for around $2 billion annually in the United States alone.[81,82] Atrial fibrillation in general has multi-factorial pathophysiology, with inflammation as one of the key factors.[83] In POAF, the role of inflammation is even more prominent.[84] Intraoperative cardiac manipulation and the introduction of cardiopulmonary bypass contribute to inflammation that is associated with cardiac surgery.[85] Accordingly, prevention of POAF through immunomodulation has been explored by others and has demonstrated acceptable results.[86,87] The most common preventive tool, however, is reduction of heart rate using β-blockers, with moderate effect.[88,89] Interestingly, despite profound recognition of its severe negative influence and following various trials, POAF occurs at a stable rate for over three decades now.[79,90] The primary distinction between AMP and other anti-inflammatory therapies is the local character of patch placement. As a result of this, there is no increased risk of side effects of corticosteroids such as post-operative infection, hyperglycemia and electrolyte disturbances.[91-94] Additionally, as opposed to suggested intrathoracic corticosteroid flushing before chest closure, the effect of AMP is sustained for multiple days. The latter is essential, as patient that underwent cardiac surgery are at risk for POAF for up to 5 days post-surgery, with a peak incidence at day 2.[85] Epicardial AMP placement is a safe-to-use preventive agent in cardiac surgery that could strongly reduce POAF incidence. Large randomized controlled trials should confirm our findings, before implementation.

Part three of this thesis outlines the exciting prospect of an experimental agent in anti-inflammatory therapy in cardiac surgery. Two exploratory studies involving a case report and a case series introduce AMP as an antagonist of inflammation, with possible application as a patch and as micronized patch injections. The third and arguably the most notable study in this part reveals a strong and distinct trend towards reduction in POAF after cardiac surgery. The low cost and ease-of-use of AMP contribute to its attractiveness.

FUTURE DIRECTIONS

Improving organ preservation through metabolic protection

The topic of organ preservation, in particular cold static storage, has been addressed in this thesis. Cold static storage perseveres as the principal form of preservation for cardiopulmonary grafts as a result of its ease-of-access and relatively low cost, combined with a great amount of clinical experience. Nonetheless, it could be speculated that this form of preservation will be gradually abandoned in the foreseeable future. Continuous

perfusion in heart and lung preservation has been under investigation for many years and has demonstrated various advantages over cold static storage. Along with the most apparent advantage of expanding the preservation timeframe, a significant number of organs could be added to the donor pool by rejuvenation during preservation. Damaged hearts and lungs could be treated with high doses of corticosteroids to minimize damage. A recent study has suggested a more prominent role for DCD organs, as it was demonstrated that DCD lung quality can be improved through reduction of inflammation and apoptosis.[95] Another benefit of continuous perfusion during preservation could be in graft quality assessment. Organ quality assessment is one of the pressing issues in heart and lung transplantation, with secondary parameters such as edema formation being at the core of clinical decision making.[96] Graft quality assessment in particular has emerged as one of the major advantages of perfused preservation in other organs, and could be implemented in cardiopulmonary transplantation accordingly.[97,98]

Without disregard to previous statements on the expected shift towards continuous perfusion graft preservation, cold static storage of cardiopulmonary grafts will continue to be an integral part of transplantation for the coming years. This is mainly attributable to its low cost and ease-of-use, allowing for accessible use for centers all over the world. The potential gain from optimizing cold static storage techniques is therefore still very significant. Ideally, static storage preservation solutions should be formulated with a combination of mitochondrial protection and provision of metabolic substrates in addition to the conventional aims of minimalizing ROS and prevention of edema. We expect cold static storage solution to continuously improve by sophisticated changes despite the gradual shift towards continuous perfusion during organ preservation.

Anti-inflammatory agents in cardiac surgery

In response to cardiac surgery and related tissue injury, mitochondrial damage associated molecular pathogens are released and trigger the immune system to produce pro-inflammatory mediators, such as C-reactive protein, interleukin 1 and interleukin 6.[99] This post-operative pro-inflammatory state is associated with an increased risk of POAF, as described before. However, there is a delicate equilibrium between pro- and anti-inflammatory agents, and in healthy subjects the interplay between the two results in optimal wound healing. The inflammatory processes should therefore not be abolished as a whole, but elegantly managed by locally acting anti-inflammatory therapeutics such as AMP. As such, epicardial application of amiodarone and/or corticosteroids prior to chest closure provide a reduction in the incidence of POAF.[100,101] These studies and the presented results in **Chapter 9** are variations on a shared future perspective of immunoregulation rather than systemic immunosuppression. Such forms of primary prevention are expected to increasingly play a role in cardiac surgery and medicine in general. Regulation of the inflamma-

tory response can be obtained through various mechanisms of action, which allows for a broad spectrum of therapeutics, such as mesenchymal stem cells as demonstrated in part 3 of this thesis and by other investigators.[102-104] Additionally, immunomodulation is emerging as therapy for patients following myocardial infarction.[105-107] Further advances are expected in the search of immunomodulators to substitute the somewhat archaic approach of systemic anti-inflammation. More specifically, personalized immunomodulation based on the circumstantial needs and the interindividual variability may provide an optimal balance between pro- and anti-inflammatory actors and improve patient outcomes.[108]

This thesis has addressed metabolism in heart failure, cardiac surgery and therapies. The role of metabolism in heart failure and cardiopulmonary transplantation is expected to continue to expand in the coming years. Following a period of fairly low interest, the attention has returned towards mitochondrial (dys)function over the past decades with emerging evidence that mitochondria are defining numerous pathologies, control the immune system, and are the gatekeepers of life and death in cells.[109-112] In this thesis' closing chapter, a range of exciting prospects that may not be within the immediate scope of this thesis are discussed, and suggestions for further research are made.

Graft quality assessment

Protection of mitochondrial bioenergetics is emerging as a key goal in cardiopulmonary graft preservation. As described in **Chapter 7**, our group has suggested that the mPTP is the crux of IRI in organ preservation and reperfusion. The mitochondrial disruption induces cell death, causing mtDNA to become freely flowing in the blood stream. Thus, since it is structurally different from nuclear DNA, circulating mtDNA could serve as a biomarker for mitochondrial (dys)function in IR injury. In an unpublished study our group hypothesized an increment in free circulating mtDNA after prolonged ischemia time. Mimicking a clinical scenario by perfusing porcine lungs via the Organ Care System after cold static storage, we aimed to test this hypothesis. Preliminary PCR assays measuring the nuclear DNA/mtDNA ratio after reperfusion of the lungs suggested more free mtDNA in the closed circulation, indicative of loss of mitochondrial content.[113,114] Might these initial and unpublished findings be confirmed in larger studies, mtDNA could provide a powerful biomarker for graft quality during perfusion before transplantation.

In addition to the evaluation of biomarkers, mitochondrial stress tests may be implemented as another graft quality assessment tool during preservation. The techniques of mitochondrial isolation and assessment described in **Chapter 2** were converted and used during cold static storage of lungs in Perfadex. Apical biopsies were taken at 4, 10 and 16 hours, and mitochondrial stress tests were performed. In this unpublished experiment, mitochondrial dysfunction was apparent after 16, but not after 10, hours of cold static

storage (Figure 6). These findings suggest gradual mitochondrial dysfunction in prolonged *ex vivo* preservation, thus providing a potential *ex vivo* graft quality assay.

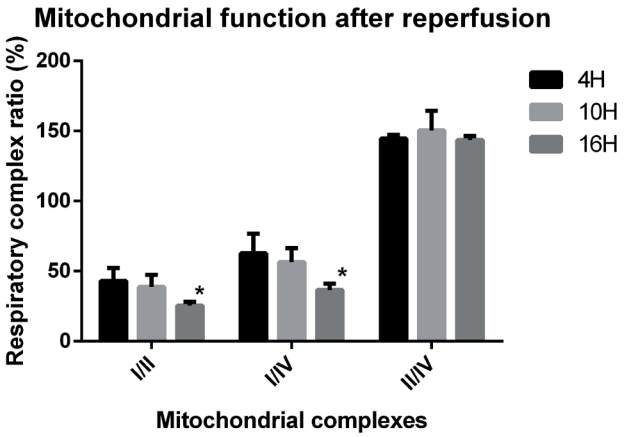

Fig. 6. Unpublished data on mitochondrial function after reperfusion of grafts that were preserved for 4, 8 and 16 hours. The data suggests a loss of mitochondrial complex I function after 16 but not after 4 or 10 hours of cold static storage of porcine lungs. Mean ± SEM, * incidates $p < 0.05$.

Stem cells as donors of bioenergy

In search of novel strategies to treat heart failure, stem cells have been of interest for many years. Historically, improved ejection fraction through cell differentiation and cardiomyocyte generation was hypothesized.[115,116] While some effect – mostly in animal models – was observed and attributed to cell differentiation, the principles on which stem cell therapy was based shifted towards paracrine effects, including anti-inflammatory properties, upregulation of growth factors and angiogenesis.[117,118] Overall, myocardial stem cell injections aiming to improve cardiac function have proven to be a safe therapeutic option, albeit with moderate and temporary effect.[119] Given the mounting evidence that bioenergetics play a vital role in heart failure, the observed but poorly understood positive outcomes of stem cell therapies may have had a different origin. Over the past decade, delivery of mitochondrial content has emerged as a therapeutic option for various bioenergetic diseases, including congenital heart disease.[120] This newly discovered mechanism of mitochondrial donation has shifted the hypothesized mechanism of stem cell therapy away from tissue regeneration and paracrine effects, even more so following the major scandal in 2019 regarding the existence of cardiac stem cells (or lack thereof).[121] Various studies have successfully demonstrated autogenic transplantation of freshly harvested and isolated mitochondria from skeletal tissue into dysfunctional or diseased sites.[122] Per-operative myocardial injections of isolated mitochondria are beneficial in protecting against IRI.[123] Similarly, post-infarction intramyocardial delivery of mitochondria has been found

to improve cellular viability in porcine model.[124] In mice, pretreatment with isolated mitochondria through the coronary arteries led to improved cardiac protection against prolonged cold ischemic storage through the replacement of damaged mitochondria.[125]

Exchange of bioenergetics without the need for mitochondrial isolation has been explored as well. This so-called mitochondrial transfer has emerged as one of the unique feature of stem cells, and it can be attributed to the enzyme Miro1.[126,127] Miro1 stimulates the formation of tunneling nanotubules, allowing stem cells to donate their mitochondria to both healthy and damaged somatic cells.[128,129] Interestingly, the mitochondrial content released from somatic cells under distress is the cue for stem cells to increase donation of the organelles, as well as to promote mitochondrial biogenesis.[130] Once transferred into somatic cells, mitochondria are capable of meeting host cells metabolic demands, while cellular function is maintained in culture for over 30 passages without impairment.[131] Furthermore, transferred mitochondria protect against acute lung injury and against oxidative stress in corneal tissue.[132,133] Further adding to the clinical value of mitochondrial transplantation and transfer, allogeneic injections of mitochondria do not appear to induce a host immune response.[134] One area, however, that remains obscure in regards to this topic is the accumulating effect on bioenergetics following transfer of mitochondria- are the mitochondria capable of producing ATP in their new host cells? In the brain, mitochondria have been shown to be functional after transfer, promoting mitochondrial transfer as a therapy for ischemic stroke.[135] A recent *in vitro* study demonstrated that co-culture of MSCs can restore mitochondrial oxygen consumption in hibernating cardiomyoblasts.[136] The list of fields that could benefit from mitochondrial transfer is quickly expanding. Such observations give rise to exciting opportunities in improving bioenergetics in the failing heart and reestablish interest in stem cell therapies.[137] Stem cells could function as donors of bioenergy and improve cardiac function significantly in the chronic ischemic patient.

Artificial organogenesis

Despite all important work in optimizing transplant outcomes and increasing life expectancy, a sustainable system of organ donation continues to pose a major challenge. In order to meet the growing demand, artificial creation of cardiopulmonary organs – organogenesis – could be a solution. New formation of organs can be subdivided into bioprinting cells onto patches and matrices, and the complete reseeding of decellularized organs, for xeno- or allograft transplantation. Bioprinting of implantable tissue is rapidly emerging as a powerful tool in various medical fields.[138] Stem cell-rich biological gels can be used as bio-ink similar to plastics in 3D printing. Stem cell regenerative properties can be fully exploited in these forms of in vitro organogenesis.[139,140] Bone and bladder have been printed and are examples of tissue well suited to be tailormade.[141] The tissue complexity of the heart and lungs impedes the same rapid development in these organs.

Nonetheless, more and more research is emerging on this topic. Cardiomyocytes have been printed onto extracellular patches, leading to a reduction of scarring and infarct size while improving ventricular wall thickness in an in vitro mouse model.[142,143] Myocardial graft bioprinting is a promising therapy for myocardial infarction.[144,145] More commonly used are printed heart valves, although their current role is mostly limited to a surgical planning tool.[146] Further research suggests the combination of autologous or allogeneic stem cells could result in durable, hybrid valves, further delivering on the promise of personalized organ regeneration.[147-149]

The second class of artificial organs consists of organs created by reseeding after decellularization. A revolutionary approach of washing out all cellular material before reseeding with stem cells could allow to rebuild organs to perfectly fit the recipient.[150] Accordingly, primary graft dysfunction and graft rejection would effectively be eliminated. Decellularized hearts and lungs, consisting solely of extracellular matrix, are proposed as ideal scaffolds for stem cells to regenerate on.[151] This is considered to be a major benefit of reseeding over 3D bioprinting, where both cellular and acellular components need to be formed from scratch. Whole heart as well as valve and vessel decellularization can be performed.[152,153] During the removal of cellular components, important characteristics such as stiffness and architecture are preserved.[154] Perfused decellularization allows for the complete and homogeneous removal of all cellular material, although the optimal detergent and enzyme combinations are a topic of debate.[155-157] It should be noted, however, that complete elimination of all host DNA is complicated, especially in larger organ scaffolds.[158] The process of decellularization could be implemented in the use of marginal, and otherwise not transplantable, heart and lungs, which then can be used as a scaffold after decellularization.[159,160] Our group has reported on methods for cardiac as well as pulmonary perfused decellularization, aiming to provide grafts acceptable for reseeding with preserved microarchitecture.[156,161] Other investigators have re-seeded progenitor cells on decellularized sheets of myocardium, improving cardiac function after sheet implantation in a rat myocardial infarction model.[162] These initial steps in regenerative medicine are promising, but the completely new formed heart and lungs are still not nearly feasible. Current major regenerative limitations include the lack of biophysical stimuli, proper oxygen delivery and the long period that cell differentiation requires for reseeding.[163-165]

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Chapter 11

Nederlandse samenvatting

In dit proefschrift worden de metabole en inflammatoire aspecten van chirurgische interventies binnen het cardiovasculaire systeem bestudeerd in drie delen. Na een algemene introductie (Hoofdstuk 1), behelst **deel één** (Hoofdstukken 2 en 3) studies naar metabolische veranderingen bij patiënten met chronische cardiovasculaire aandoeningen, zowel op mitochondriaal als systemisch niveau (Hoofdstukken 2 en 3). In Hoofdstuk 2 wordt aangetoond dat zuurstofverbruik verminderd is bij mitochondria die geïsoleerd werden uit weefsel van het linker hartoor van patiënten die een *coronary artery bypass grafting* (CABG) ondergingen, in vergelijking met mitochondria uit hetzelfde weefsel bij patiënten die een operatie aan een hartklep ondergingen. Het geobserveerde verschil in deze studie kan primair worden toegeschreven aan een verlies van functionaliteit van complex I. Hoofdstuk 3 onderzoekt de invloed van steunhart implantatie op het metabolisme van het gehele lichaam binnen een kleine onderzoeksgroep van patiënten met eindstadium hartfalen. Hier is de implantatie van een totaal artificieel hart (TAH), in tegenstelling tot een implantatie van linksventriculair steunharten (LVAD), geassocieerd met een toename van metabolisme in rust. Dit wordt voornamelijk toegeschreven aan het mechanistische verschil tussen beide kunstharten en de mate van chirurgisch trauma.

Deel twee (Hoofdstukken 4-6) adresseert verscheidene uitdagingen binnen de cardiopulmonale transplantatie. Hoofdstuk 4 geeft een overzicht van de huidige standaarden in donorhart preservatie, waarbij het belang van bescherming van *'bioenergetics'* gedurende de preservatie periode wordt onderstreept. Meerdere preservatie vloeistoffen en -technieken worden geanalyseerd waaruit aanbevelingen zijn geformuleerd ter verbetering van de huidige preservatie standaard. Dit overzicht bevat in het bijzonder aandacht voor de rol van mitochondria en de mitochondriële *permeability transition pore* en hun beider bijdrage aan ischemie-reperfusieschade. In Hoofdstuk 5 wordt verbeterde mitochondriële bescherming *in vitro* aangetoond bij toepassing van een nieuwe preservatievloeistof op een primaire cellijn van cardiomyoblasten in een rat. In Hoofdstuk 6 blijkt deze innovatieve preservatievloeistof een superieure omgeving te vormen voor varkenslongen tijdens *cold static storage* op basis van metabolomics, het ge- en verbruik van substraten in zowel weefsels als preservatievloeistof en het behoud van redox potentieel.

In **deel drie** van dit proefschrift (Hoofdstukken 7-9) worden de anti-inflammatoire eigenschappen van *amniotic membrane patches* (AMP) bestudeerd binnen een selectie van cardiochirurgische scenario's. Allereerst presenteren we een klinische casus van complexe pericarditis in Hoofdstuk 7. In dit *case report* wordt AMP geacht een bijdrage te hebben geleverd aan de inhibitie van de ontstekingsrespons en derhalve te zijn ingezet als een therapeuticum. Een vergelijkbaar immuun sturend effect wordt geobserveerd in Hoofdstuk 8, waarbij vermalen AMP in het myocard geïnjecteerd werd na steunhart implantatie bij een *case series* van 16 patiënten. Waar steunhart implantatie doorgaans leidt tot toename van

sensitisatie van anti-HLA antilichamen en dientengevolge de geschiktheid om een orgaan ter donatie te ontvangen voor patiënten beperkt, resulteert de injectie van vermalen AMP in meerderheid van de casus tot een onderdrukking van anti-HLA antilichaam vorming. Ten slotte, in Hoofdstuk 9, wordt het anti-inflammatoire vermogen van AMP beoordeeld in het voorkomen van postoperatief atriumfibrilleren, een van de meest voorkomende complicaties van cardiochirurgie. Aansluitend op de chirurgische procedure, nog voor het sluiten van de thorax, werden AMP's over het epicardium geplaatst, leidend tot een aanmerkelijke trend van reductie van postoperatief atriumfibrilleren. Het geobserveerde effect zou kunnen worden toegeschreven aan lokale onderdrukking van de immuunrespons die een essentiële rol speelt bij de ontwikkeling van postoperatief atriumfibrilleren.

Chapter 12

Acknowledgements
List of publications
Curriculum vitae
Ph.D portfolio
List of abbreviations

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Toen ik in 2012 mijn eerste mailtje ter kennismaking naar jou typte, **Jon**, om te vragen of je mijn masteronderzoek vanuit Nederland wilde begeleiden, had ik niet kunnen voorspellen dat je aan de wieg zou staan van dit alles. Je motivatie, sturing en steun waar nodig hebben dit werk mede mogelijk gemaakt. Erg dierbaar is mij de herinnering aan jouw bezoek in Arizona. Voor mij tekent het je toewijding. Dank je wel voor alle keren dat je 's avonds laat nog proof reads hebt gedaan, en de vele malen dat je ondanks drukke schema's toch nog vóór het ontbijt of ná het avondeten tijd voor me kon vrijmaken. En juist die meetings namen vaak een hoop meer tijd in beslag dan gepland, omdat we naast de serieuze kant ook alle luchtige zaken des levens uitgebreid bespraken. Ik hoop uit de grond van mijn hart dat we dat vooral nog lang blijven doen.

Na de introductie via Jon, hebben we de eerste 2 jaar voornamelijk op Skype de ontwikkelingen door genomen, **Dirk**. Ook dit gebeurde vaak op de meest onmogelijke tijdstippen vanwege het tijdsverschil. En toch wist je iedere keer met de juiste vragen en opmerkingen een nieuw licht te schijnen op gedachten en resultaten. Je hebt me wel eens verteld dat naarmate de jaren verstrijken, je tot het besef komt dat je nalatenschap in de wetenschap niet de door jou geschreven artikelen zijn, maar de positieve invloed die je op studenten en jonge wetenschappers kunt hebben. Ik kan met grote stelligheid zeggen dat jouw verhelderende visies mij als wetenschapper vorm hebben gegeven. Ook wil ik je expliciet danken voor je flexibiliteit en ruimdenkendheid in onze toch wat ongebruikelijke constructie. We gaan elkaar hopelijk nog vaak zien en spreken!

Lieve **papa**, hoewel je het zelf helaas niet meer kan lezen, wil ik je bedanken voor de onvoorwaardelijke steun. Vanaf de eerste plannen om naar de VS te gaan, heb jij op alle mogelijke manieren bijgedragen om dit te laten slagen. Het was erg fijn om zo nu en dan (of best vaak) mijn beklag te kunnen doen als er iets niet naar wens verliep, binnen of buiten het lab. Je betrokkenheid, ook als je geen idee had waar het nou eigenlijk over ging, was een enorme steun. In mijn 3 jaar in Arizona is er veel gebeurd, en juist jij wist de ratio te bewaren op de momenten dat nodig was. Je bent daarom een hoeksteen van dit werk geweest. Ik hou van je en dank je wel voor alles.

Katie, thank you for all the help drafting manuscripts, running assays in your spare time, and your time and effort you put into everything. Thanks for teaching me about seeing a cow every day! For wearing orange and cheering with me during the World Cup, and then cooling down in the cold room. For all the fun and laughs we had, you are a great friend!

Destiny, you made life in the lab so much easier. Thank you for all the ordering, editing, arranging, and patiently listening whenever I was frustrated about something. Which clearly was very rare.. I cherish all the great moments we had, even the dreadful drives to the animal lab every week and the late night experiments (why didn't we peak more convenient time points??). Thank you!

You were undoubtedly the strongest of forces behind the scenes, **Kitsie**. Without your organizational skills not much would have ever gotten done. I remember all the paperwork and administrative issues you had to deal with for me to be able to come, and I thank you for that.

Anthony, my most remarkable dude, we sure had our moments in lab didn't we? You were very welcoming to me from the start (monkey burger, Halloween parties) and I really appreciate you for it. It was great to see you in Holland as well, and I really hope to see you again soon!

Tia, Alice, Jessika, thank you for all the constructive feedback and discussion on work (and life of course..)! It was a pleasure working with you all.

Foeke en Arthur, aan jullie was de (on)dankbare taak van paranimf toebedeeld. Niet alleen zijn jullie beiden afzonderlijk al heel lang in mijn leven, maar met Foeke als voormalig kind aan huis kennen jullie elkaar ook al lang. **Foeke**, we hebben een bestaan in de VS in de zelfde periode met elkaar gehad, en beiden werkend aan een onderzoek. Dat leverde vele telefoontjes op waarbij we allebei onze verbazing en ergernissen breeduit hebben kunnen delen, maar toch ook de mini succesjes binnen het lab hebben besproken. Met natuurlijk een mooi trippie naar Vegas als hoogte- (of diepte-?) punt. Dank voor je hulp in alles!

Arthur, grote, kleine broer en bovenal grote vriend, wat fijn dat je 2 keer bij mij op bezoek bent geweest in de jaren dat ik je erg heb gemist. We hebben toch wel een aantal mooie dagen meegemaakt, van pool parties (start: 09.00 AM) tot schieten met geweren in de woestijn. Dank je wel dat je paranimf bent, dat vind ik heel bijzonder.

Lieve **mama**, de eerste keer moest je nog een beetje wennen aan de hitte en de lege vlaktes, maar de tweede keer was je toch wel overtuigd! Het was heel fijn om je ook in Amerika bij

me in de buurt te hebben gehad. De prachtig geposeerde foto´s van de pink jeep tour zijn een mooie herinnering. Ik hou van je.

Margot, lieve zus, om de paar dagen hadden we een Skype sessie terwijl ik aan het ontbijt zat. Met Boris die iedere keer weer een volledige rondleiding van mijn huis (inclusief inhoud van ALLE kastjes) wenste. Aan de andere kant van de wereld zitten voelde nooit zo dicht bij huis! Door alle omstandigheden heb je helaas nooit langs kunnen komen, maar we houden het vol: we gaan nog een keer naar Arizona en dan laat ik je alsnog alles daar zien!

Rob, Danny, Chris, Alex, a.k.a. 'dem boyz', I'm pretty sure the sales at Trident must have halved after we left Tucson. We made a lot of great memories. Thank you for a lot of things: celebrating Thanksgiving like a real American (dinner starts at 2pm!), understanding that ball is life, a bizarre day at the Waste Management Open Golf, many tailgates, but most importantly: thanks for ruining my lunch!

Adriaan, Maarten, Frank, Benjamin, Sander, Koen (2x!!), jullie hebben allemaal mogen proeven van de zoete nectar die het leven in Arizona heet. Voor de een smaakte het misschien beter dan voor de ander. Desalniettemin: dank jullie wel dat jullie mij zijn komen opzoeken. Het waren stuk voor stuk bijzonder fijne bezoekjes, een onderstreping van onze vriendschap en ik waardeer dat enorm.

Last but not least: lieve **Sophie**, jij verdient een bijzonder dankwoord. Tijdens mijn tijd in Arizona 'ontmoetten' wij elkaar, en toen ik terug kwam in Nederland, zijn we elkaar ook niet meer uit het oog verloren. Dat proefschrift zou ik zo wel even in een paar maandjes tijd schrijven, toch? Het duurde allemaal iets langer, en de vele avonden en weekenden dat ik weer achter mijn computer ging zitten stelden jouw geduld soms op de proef. Maar zonder jouw steun en motivatie had ik de moeilijke periode van het schrijven niet kunnen volbrengen. Ik ben je eeuwig dankbaar voor alles wat je me in het leven hebt gegeven. Ik hou van je.

LIST OF PUBLICATIONS

Published

Schipper DA, Marsh KM, Ferng AF, Duncker DJ, Laman JD, Khalpey Z.
'The critical role of bioenergetics in donor cardiac allograft preservation'
Journal of Cardiovascular Translational Research; 2016 Jun;9(3):176-83

Schipper DA, Louis AV, Dicken D, Johnson K, Smolenski R, Black S, Garcia JM, Khalpey Z.
'Improved metabolism and redox state with a novel preservation solution: implications for donor lungs after cardiac death (DCD)'
Pulmonary Circulation; 2017 Apr-Jun;7(2):494-504

Schipper DA, Palsma R, Marsh KM, O'Hare C, Dicken D, Kazui T, Johnson K, Smolenski T, Duncker DJ, Khalpey Z.
'Chronic myocardial ischemia leads to loss of maximal oxygen consumption and complex I dysfunction'
Annals of Thoracic Surgery; 2017 Oct;104(4):1298-1304

Marsh KM, **Schipper DA**, Ferng AS, Johnson K, Fisher J, Knapp SM, Dicken D, Khalpey Z.
'Metabolic Impact of Rapamycin (Sirolimus) and B-Estradiol using Mouse Embryonic Fibroblasts as a model for Lymphangioleiomyomatosis'
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Ferng AS, **Schipper DA**, Connell AM, Marsh KM, Knapp S, Khalpey Z.
'Novel vs Clinical Organ Preservation Solutions: Improved Cardiac Mitochondrial Protection'
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'The evidence for pharmacologic treatment of neuropathic cancer pain'
Journal of Pain and Symptom Management; 2013 Oct;46(4):581-590

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'Adipose-derived human stem/stromal cells: comparative organ specific mitochondrial bioenergy profiles'
Springerplus; 2016 Dec 1;5(1):2057

Marsh KM, Ferng AS, Pilikian T, Desai A, Avery R, Friedman M, Oliva I, Jokerst C, **Schipper DA**, Khalpey Z.
'Anti-inflammatory properties of amniotic membrane patch following pericardiectomy for constrictive pericarditis'
Journal of Cardiothoracic Surgery; 2017 Jan 26;12(1):6

Teves JMY, Bhargava V, Kirwan KR, Corenblum M, Justiniano R, Wondrak GT, Annadurai A, Flores A, **Schipper DA**, Khalpey Z, Sligh J, Curiel-Lewandrowski C, Sherman S, Madhavan L
'Parkinson's disease skin fibroblasts display signature alterations in growth, redox homeostasis, mitochondrial function and autophagy'
Frontiers in Neuroscience; 2018 Jan 12;11:737

Submitted/in preparation

Schipper DA, Bursey J, Bashar A, Danielson H, Pilikian T, Tran P and Khalpey Z
'Metabolic shift following mechanical circulatory device implantation in heart failure patients'

Schipper DA, Premyodhin N, Ashbeck JD, Johnson K, Kovacs KL, Skaria RS, Pilikian TR, Laman JD and Khalpey Z
'Micronized human amniotic stem cell matrices improve panel reactive antibody (PRA) against HLA-I and II in patients undergoing left ventricular assist device placement (LAVD)'

Schipper DA, Hallmark B, Marsh KM, Johnson K, Duncker DJ and Khalpey Z
'Reduced post-operative atrial fibrillation following amniotic membrane patch placement'

Posters and abstracts

Schipper DA, Bashar A, Danielson H, Son T, Tran P, Slepian M, Khalpey Z.
'Comparative metabolism in patients with heart failure and mechanical circulatory devices'
Oral presentation ISRBP 22nd annual meeting, San Francisco, CA. September 25-27, 2014

Schipper DA, Khalpey Z.
'Organ preservation & 3D printing'
Oral presentation 'Spotlight on Science 2015', The Gregory School, Tucson, AZ. February 18, 2015.

Schipper DA, Marsh K, Ferng A, Medina A, Runyan R, Neilson A, Konhilas J, Johnson K, Khalpey Z
'Amniotic stem cells as donors of bioenergy in the heart'

Poster presentation Keystone symposium 'Heart diseases and regeneration: insights from development (X1)', Copper Mountain, CO. March 1-6, 2015.

Schipper DA, Louis A, Dicken D, Johnson K, Michelotti G, Smolenski T, Black S, Garcia J, Khalpey Z
'Improved Metabolism & Redox State with a Novel Preservation Solution: Implications for Donor Lungs after Cardiac Death (DCD)'
Poster presentation, Resident Research Symposium, University of Arizona, Department of Surgery. May 18, 2016.

Schipper DA, O'Hare C, Palsma RP, Dicken D, Kazui T, Khalpey Z
'Ischemic patients undergoing cardiac surgery have significant complex I dysfunction'
Oral presentation International Academy of Cardiology, 21st World Congress on Heart Disease, Boston, MA. July 30-August 1, 2016.

Schipper DA, Louis A, Dicken D
'*Animal and Comparative Biomedical Science 102: Introduction to Animal Science Laboratory*'
Lectures, University of Arizona Food Products and Safety Laboratory. February 29-March 2, 2016.

Schipper DA
'The pivotal role of mitochondria in cardiac ischemia reperfusion injury'
Scientific meeting, department of cardiothoracic surgery, Academic Medical Center (AMC) Amsterdam. January 17, 2018.

Awards and funding

The Dutch Heart Foundation
Student's grant, 2014

Seahorse Bioscience
Travel Award, 2015

Stichting Bekker-la Bastide-Fonds
Scientific stipend, 2018

Stichting Hippocrates Studiefonds
Hippocrates study award, 2019

CURRICULUM VITAE

David Alexander Schipper was born on August 12, 1986 in Rotterdam, the Netherlands. He attended Erasmiaans Gymnasium high school and moved to Amsterdam, while obtaining his Bachelor's degree in medicine at the Erasmus University, Rotterdam. In the fall of 2013, David was offered an exchange student position at dr. Zain Khalpey's laboratory at the University of Arizona in Tucson, AZ, under supervision of prof. Jon Laman. After having gained extensive laboratory experience, David was offered to fulfil the position of research specialist under the guidance of dr. Zain Khalpey. At the same time, prof. dr. Dirk-Jan Duncker invited him to enter the PhD program at the department of experimental cardiology at Erasmus University, making this a collaborative appointment. The Khalpey lab focused on clinically translational research, in order to help as many patients with in vitro, ex vivo and ultimately in vivo approaches. During his time in Tucson, David presented his work at several scientific conferences, including the International Academy of Cardiology conference in 2016 and the Keystone symposium on Heart disease and regeneration in 2015. David was awarded the Hippocrates price in 2019 for his scientific work, as well as funding from Bekker-la Bastide-fonds and the Dutch Heart Foundation.

After finishing his laboratory work in the summer of 2016, David returned to the Netherlands to obtain his Master's degree in medicine and continue to publish his scientific work. He is currently a cardiology intern at the Erasmus MC and aims to apply for cardiology residency in the foreseeable future. He is married to Sophie Schipper-Bargmann, and together they have a son, Raphael.

PH.D PORTFOLIO

Congres	**ECTS**
ISRBP	1
Gregory school	0,3
Keystone	0,6
U of A Resident symposium	0,4
IAC Boston	1
AMC Amsterdam	0,2
Onderwijs krijgen	
Seahorse advanced course	1,2
Cardiac research labs lectures	3
Onderwijs geven	
Begeleiding student	5
Biomed science 102	1
Cardiac research labs lectures	1,5
Training lab techs	4
Totaal	**19,2**

LIST OF ABBREVIATIONS

AF	- atrial fibrillation
AMP	- amniotic membrane patch
ALM	- adenosine lidocaine magnesium
ATP	- adenosine-tri-phosphate
BTT	- bridge-to-transplant
CABG	- coronary artery bypass grafting
DCD	- donated after cardiac death
DT	- destination therapy
ETC	- electron transport chain
IRI	- ischemia reperfusion injury
LV	- left ventricle
LVAD	- left ventricular assist device
mPTP	- mitochondrial permeability transition pore
mtDNA	- mitochondrial DNA
OCR	- oxygen consumption rate
ORP	- oxidation reduction potential
OXPHOS	- oxidative phosphorylation
PCI	- percutaneous coronary intervention
POAF	- post-operative atrial fibrillation
PRA	- panel-reactive antibodies
ROS	- reactive oxygen species
RCR	- respiratory control rate
REE	- resting energy expenditure
TAH	- total artificial heart
VAD	- ventricular assist device