DEVELOPMENT OF A NON-DEPOLARISING NORMOKALAEMIC CARDIOPROTECTIVE PRESERVATION SOLUTION FOR USE IN ORTHOTOPIC HEART TRANSPLANTATION

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Declaration

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DECLARATION OF ETHICS

The research presented and reported in this thesis was conducted within the guidelines for research and ethics outlined in the *National Statement on Ethics Conduct in Research Involving Human* (1999), the *Joint NHMRC/AVCC Statement and Guidelines on Research Practice* (1997), the *James Cook University Policy on Experimentation Ethics Standard Practices and Guidelines* (2001), and the *James Cook University Statement and Guidelines on Research practice* (2001). Relevant research methodology reported in this thesis received clearance from the James Cook University Experimentation Ethics Review. Animal Ethics numbers A781, A1084 and A1515.

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Signature Date

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ABSTRACT

Background

Since the 1970s preservation solutions for donor hearts for transplantation have relied on hypothermic depolarising potassium to arrest and protect the heart. Despite many studies investigating improvements in composition, no preservation solution has been able to provide adequate protection of the human donor heart beyond 4 – 6 hours. Suboptimal preservation and use of marginal hearts, from a diminishing donor pool, have both been linked to poor clinical outcomes. In 2004, our laboratory developed a new normokalaemic polarising cardioplegia, comprising adenosine and lignocaine (AL) for use at warmer temperatures (28 - 30°C). Subsequent studies have reported superior functional recoveries in the isolated rat heart when compared with depolarising St Thomas solution. However, the efficacy of AL cardioplegia for use at colder temperatures has not yet been investigated. The aim of this research was to develop a normokalaemic AL cardioplegic solution for use during donor heart harvest, cold storage and implantation, to possibly extend safe cold storage times to 8 hours. The performance of this normokalaemic AL preservation solution was compared with gold standard heart preservation solutions, Celsior and Histidine Tryptophan Ketoglutarate solution (Bretschneider's solution, HTK or Custodial).

Methods and Experimental Design

All studies employed the isolated working rat heart following 2, 6 and 8 hours of cold storage. Primary endpoints include aortic flow, coronary flow, cardiac output, heart rate, rate pressure product, systolic and diastolic pressures. Metabolic studies include the determination of lactate levels, oxygen consumption rates, pH and troponin T release.

Experimental Design

The first of the studies presented here investigated the effect of temperature on AL preservation. Functional recoveries of hearts stored in AL solution for 6 hours (4°C and 28 -30°C) were compared to those stored in Celsior. The second study was designed to evaluate a new oxygenated AL arrest/reperfusion strategy. Hearts stored in AL solution or Celsior (4°C) for 6 hours were rewarmed with oxygenated AL arrest/reperfusion strategy, and their functional recoveries were compared to hearts rewarmed in Celsior solution or Krebs Henseleit alone. The third study investigated modifications to the AL solution. Various calcium, magnesium, adenosine and lignocaine concentrations were tested over 6 hours (4°C) followed by rewarming with the oxygenated AL arrest/reperfusion strategy to determine the best solution for extended storage. The fourth study then combined higher adenosine and lignocaine concentrations (2x AL), low calcium/high magnesium (0.22 mmol/l/2.6 mmol/l) with melatonin (100 μM) and insulin (0.01 IU/ml) for storage of hearts over 8 hours followed by the oxygenated AL arrest/reperfusion strategy.

Results

Following 6 hours of cold storage (4°C) AL hearts recovered 68 ± 5% of pre-arrest cardiac output compared to 47 ± 14% for hearts stored in Celsior (4°C) ($p<0.05$). At warmer temperatures (28 - 30°C) AL stored and flushed hearts recovered 55 ± 3% of pre-arrest cardiac output compared to Celsior hearts which failed to recover. These results showed that AL preservation was versatile at both cold and warm temperatures in the working isolated rat heart. The second study, revealed that hearts stored for 6 hours of cold storage (4°C) in AL solution and reperfused with warm oxygenated AL arrest/reperfusion strategy recovered a significantly higher cardiac

output (66 ± 4%) compared with those reperfused with warm oxygenated Krebs-Henseleit vehicle (45 ± 2% ; $p<0.05$). Cold Celsior hearts reperfused with warm oxygenated Celsior had 9 times higher effluent lactate values with acidosis (pH 6.5) during the last minute of rewarming compared with all groups, and this was associated with early myocardial, vascular, and electrical stunning. A surprising finding was that reperfusing cold Celsior hearts with warm oxygenated AL arrest/reperfusion strategy improved cardiac output in early reperfusion (first 10 minutes) ($p<0.05$) and reduced stunning. The third study showed that lowering the calcium (0.22 mM) and increasing the magnesium (2.6 mM) of the AL preservation solution for 6 hours cold storage (4°C) of isolated rat hearts followed by warming in oxygenated AL arrest/reperfusion strategy (physiological calcium and magnesium) resulted in improved functional recoveries. The cardiac output (74%) at 60 mins reperfusion was significantly better than rewarming in AL solution with low calcium/high magnesium (63%) or Krebs Henseleit solution alone (53%)($p<0.05$). Interestingly this strategy also provided significantly better recoveries in early reperfusion (10 to 30 minutes), than storing hearts in AL solution with physiological levels of calcium and magnesium. Doubling the adenosine and lignocaine concentrations in the presence of low calcium/high magnesium followed by reperfusion in warm oxygenated adenosine and lignocaine with low calcium/high magnesium arrest/reperfusion strategy further improved functional recoveries. Recovery of aortic flow improved by 15% (54 to 69%, $p<0.05$), cardiac output by 12% (63 to 75%, $p<0.05$) and there were equivalent recoveries of coronary flow. In the fourth study, hearts preserved for 8 hours in AL preservation solution with low calcium/high magnesium returned 55% CO, 101% HR, and 90% to 105% systolic and diastolic pressures following 8 hours of cold storage (4°C). Combining this

strategy of 2x adenosine and lignocaine concentrations with low calcium/high magnesium, melatonin (100 μmol/L) and insulin (0.01 IU/ml), for an extended arrest time of 8 hours resulted in an increased return of cardiac output (78%) with no detectable troponin T (<0.03 mg/ml). In contrast, HTK (Custodial) hearts returned 4% cardiac output, 16% heart rate, and 11% to 17% developed pressures, and troponin T was 0.13 mg/ml in effluent 5 minutes after the rewarm. Celsior hearts, despite an 86% return of heart rate, also failed to return sufficient left ventricular function (~10%) with a return of 25% cardiac output and a troponin T level of 0.24 mg/ml.

Conclusions

AL cardioplegia is versatile for both cold (4°C) or warm (28 - 30°C) storage of the isolated rat heart for up to 6 hours. Hearts stored in AL generated significantly higher aortic flow, coronary flow and cardiac output than than those stored in Celsior under the same conditions. Furthermore, reperfusing and rewarming isolated rat hearts with the oxygenated AL arrest/reperfusion strategy significantly improved functional recoveries of hearts stored for 6 hours in cold adenosine and lignocaine solution and reduced stunning in the Celsior stored hearts. Clinically this finding may be of value for providing improved cardioprotection of the donor heart through the cold to warm transitions during implantation and reperfusion.

Doubling the adenosine and lignocaine concentrations of the AL cardioplegia, lowering the calcium concentration to 0.22 mmol/l and increasing the magnesium concentration to 2.6 mmol/l, further improved the aortic and coronary flowsfollowing 6 hours of cold static storage; thus implying improved myocardial

and coronary vascular recovery. Extended storage (8hours) of hearts in the modified AL solution augmented with melatonin and insulin, significantly improved recoveries of hearts (78% of cardiac output and no troponin T release) when compared to hearts stored in both Celsior and HTK (Custodial) which recovered less than 21% of their pre-arrest cardiac output. In conclusion, the new AL preservation solution combined with oxygenated AL arrest/reperfusion strategy may provide a new paradigm for the donor heart harvest, storage through to implantation.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

Units	
bpm	Beats per minute
Da	Daltons
g	Grams
mg	Milligrams
L	Litre
ml/min	Millilitres per minute
mmol/l	Millimole per litre
mM	Millimolar
M	Molar
nM	Nanomolar
µM	Micromolar
mm/Hg	Millimetres of mercury
Abbreviations/Acronyms	
ΔG	Gibbs free energy
ADP	Adenosine di phosphate
AF	Aortic flow
αO_2	Bunsen solubility coefficient
AL	Adenosine and lignocaine cardioplegic solution
ANT	Adenine nucleotide transporter
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
AV	Atrioventricular
B_p	Barometric pressure
Ca^{2+}ATPase	Calcium ATPase
CABG	Coronary artery bypass graft
CAV	Cardiac allograft vasculopathy
CF	Coronary flow
CMV	Cytomegallo Virus
CO	Cardiac output
CPD	Citrate phosphate dextrose
CVR	Coronary vascular resistance
DP	Diastolic pressure
EC	EuroCollins Solution
Em	Resting membrane potential
FDA	US Food and Drug Administration
HIF-1	Hypoxia inducible factor-1
HES	Hydroxyl ethyl starch
Hrs	Hours
HR	Heart rate
HTK	Histidine tryptophan ketoglutarate or Custodial
ICAM-1	Intracellular adhesion molecule-1
IPC	Ischaemic Preconditioning
IRI	Ischaemic reperfusion injury
K_{ATP}	ATP sensitive potassium channels

KCl	Potassium chloride
KH	Krebs Henseleit solution
LDH	Lactate dehydrogenase
MAP Kinase	Mitogen activate protein kinases
MHC	Major histocompatibility complex
MI	Myocardial infarction
Mins	Minutes
Mito	Mitochondrial
Mito K_{ATP}	Mitochondrial ATP sensitive potassium channel
MMP	Matrix metallo proteinase
MPTP	Mitochondrial permeability transition pore
MVO_2	Myocardial oxygen consumption
NAD^+	Nicotine adenine di nucleotide
NADH	Nicotine adenine di nucleotide hydride
Na^+/Ca^{2+} exchanger	Sodium /calcium exchanger
Na^+/H^+ exchanger	Sodium/hydrogen exchanger
$Na^+/K^+/2Cl^-$ cotransporter	Sodium, potassium, chloride co-transporter
NHBD	Non Heart-Beating Donor
NO	Nitric oxide
P_aO_2	Arterial partial pressure of oxygen
Pi	Inorganic phosphate
P_vO_2	Venous partial pressure of oxygen
PDH	pyruvate dehydrogenase
PEG	Poly ethylene glycol
PGF	Primary graft failure
PKC	Protein kinase C
PTCI	Percutaneous coronary intervention
Pyr	L-pyruvate
ROS	Reactive oxygen species
RPP	Rate pressure product
Sarc K_{ATP}	Sarcolemmal ATP sensitive potassium channel
SEM	Standard error of mean
SP	Systolic pressure
STH	St Thomas hospital solution No. 2
STP	Standard temperature and pressure
SV	Stroke volume
SVT	Supra ventricular tachycardia
%TTW	Percent total tissue water
TTX	Tetrodotoxin
VDAC	Voltage dependent anion channel
V_p	Water vapour pressure

CHAPTER 1: INTRODUCTION

Heart transplantation as a treatment for patients in end stage heart failure has been an outstanding success. Today over 80% of patients undergoing heart transplantation will survive at least one year and most will return to normal activity levels (Excell *et al.*, 2004; Taylor *et al.*, 2009). Cardiac transplantation, however, is facing unprecedented challenges and pressures from donor heart shortages and the use of marginal donors (Jamieson and Friend, 2008). Suboptimal cardioprotection strategies used during the harvest and storage of these marginal hearts lead to ischaemia reperfusion injury (IRI) upon implantation and acute and chronic rejection in the recipient (Jahania *et al.*, 2000; Russo *et al.*, 2010).

Despite the availability of over 167 different solutions and a myriad of studies demonstrating beneficial combinations and additives, the safe storage time for human hearts remains at 4 - 6 hours (Demmy *et al.*, 1994). Most preservation solutions arrest and store the donor heart in a hypothermic hyperkalaemic environment (Chambers, 1999). Hyperkalaemic arrest and preservation solutions depolarise the myocyte and endothelial cell membrane from -83 mV to around -30 mV (Chambers, 1999); this leads to ongoing cellular processes which ultimately cause myocyte damage. Effects from damage to the myocardial tissue include microvascular injury, coronary vasoconstriction and spasm, arrhythmias and ventricular stunning (Attwell *et al.*, 1979; Sellke *et al.*, 1993b; Askenasy, 2001).

In 2004, Dobson and Jones reported superior recovery of isolated rat hearts following 2 and 4 hour warm arrest with intermittent flushes with a novel normokalaemic cardioplegic arrest solution, containing adenosine and lignocaine, when compared to a traditional hyperkalaemic depolarising cardioplegic solution (Dobson, 2004). The aim of the studies presented in this thesis was to continue the development of a normokalaemic adenosine and lignocaine cardioplegic solution for donor heart harvest, cold storage and implantation in an effort to extend the cold storage times to 8 hours. The AL preservation solution will be compared with gold standard heart solutions, Celsior and HTK (Bretschneider's solution or Custodial).

The overall aims, study design and goals of the thesis are presented in table 1.1 below.

Table 1.1: Overall aims, study design and goals of the thesis

Study 1	**Aim** To investigate the effect of AL cardioplegia following 6 hours at both warm and cold temperatures. **Study Design** Hearts will be arrested for 6 hours in warm intermittent (28 – 30 °C; 2 mins flush every 18 mins) and cold static storage (4°C) and compared with Celsior solution. L-pyruvate will be tested as a cardioprotectant. **Goal** To establish the efficacy of AL cardioplegia for cold static storage in rat hearts.
Study 2	**Aim** To investigate the effect of rewarming cold hearts following storage with oxygenated AL arrest/reperfusion strategy. **Study Design** Cold hearts will be reperfused with warm oxygenated AL arrest/reperfusion solution following 6 hours static storage (4°C) in either AL cardioplegia or Celsior solution. Functional recovery will be compared with reperfusing with oxygenated modified Krebs Henseleit (KH) and Celsior. **Goal** To establish a new reperfusion and rewarming paradigm for heart implantation.
Study 3	**Aim** To test the effect of lowering Ca^{2+} and increasing Mg^{2+} and the effect of increasing AL concentrations in the presence of low Ca^{2+} /high Mg^{2+} along with the new oxygenated AL arrest/reperfusion strategy. **Study design** The effects of low Ca^{2+} and high Mg^{2+} (0.22 mM/2.6 mM) and increased AL concentrations over 6 hours cold static storage followed by the oxygenated AL arrest/reperfusion strategy will be investigated. **Goal** To modify the AL cardioplegia for extended cold static storage times.
Study 4	**Aim** To investigate the effect of the addition of melatonin and insulin to the modified AL solution over 8 hours of cold static storage and compare this with Celsior and HTK (Custodial). **Study Design** Optimized AL concentrations (2xAL) and low Ca^{2+}/ high Mg^{2+} will be combined with 100 μM melatonin and 0.01 IU/ml insulin and functional recoveries of hearts stored for 8 hours followed by the oxygenated AL arrest/reperfusion strategy will be compared with the functional recoveries of hearts arrested and reperfused in Celsior and HTK (Custodial). **Goal** To evaluate the new AL cold preservation solution and AL oxygenated arrest reperfusion as an approach towards therapeutic suspended animation.

CHAPTER 2: LITERATURE REVIEW

> *The cell is immortal. It is merely the fluid (water) in which it floats that degenerates. Renew this fluid at intervals, give the cells what they require for nutrition and, as far as we know, the pulsation of life may go on forever.*
>
> *Alexis Carrel (1912)*

2.1 Heart Transplantation: A Short History

The history and development of heart transplantation has been an exciting journey involving scientific and surgical innovation, technology and discovery (DiBardino, 1999). In a little over a century, the field has grown from an idea into practice through the work of a number of pioneers such as Alexis Carrel, Charles Guthrie, Charles Lindbergh, Frank Mann, Wilfred Bigelow, Norman Shumway, C. Walton Lillehei, Richard Lower and Christian Barnard (Barnard, 1968). Barnard is well known for performing the first human-to-human transplant in Cape Town, South Africa in 1967 (Barnard, 1968). However, the first heart transplant would not have been possible if it were not for the surgical techniques developed by Alexis Carrel, who, in the late 19th century and early 20th century, developed a triangulation technique for vascular anastomoses that could be used to successfully join the donor and recipient blood supplies (Dutkowski *et al.*, 2008). During this time, Carrel and his colleague Charles Guthrie performed a series of transplants including the first canine heterotrophic heart transplant (Carrel, 1905; Carrel, 1912b). Carrel also investigated strategies of organ and tissue preservation from 1906 to 1912 which are

summarised in his 1912 paper entitled "The preservation of tissues and its application in surgery" (Carrel, 1912a). Alexis Carrel received the Nobel Prize in Physiology and Medicine in 1912 in recognition for his work on vascular suture and the transplantation of blood vessels and organs, which not only provided proof-of-principle for future transplantation research but anticipated many of the basic science and clinical discoveries that were to come in the 20th century.

2.1.1 From animal experimentation to clinical experimentation

The next major advance in the field of heart transplantation occurred in 1933 where Mann and colleagues simplified the anastomoses of Carrel and colleagues to facilitate coronary perfusion in two heterotropic models, auto transplantation and homo-transplantation. Mann also anticipated the problem of rejection by commenting that the reasons for graft failure appeared to be biological rather than technical (Mann *et al.*, 1933). The work of Russian surgeon Vladimir Denikhov who performed the world's first animal heart and lung transplants in the 1940s and 50s was also a contributor although his work went unknown to the western world until it was published in the 1962 (Shumacker, 1994).

2.1.2 Clinical experimentation to translation into practice

In the 1950s major advances in cardiac and transplant surgery were steered by Wilfred Bigelow, Norman Shumway, C. Walton Lillehei and Richard Lower. Hypothermia and temperature control, a key factor in organ protection, was pioneered by Bigelow and later utilised by Shumway, Lillehei and Lower (Bigelow *et al.*, 1950; Lower *et al.*, 1962; Lillehei *et al.*, 1964). Perfusion devices were also being developed culminating in the first heart-lung bypass machine by Gibbon in

1954 (Gibbon, 1954) which helped to overcome the detrimental effects of circulatory arrest and allowed surgeons to work in a bloodless operating field while the systemic circulation was maintained. High potassium citrate cardioplegia was developed and the first warm blood high potassium arrest was performed in 1955 on a dog by Melrose and colleagues (Melrose *et al.*, 1955). Unfortunately, potassium citrate was soon found to be damaging to humans and there was a 15 year moratorium on its use until the late 1960s.

2.1.3 Early clinical successes to dampened enthusiasm

In the 1960s there was great excitement in the field of transplantation culminating in the first human heart transplant by Barnard's team in 1967 in South Africa (see above). A month later, the first heart transplant in the US was performed by Norman Shumway (Hunt and Haddad, 2008), and Donald Ross performed UK's first transplant at the National Heart Hospital, Marylebone on 3rd May 1968. In Australia, the first heart transplant was performed in 1968 at St Vincent's hospital Sydney by Dr Harry Windsor (Windsor, 1969). However, like the early problems with clinical adoption of high potassium cardioplegia in cardiac surgery, the initial enthusiasm for the procedure was dampened by major post-operative complications and allograft rejection (Hunt, 2006).

2.1.4 Dampened enthusiasm to a successful treatment

Poor outcomes led to the search for immunosuppressive agents, and the next turning point was Borel's 1972 discovery of cyclosporine (Borel and Kis, 1991). In the 1980s the introduction of cyclosporine partially solved the problem of rejection with improved survival rates and this allowed for a resurgence of interest in

transplantation of all organs (Kirklin *et al.*, 2004). Over the years as the surgical technique, basic immunology and post transplant management strategies have been refined; heart transplantation has matured into a successful treatment for end-stage heart failure.

Today there are over 100,000 solid organ transplants performed worldwide each year (Taylor *et al.*, 2007). Of these over 70,000 are kidney transplants, over 20,000 are liver transplants and approximately 5000 are heart transplants. Expected survival following cardiac transplant is on average 13 years with a half life of 10.0 years (Taylor *et al.*, 2009), whilst survival for patients with end stage disease without a transplant is 8% after 2 years (Rose *et al.*, 2001; Levy *et al.*, 2002).

2.2 Problems Currently Facing Heart Transplantation

The extraordinary advances in cardiac transplantation over the past four decades and its success as a treatment for end stage heart disease has meant that there are growing waiting lists for organs (Jamieson and Friend, 2008). In 2009, it was estimated that over 100,000 patients were on waiting lists for organ transplant and of these over 3000 were awaiting a heart transplant (United Network for Organ Sharing, 2008). However, the number of heart transplant procedures performed is limited by the availability of viable organs.

As donor shortages worsen there has been an increase in waiting lists and waiting list deaths. In the USA the transplant waiting list mortality remains high at 17% per year for adults, 45% for status 1 patients and 25% for children (Zaroff *et al.*, 2002; Herrington and Tsirka, 2004; Magee, 2004; Boucek *et al.*, 2008); the statistics are similar for Australia (Excell *et al.*, 2009).

2.2.1 Donor shortages leading to the use of marginal donors

In response to donor shortages, a reappraisal of donor selection criteria (Rosendale *et al.*, 2002; Eisen, 2004; Excell *et al.*, 2009) has resulted in a growing consensus to include the use of marginal donors: those smaller in size than the recipient; those affected by coronary artery disease; those with left ventricular dysfunction and those of an older age group. Where previously donors were restricted to an age less than 35 years, now organs are accepted from donors up to and over fifty years of age (Rosendale *et al.*, 2002; Zaroff *et al.*, 2002). The use of hearts from extended criteria donors has resulted in an increased number of organs discarded due to poor organ function or biopsy findings (Rosendale *et al.*, 2002). In fact an American study reported that during the years 1995 to 2000, over 5000 (17%) donated hearts were discarded (Rosendale *et al.*, 2002; Zaroff *et al.*, 2002).

The benefit of using hearts from marginal or older donors has been widely debated (McCarthy *et al.*, 1998; Argenziano *et al.*, 1999; Abouna, 2004; Lima *et al.*, 2006; Russo *et al.*, 2007). McCarthy and colleagues (1998) found in a small single centre study that the use of marginal donors did not appear to diminish the outcomes for recipients. In contrast, Russo and colleagues (2007) in a larger study found that transplanted older donor hearts were more susceptible to ischaemic damage and carried a greater risk of early graft failure and death. However, the risk of poorer outcomes following the use of marginal organs must been weighed up against the risk of death while waiting for transplantation (Lietz *et al.*, 2004).

If the number of viable organs used for transplantation could be increased then more patients could be given a chance at a new life (Abouna, 2004); however, use of these

organs has been associated with poorer outcomes such as primary graft failure and acute and chronic rejection (Eisen, 2004).

2.2.2 Primary graft failure and chronic allograft vasculopathy: Still limit the success of heart transplantation

2.2.2.1 Primary graft failure

Primary graft failure (PGF) has been identified as the most frequent postoperative complication in both adult and pediatric transplant patients (Jahania *et al.*, 2000; Aliabadi *et al.*, 2007; Russo *et al.*, 2007; Zuppan *et al.*, 2009; Russo *et al.*, 2010). It accounts for almost 40% of deaths in adults (Lima *et al.*, 2006; Taylor *et al.*, 2007), 30% in children (Huddleston, 2000) and 5 – 10% of all heart transplantation recipients in the first 30 days (Jahania *et al.*, 2000; Taylor *et al.*, 2007). Figure 2.1, taken from the International Society for Heart and Lung Transplantation (ISHLT) 2009 statistics, shows that primary graft failure is among the leading causes of death following heart transplantation.

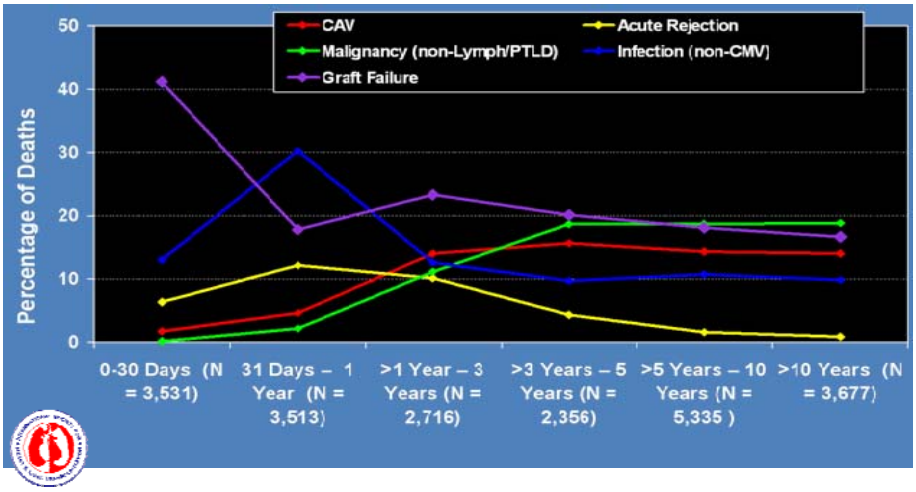

Figure 2.1: Leading causes of death post transplantation (Jan 1992 – June 2008) International Society for Heart and Lung Transplantation (ISHLT, 2009).
During the first 30 days graft failure, infection (Cytomegallo virus) and acute rejection are the leading causes of death following heart transplantation. Infection and primary graft failure lead during the first year. After five years chronic allograft vasculopathy (CAV) and malignancy (non-lymphoproliferative and post transplant lymphoproliferative disorders (PTLD).

In the absence of immunological and anatomical evidence for failure, primary graft failure is characterised by prolonged post ischaemic ventricular dysfunction or myocardial stunning (Jahania *et al.*, 2000). The severity of primary graft failure appears to be dependent on the type and severity of the ischaemic insult and can persist for 7 – 10 days post-transplant despite adequate coronary flow (Jahania *et al.*, 2000; Kirklin *et al.*, 2004). Myocardial stunning, one of the most common causes of graft failure, is often associated with the haemodynamic changes following brain death and subsequent ischaemia (Obadia *et al.*, 1997). Donor organ injury comes from inadequate protection of the donor organ during procurement (pre-harvest, harvest, storage and implantation), organ rejection and various donor-recipient-related factors (Jahania *et al.*, 2000);(Valantine, 2003);(Allen, 2004);(McCrystal *et al.*, 2004);(Rahmani *et al.*, 2006);(Stoica *et al.*, 2006);(Rudd and Dobson, 2009).

2.2.2.1.1 Risk factors for developing primary graft failure

Risk factors for primary graft failure have not been characterised (Lima *et al.*, 2006). However, emerging evidence includes; donor-related factors and pre-harvest drug treatments, such as inotrope use to improve neurohormonal haemodynamic deficits occurring during brain death, and subsequent ischaemia (Kirklin *et al.*, 2004; Santise *et al.*, 2009; Iyer *et al.*, 2011). The incidence of post transplantation PGF could be exacerbated by an increased use of organs from marginal donors (Oto *et al.*, 2008). A number of studies have investigated the use of marginal donors, and their contribution to the incidence of primary graft failure is still undecided. Lima and colleagues (2006), for example, found that organs from marginal donors did not show an increased incidence of primary graft failure. Interestingly, however, they found that prolonged ischaemic times (>4.5 hrs) were a more accurate indicator of

the probability for developing primary graft dysfunction. This factor has also been highlighted by other studies. In a retrospective study, Russo and colleagues (2007) found that prolonged ischaemic times particularly in older donor hearts were an independent risk factor for developing primary graft failure.

2.2.2.2 Cardiac allograft vasculopathy

Along with primary graft failure, an accelerated form of coronary artery disease known as cardiac allograft vasculopathy (CAV) limits the long-term success of heart transplantation (see Figure 2.1) (Kirklin *et al.*, 2004; Perrault and Carrier, 2005; Schmauss and Weis, 2008). CAV affects the entire coronary tree in a diffuse fashion, leading to coronary endothelial dysfunction, injury and eventual graft failure (Hollenberg *et al.*, 2001; Perrault and Carrier, 2005). According to recent statistics, CAV is detectable in 8% of heart transplant recipients surviving one year and in 32% and 43% of those surviving five and eight years respectively. Survival following detection of CAV is diminished (Taylor *et al.*, 2007; Taylor *et al.*, 2009).

2.2.2.2.1 Risk factors for developing cardiac allograft vasculopathy

Factors that are implicated in the development of primary graft failure and CAV occur at multiple points in the transplantation process and result in cumulative endovascular injuries (Schmauss and Weis, 2008). See Figure 2.2 below. These include; factors related to the donor and recipient, including gender and increasing donor age (Eisen, 2004; Kirklin *et al.*, 2004; Taylor *et al.*, 2007; Taylor *et al.*, 2009); rejection and infections (Perrault *et al.*, 1997; Valantine, 2004b); neuro hormonal changes of brain death and mechanical injury during harvest (Valantine, 2003; Schmauss and Weis, 2008). Other factors such as exposure to hyperkalaemic

depolarising cardioplegic solutions (Drinkwater *et al.*, 1995; He *et al.*, 1997), prolonged ischaemic times (Valantine, 2004a) and ischaemia reperfusion injury upon implantation (Murphy *et al.*, 1997; Beyersdorf, 2004).

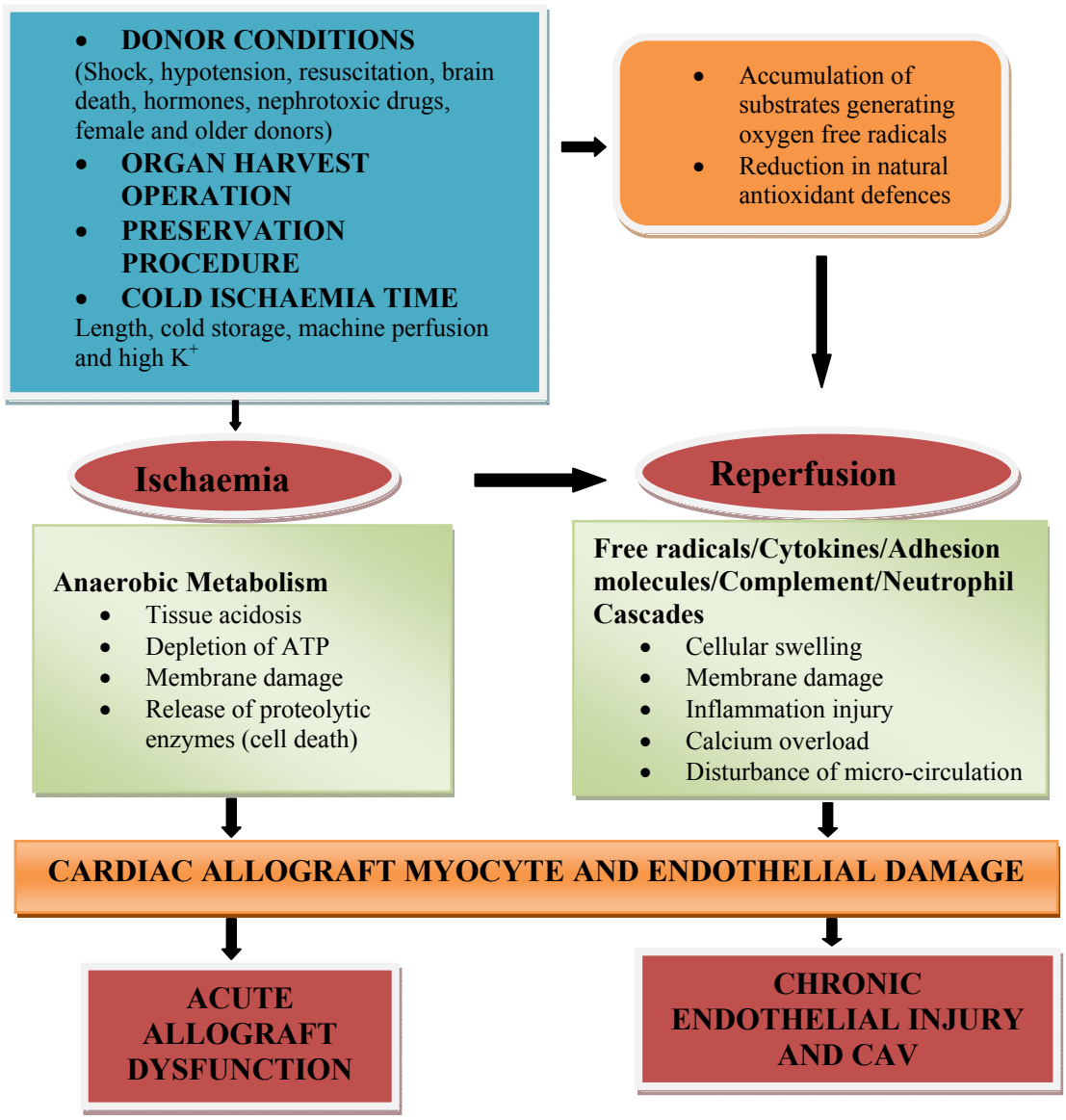

Figure 2.2: Pathogenesis of acute and chronic allograft dysfunction
The damaging effects of ischaemia occuring during harvest and storage of the donor heart are typically manifest in reperfusion. Free radical generation and the depletion of naturally occurring free radical scavengers set the scene for free radical injury and calcium loading. Subsequent myocyte and endothelial damage may allow for acute and chronic allograft dysfunction.
(Modified from (Kirklin and McGiffin, 1999)

2.3 Phases of Cardiac Transplantation: Opportunities for Damage to the Donor Organ

In addition to the major physiological changes that arise from brain death and the drugs administered to the donor patient prior to organ retrieval, the success of the organ transplant may also be affected by the conditions of harvest, retrieval, storage and implantation of the heart (Stoica *et al.,* 2002). The injury sustained is an accumulation of insults suffered during the warm and cold ischaemic periods (Jahania *et al.,* 1999). Techniques for organ preservation serve to minimise this damage and promote graft survival and function (McCrystal *et al.,* 2004).

Damage to the donor heart during transplantation occurs in 3 phases: phase 1) The 'arrest and harvest' or warm ischaemic phase which includes the time from interruption of circulation to the donor heart to the time the heart is flushed with hypothermic preservation solution; phase 2) cold ischaemic phase, while the donor heart is preserved in hypothermic preservation solution until implantation and phase 3) implantation phase, warm ischaemia followed by reperfusion.

A brief history and introduction to the current methods of protecting the heart during these three phases follows.

2.4 Phase 1: Arrest and Harvest of the Donor Heart

Modern practice in cardiac transplantation is to achieve electro-mechanical arrest of the heart using cardioplegia. Most commonly used cardioplegic solutions are designed to arrest the heart at depolarising potentials, provide a bloodless operating field and prevent further damage of the heart (Chambers and Fallouh, 2010).

2.4.1 History of arrest and cardioprotection

2.4.1.1 Development of cardioplegia

2.4.1.1.1 Early experimental studies: Potassium as an arresting agent

The discovery of potassium as a powerful arresting agent for the heart can be traced back to the pioneering work of Ringer during the late 19th century and early 20th century. During his time at University College in London he published seminal papers that described the antagonistic effects of calcium and potassium (Ringer and Sainsbury, 1882; Ringer, 1883; Toledo-Pereyra, 1986; Miller, 2004). Ringer described the effects of incremental doses of potassium on the heart, describing smaller and smaller beats until paralysis of the muscle (Ringer and Sainsbury, 1882).

In 1929, Hooker demonstrated that an infusion of a high potassium solution (1 ml of 2.5% KCl or 336 mM) could resuscitate a canine heart in ventricular fibrillation and which upon washout of the potassium solution could resume normal rhythm (Hooker, 1929). In 1954 Montgomery used a similar approach to defibrillate human hearts (Montgomery *et al.*, 1954) as did Lam and colleagues (Lam *et al.*, 1955) who

used an intraventricular injection of KCl (667 mM) to induce cardiac arrest for heart surgery in dogs.

2.4.1.1.2 Clinical experimentation to dampened enthusiasm

This foundational work, inspired Melrose and colleagues (1955) to introduce the concept of elective reversible cardiac arrest. An intracoronary infusion of potassium citrate (1mg/ml) added to the blood at 37 °C could effectively arrest the heart in diastole. Arrest could be maintained provided the concentration of potassium citrate remained high enough in the coronary arteries; cardiac arrest could be reversed by washout of the solution (Melrose *et al.*, 1955). This animal experimentation led to a standardized method for cardiac arrest which was used clinically (Gerbode and Melrose, 1958; Shiroishi, 1999). As early as 1956, there were reports of unfavourable outcomes following cardiac surgery and frequent dysrhythmias following potassium citrate arrest (Brown *et al.*, 1956; Schramel *et al.*, 1957; Nunn *et al.*, 1959) and in 1957, Allen and Lillehei advised caution with the use of the Melrose technique for elective cardiac arrest in patients with cardiac damage (Allen and Lillehei, 1957).

The Melrose technique was abandoned following the finding of focal areas of necrosis in cardiac tissues on autopsy following several post-operative deaths (McFarland *et al.*, 1960; Cordell, 1995). This delayed progress in the field of cardiac arrest infusions until the mid 1960s. During this time a number groups experimented with techniques, including; continuous coronary perfusion, topical hypothermia with simultaneous aortic cross-clamping, various drugs and electrically induced ventricular fibrillation to avoid damage caused by ischaemia (Bigelow *et al.*, 1950;

Bigelow *et al.*, 1954; Gibbon, 1954; Lillehei *et al.*, 1957; Chambers and Hearse, 2001).

2.4.1.1.3 Renewal of enthusiasm to clinical translation

Gradually however, groups in Europe and America came back to cold chemical cardioplegia containing potassium, which are still in use today. As early as 1957, Around the same time Bretschneider and colleagues (Bretschneider *et al.*, 1975) developed a solution, Bretsneider's solution (HT or Custodial) which stilled the heart because it was sodium poor and calcium free. In the USA, Gay and Ebert (Gay and Ebert, 1973) demonstrated that a solution containing 25 mmol/L potassium, could afford good cardioprotection for up to one hour in dog hearts. Roe and colleagues then followed with the development of Roe's solution (Roe *et al.*, 1977) containing a potassium concentration of 20 mmol/L. In 1976, Hearse and colleagues (Hearse *et al.*, 1976), developed St Thomas Hospital Solution no.1 after a thorough investigation of ischaemic arrest and reperfusion in the isolated rat heart model. In 1978, Gerald Buckberg and his group (Follette *et al.*, 1978) reported increased recoveries after modifications of the calcium, pH, potassium and osmolality of the blood cardioplegic mixture. His group also investigated additives for the enrichment of the depleted energy supplies of the myocardium, which led to advances in long-term preservation solutions (Cordell, 1995).

For over forty years hyperkalaemic cardioplegic solutions have remained the most popular method for stilling the heart during open heart surgery and harvest of the donor heart for transplantation.

2.5 Phase 2: Cold Ischaemic Phase of the Donor Heart

Due to its simplicity, cold static storage remains the most commonly used method for preserving donor organs during storage until implantation (McLaren and Friend, 2003). The donor heart is flushed, arrested and placed in a sterile bag containing either cardioplegic solution or a crystalloid preservation solution for cold storage. Cold storage is required to delay cell death and preserve energy in the donor heart. A 1.5-2.0 fold decrease in most metabolic enzyme activities is seen for every 10°C decrease in temperature, this follows the van't Hoff rule, which can be expressed as $Q_{10}=K_2/K_1^{(10/t_2-t_1)}$ where Q_{10} is van't Hoff's coefficient for a 10ºC drop and K_1 and K_2 are the reaction rates at temperature $t_1$and t_2, respectively (Bigelow *et al.*, 1954; Jahania *et al.*, 1999). In fact Belzer argued that dropping the organ's temperature from 37ºC to 0ºC should reduce the heart's metabolism by 12 – 13 fold and extend safe preservation times by 12 – 13 hours (Bigelow *et al.*, 1954; Belzer and Southard, 1988; Jahania *et al.*, 1999). Despite these bold predictions, cold ischaemic storage of hearts remains limited to 4-6 hours (Jahania *et al.*, 1999).

2.5.1 Limitations of cold ischaemic storage

Three fundamental problems still limit the effectiveness of current methods of cold ischaemic storage. Firstly, ongoing ionic imbalances lead to cell swelling, oedema formation and calcium loading (McMurchie *et al.*, 1973; Hendry *et al.*, 1989; Conte and Baumgartner, 2000). Secondly ongoing cellular processes and less efficient anaerobic respiration lead to ATP depletion and an accumulation of metabolic by-products (Bretschneider, 1980; Rousou *et al.*, 1982). Lastly, damage to the endothelium and vasculature lead to further damage from ischaemia/reperfusion

injury (Amrani *et al.*, 1992; Cartier *et al.*, 1993; Yang and He, 2005);(Jamieson and Friend, 2008).

2.5.2 Design of current preservation solutions

Current preservation solutions have been designed to limit damage to the donor organ caused by cold ischaemic storage and their composition is based on three basic principles first described by Belzer and Southard (1988): 1) rapid mechanical and hypothermic arrest of metabolism; 2) provision of physical and biochemical environment to maintain the viability of the structural components of the tissue; 3) minimisation of reperfusion injury.

As a consequence, ionic concentrations of current preservation solutions are designed to rapidly arrest the heart, limit cell swelling, and mimic either intracellular or extracellular environments. Other additives include metabolic substrates for the production of high energy phosphates, impermeants to provide osmotic and oncotic balance, buffers for pH changes and anti-oxidants to limit reperfusion injury upon reperfusion, as well as agents for membrane stabilization. The composition of commonly used preservation solutions is shown in Table 2.1 below.

2.5.3 Preservation solutions for cardiac transplantation

The development of cold storage solutions for cardiac preservation arose from three different areas of research. The first was the extension of hyperkalaemic cardioplegic solutions to preservation solutions, such as St Thomas Hospital Solution No. 2 described above (Galinanes *et al.*, 1992b). Secondly the use of preservation solutions designed for the preservation of intra-abdominal organs such as Euro-Collins solution (EC) or the University of Wisconsin (UW) solution (Belzer *et al.*, 1992).

Lastly the development of solutions dedicated to solid organ preservation such as Celsior (Menasche *et al.*, 1994).

2.5.3.1 Intracellular vs Extracellular Ionic Compositions

Preservation solutions like the cardioplegic solutions described earlier rely on high extracellular potassium concentration to induce arrest of the donor heart. Therefore, preservation solutions contain varying concentrations of potassium from 10 mM to 125 mM and based on their ionic composition, are termed either intracellular or extracellular. Intracellular solutions mimic the ionic concentration of the cytosol of the cell in an effort to abolish ionic gradients responsible for passive exchanger activities and limit cell swelling (Michel *et al.*, 2002). Extracellular- type solutions mimic the extracellular fluid ionic concentrations and were originally designed for cardioplegia during open heart surgery and not long term preservation solutions (Michel *et al.*, 2002). See Table 2.1.

2.5.3.2 Minimise hypothermic induced cell swelling and prevent expansion of the interstitial space

The addition of an impermeant, colloid or oncotic agent to maintain osmotic balance and therefore ultra cellular structure is often warranted to counteract the effects of cell swelling seen in long term preservation with crystalloid solutions. Impermeants are unable to enter the cell but able to escape the vascular bed and therefore remain in the extracellular space and counteract intra cellular swelling (Menasche *et al.*, 1993). Commonly used impermeants include saccharides (Wahlberg *et al.*, 1989; Sumimoto *et al.*, 1990), and non-saccharides or negatively charged anions such as albumin (Dunphy *et al.*, 1999), lactobionate (Burgmann *et al.*, 1992), chloride and citrate. Colloids or oncotic agents are large molecules that cannot leave the vascular

bed and therefore, limit interstitial oedema. Commonly used colloids include mannitol, hydroxyethyl starch (HES), dextrans and poly-ethyleneglycol (PEG) (Wicomb *et al.*, 1984; Hauet and Eugene, 2008).

2.5.3.3 Prevention intracellular acidosis

Another important feature of preservation solutions is the buffering of cellular acidosis (Baicu and Taylor, 2002). Hydrogen ions accumulate during hypothermic ischaemia signalling metabolic imbalance, in fact pH of mammalian cells has been recorded to drop to 6.5 – 6.8 during the first few hours of hypothermia (Fuller *et al.*, 1988). This acidity contributes to a number of deleterious processes, such as a reduction of the efficiency of enzymatic processes and a reduction in contractility (Fabiato and Fabiato, 1978), a metabolic block of glycolysis (Das and Maulik, 1996) and may cause structural damage through the destabilisation of lysozomes containing proteases and other catalytic enzymes (Baicu and Taylor, 2002).

Frequently used buffering agents include histidine, sodium bicarbonate, citrate and phosphate (Baicu and Taylor, 2002; McCrystal *et al.*, 2004). Buffers to counteract acidosis are often also chosen for their secondary effects. For example histidine a component of Celsior and University of Wisconsin solution (UW), is included in high concentrations to buffer hydrogen ions (pKa 6.8) in an effort to inhibit the Na/H exchanger (Hoenicke *et al.*, 2000) limit Na^{+}/Ca^{2+} exchange (Piper *et al.*, 1998) and is also reported to have calcium chelating effects (Burgmann *et al.*, 1992), to be an inhibitor of matrix metalloproteinase (MMP) ('t Hart *et al.*, 2002) and to act as an impermeant (Baicu and Taylor, 2002).

2.5.3.4 Provision of substrates for maintenance of high energy phosphates during reperfusion

Maintenance of high energy phosphates becomes important when extending ischaemic times in preservation (Wicomb *et al.*, 1986). During ischaemia the heart is rapidly depleted of ATP (Neely *et al.*, 1973). In fact, Stringham and colleagues (Stringham *et al.*, 1992) demonstrated in rabbit hearts that following 12 hours of storage in UW ATP levels dropped to 85% of pre-arrest and this decreased to 78% by 18 hours and 35% after 24 hours. Development of contracture increases the rate of ATP consumption by 3 to 6 times (Stringham *et al.*, 1992). Depletion of ATP may also result in the disassembly of cytoskeletal elements making the myocyte more vulnerable to damage during reperfusion (Ganote and Armstrong, 1993).

Supplementation of preservation solutions with Krebs-cycle intermediates such as glutamate, aspartate (Choong *et al.*, 1988; Choong and Gavin, 1990); (Galinanes *et al.*, 1992a; McCrystal *et al.*, 2004), lactate (Teoh *et al.*, 1990); (Olivencia-Yurvati *et al.*, 2003) and more recently pyruvate, have been shown to prevent ATP depletion, enhance the generation of high energy phosphates and enhance post ischaemic recovery of the donor heart. At supra-physiological concentrations, pyruvate has been shown to improve contractile function in hearts metabolizing glucose, in reversibly injured hearts and stunned hearts (DeBoer *et al.*, 1993; Dobsak *et al.*, 1999; Mallet and Sun, 1999). Pyruvate oxidation generates large amounts of ATP (Jahania *et al.*, 1999), activates pyruvate dehydrogenase (PDH) (Bunger *et al.*, 1989) and pyruvate has also been shown to be a powerful cytosolic anti-oxidant increasing NAD^+/NADH ratio (Bassenge *et al.*, 2000; Mallet and Sun, 2003). Dichloroacetate,

a non-metabolizable pharmacological PDH kinase inhibitor has also been shown to improve post ischaemic contractile function and improve oxygen utilisation efficiency (Smolenski *et al.*, 2001).

2.5.3.5 Prevention of injury from oxygen free radicals

There is strong evidence that reactive oxygen species (ROS) play an important role in ischaemia reperfusion injury (Hess and Kukreja, 1995; Das and Maulik, 1996) and strategies established prior to or at the onset of reperfusion can improve out comes for donor hearts subjected to prolonged ischaemia (Ryan *et al.*, 2003; Gao *et al.*, 2007; Beyersdorf, 2009) while interventions initiated later in reperfusion fail to provide significant protection (Bolli *et al.*, 1989).

The efficacy of the addition of free radical scavengers and antioxidants to preservation solutions is well documented. Allopurinol (xanthine oxidase inhibitor), mannitol, histidine, superoxide dismutase, prostaglandin synthesis inhibitors, vitamin A and E, ascorbate, methionine, N-acetyl cysteine (Dhalla *et al.*, 2000) catalase, deferoxamine and reduced glutathione (Myers *et al.*, 1986; Chambers *et al.*, 1989); (Dhalla *et al.*, 2000) have all been demonstrated to provide protection from ischaemia reperfusion injury, particularly if applied during reperfusion (Chambers *et al.*, 1987; Mei *et al.*, 2009).

Reduced glutathione, a naturally occurring low molecular weight antioxidant, has been shown to improve recoveries when administered as a component of the cardioplegia (Chambers *et al.*, 1989; Southard *et al.*, 1990; Vreugdenhil *et al.*, 1991; Menasche *et al.*, 1993) or during reperfusion (Julia *et al.*, 1991; Cheung *et al.*, 2000).

Glutathione is an important component of a number of preservation solutions including UW and Celsior, however, it is problematic as in its reduced form it is susceptible to oxidation (Gnaiger *et al.*, 2000). In a clinical model of renal transplantation, superoxide dismutase has been shown to reduce both acute and chronic rejection although without impact on delayed graft rejection (Land *et al.*, 1994).

2.5.4 Preservation solutions in common use

2.5.4.1 University of Wisconsin Solution

In 1967 Belzer and colleagues (Belzer *et al.*, 1967) developed a storage solution for the continuous perfusion of kidneys that has become one of the most widely used preservation solutions. The composition of the UW solution is based on scientific evidence and contains 13 components thought to enhance organ preservation (See Table 1.1) (Southard and Belzer, 1993).

In 1990 Wicomb and colleagues (Wicomb *et al.*, 1990) developed Cardisol, a solution similar to the UW solution specifically for heart preservation, replacing HES with poly ethylene glycol (PEG). The superiority of UW for long term preservation has been demonstrated experimentally in rabbit hearts (Kajihara *et al.*, 2006), blood perfused pig hearts (Drinkwater *et al.*, 1995) and in humans (Stein *et al.*, 1991).

2.5.4.2 Euro-Collins Solution

In 1969 Dr Collins and his colleagues described an intracellular type solution for the preservation of kidneys using cold static storage. They demonstrated preservation of canine kidneys on ice for up to 30 hours (Collins *et al.*, 1969). This preservation

solution and mode of storage became the procedure of choice over machine perfusion because of its simplicity of use and low cost. The Collins solution was modified by the Eurotransplant Organization by removing the magnesium (Muhlbacher *et al.*, 1999) in 1967 to develop the Euro-Collins solution. Eurocollins solution was made hypertonic with the addition of glucose and was the standard in Europe for about 15 years (Michel *et al.*, 2002).

2.5.4.3 Celsior Solution

While UW had proved to be a widely accepted and popular preservation solution for donor hearts Celsior, a solution specifically designed for the storage of the metabolically demanding heart (Menasche *et al.*, 1993) has also found wide acceptance. Celsior solution was designed to be used as an arresting solution, storage solution and perfusion fluid (Boku *et al.*, 2006). In his initial investigations Menasche compared the performance of Celsior solution St Thomas Hospital no.2 (Menasche *et al.*, 1993). In his studies on an isolated rat heart non-working Langendorff model he was able to demonstrate superior protection using Celsior following 5 hours of cold static storage. While he found there to be no difference between the groups with respect to recovery of coronary flow, the St Thomas group were twice as likely to develop post ischaemic contracture, higher post-ischaemic diastolic pressures and contractility was significantly depressed. Menasche's group also compared the performance of Celsior and St Thomas solution in a heterotrophic transplantation model in rabbits. They found that hearts stored in Celsior demonstrated significantly smaller losses of compliance, when the pressure –volume

curves from the two cohorts were compared. He concluded that differences in the two solutions were likely due to calcium overload.

2.5.4.4 Bretschneider solution

Developed in the 1960s, Bretschneider's or Histidine-Tryptophan-Ketoglutarate (HTK or Custodial) solution has become a well established and widely used solution in organ preservation. Based on an intracellular ionic make-up with low calcium (0.015 mM), HTK (Custodial) has a very low viscosity and according to Bretschneider should allow for rapid equilibration with tissue. This however, can lead to the use of large infusion volumes which can present problems clinically (Fallouh *et al.*, 2009). HTK (Custodial) has been largely associated with successful intra abdominal organ preservation (Corps *et al.*, 2009) but has also been shown to be safe and efficacious for donor heart in both experimental studies of rat hearts (Kober *et al.*, 1997; Lee *et al.*, 2010), canine hearts (Dyszkiewicz *et al.*, 1990; Ackemann *et al.*, 2002) and clinical trials (Reichenspurner *et al.*, 1993).

2.5.5 Preservation solutions used for comparison in these studies

While Cardisol (UW) is the most widely used solution in the US it was decided to limit comparison to solutions containing an extracellular composition. In these studies the solutions chosen for comparison are Celsior and Bretsneider (HTK). AL is a normokalaemic solution containing 5.4 mmol/L of potassium and it was decided to limit comparisons to those solutions that were most similar to AL in terms of potassium concentration.

Table 2.1: Components of Commonly available Preservation Solutions.
Including St Thomas Hospital Solution No.2 (STH-2); University of Wisconsin Solutions (UW-1&2), Histadine, tryptophan ketoglutarate solution (HTK or Custodial) and Eurocollins solution (EC). Adapted from (Michel *et al.*, 2002).

Components	Celsior (mmol/L)	STH-2 (mmol/L)	UW-1 (mmol/L)	UW-2 (mmol/L)	HTK (mmol/L)	EC (mmol/L)
TYPE	Extra	Extra	Extra	Intra	Intra	Intra
Ionic Concentrations						
Na^+	100	120	125	30	15	15
K^+	15	16	30	125	9	15
Ca^{2+}	0.25	1.2			0.015	
Cl^-	41.5	160			32.0	15
Mg^{2+}	13	16	5	5	4	
SO_4^{2-}			5	5		
HPO_4^{3-}						42
HPO_4^{2-}			25	25		
HCO_3^-		10				15
Metabolic Substrates						
Glucose						194
Glutamate	20					
α-Ketoglutarate					1	
Tryptophan					2	
Adenosine			5	5		
Impermeants						
Lactobionate	80		100	100		
Mannitol	60				30	
D-Raffinose			35.36	35.36		
Hydroxyl Ethyl starch (HES)			50	50		
Buffers						
Histidine	30				180	
Histidine-HCl					18	
Antioxidants						
Allopurinol			1	1		
Reduced glutathione	3		3	3		
Membrane Stabilization						
Lignocaine		1				

2.6 Phase 3: Implantation of the Donor Heart

Early reperfusion is an often over-looked opportunity for protecting the donor heart from ischaemia reperfusion injury (IRI), what follows is a review of the implantation phase of cardiac transplantation. Implantation involves removal of the heart from the cold ischaemic storage solution and flushing the heart with cardioplegic solution,

returning the heart temperature to normal during implantation and early reperfusion prior to and following the removal of the aortic clamp (Beyersdorf, 2004). Transferring the often pre-injured donor heart from a cold, ischaemic storage environment to a warm, oxygenated environment for arousal and implantation predisposes the myocardium and vascular endothelium to IRI, which can contribute to graft injury, and possibly lead to acute and chronic rejection (Jahania *et al.*, 1999; Jahania *et al.*, 2000; Stoica *et al.*, 2001; Parolari *et al.*, 2002; Schmauss and Weis, 2008).

2.6.1 Ischaemia reperfusion injury

Ischaemia reperfusion injury is defined as myocardial injury caused by the restoration of blood flow (oxygen) following a period of ischaemia, culminates in the death of myocytes which were viable immediately before the onset of reperfusion (Piper *et al.*, 1998). Jennings and colleagues (1960) first described the histological changes seen in reperfused canine myocardium following 40 minutes of regional ischaemia and uncontrolled reperfusion. They described changes including cell swelling, contracture of myocytes, disruption of the sarcolemma, and the accumulation of intra-mitochondrial calcium phosphate particles. These changes were not present before the onset of reperfusion (Jennings *et al.*, 1960; Beyersdorf and Buckberg, 1992; Yellon and Hausenloy, 2007).

Cerra and colleagues (Cerra *et al.*, 1975) later found that similar changes in humans were linked to poor survival following cardiac surgery.

IRI is now recognized as a highly complex series of events mediated by inflammation and involving the vascular endothelium, intracellular spaces, numerous

cytokines, chemical markers and immune cells (Linfert *et al.*, 2009). Clinically, IRI results in a spectrum of post ischaemic events such as reperfusion arrhythmias, vascular and microvascular damage leading to the "no-reflow" phenomenon and myocardial functional stunning (Opie, 1989; Vinten-Johansen *et al.*, 2007), the extreme of which is termed the "stone heart" (Cooley *et al.*, 1972).

2.6.1.1 Mechanism of ischaemia reperfusion injury

Despite numerous investigations the mechanism of IRI, is still not completely understood but appears to include: Release of reactive oxygen species (ROS); Cytosolic and mitochondrial calcium overload and acute and chronic inflammatory response upon reperfusion and implantation. Together these perturbations lead to irreversible damage and rejection of the donor organ. The proposed mechanism is shown in Figure 2.3 below.

2.6.1.2 Reactive oxygen species and ischaemia reperfusion injury

There is strong evidence that reactive oxygen species (ROS) play an important role in ischaemia reperfusion injury (Hess and Kukreja, 1995; Das and Maulik, 1996). Whilst ROS generation is small and limited during ischaemia (mainly signalling to trigger protective responses) (Becker, 2004) there is a marked burst of ROS occurring during the first few seconds to minutes of reperfusion which may overwhelm the cardiomyocyte's ubiquitous antioxidant protection already depleted from the ischaemic period (Kloner *et al.*, 1989; Ambrosio *et al.*, 1991; Holmberg *et al.*, 1991; Kevin *et al.*, 2003).

Damage from ROS comes via both direct attack on cellular components, principally membrane lipids and proteins and indirect damage resulting from ROS mediated calcium handling defects (Hearse, 1991; Bolli and Marban, 1999; Gross *et al.*, 1999; Saini-Chohan and Dhalla, 2009; Suleiman *et al.*, 2011). ROS have been shown to directly oxidize thiol groups on the myofilaments causing decreased responsiveness of the myofilaments to calcium, a reduction in maximal calcium-activated force and damage to the contractile apparatus through selective proteolysis of troponin I (MacFarlane and Miller, 1992; Gao *et al.*, 1995; Marban and Gao, 1995; Perez *et al.*, 1998; Van Eyk *et al.*, 1998). The generation of ROS into the extracellular space has also been to the inflammatory response which occurs as a consequence of IRI (Boros and Bromberg, 2006).

2.6.1.3 Maintaining calcium homeostasis: The key for optimal preservation

In resting cells the calcium concentration of the mitochondrial matrix is usually low, however; Jennings and colleagues (1960) demonstrated a 10 fold increase in calcium uptake following reperfusion of ischaemic myocardium with the development of contraction bands and inter-mitochondrial dense bodies possibly calcium phosphate. They argued that ischaemia followed by uncontrolled reperfusion caused an excess uptake of calcium by the cytosol, in turn leading to a mitochondrial overload. This has since been demonstrated in both heart (Nayler, 1981) and liver cells (Farber, 1982). While ischaemia itself causes damage to myocytes, abrupt reperfusion and the associated ionic shifts during the first few minutes determines the fate of the ischaemically damaged cells (Piper *et al.*, 2004; Bers and Ginsburg, 2010).

2.6.1.3.1 Calcium loading during ischaemia

During ischaemia the rise of intracellular calcium comes about via well-defined pathways. The concentration of intracellular sodium rises due to increased sodium entry through Na^+/H^+ exchange of accumulated hydrogen ions as a consequence of anaerobic metabolism. Sodium entry also comes about through the sodium fast channels which open due to depolarisation of the cell membrane from increasing extracellular potassium (Suleiman and Chapman, 1990; Murphy *et al.*, 1991). There is reduced extrusion of sodium via Na^+/K^+ ATPase as ATP stores become depleted (Haigney *et al.*, 1994); calcium entry into the cell then comes about via the reversal of the sodium-calcium exchanger (Powell *et al.*, 1984; Haigney *et al.*, 1992; Ziegelstein *et al.*, 1992; Barry and Bridge, 1993; Labow *et al.*, 1993; Ziegelstein *et al.*, 1994; Tsukube *et al.*, 1996b), inhibition of sarcoplasmic reticulum calcium uptake by the Ca ATPase induced by the acidosis of ischaemia (Kihara *et al.*, 1989; Steenbergen *et al.*, 1990; Valverde *et al.*, 2010) and a reduction in the number of ryanodine receptors (Zucchi *et al.*, 1994; Zucchi *et al.*, 1995).

2.6.1.3.2 Calcium loading during reperfusion

The dramatic increase in cytosolic calcium upon reperfusion is thought to come about by influx (Bolli and Marban, 1999; Piper *et al.*, 2004). Reperfusion causes an abrupt normalisation of extracellular pH, which drives a further increase in Na^+/H^+ exchanger activity and the subsequent influx of sodium is thought to further increase intracellular calcium (Kusuoka *et al.*, 1987; Piper *et al.*, 2004). The increase in intracellular calcium also comes about secondary to ROS mediated sarcolemmal-membrane damage, dysfunction of the sarcoplasmic reticulum (Kaneko *et al.*, 1989a; Kaneko *et al.*, 1989b) and reversal of the Na^+/Ca^{2+} exchanger (Reeves *et al.*, 1986).

Evidence of a brief transient calcium overload immediately following commencement of reperfusion was demonstrated by Kusuoka in isolated isovolumic ferret hearts following 15 minutes of global ischaemia (Kusuoka *et al.*, 1987). Valverde and colleagues (Valverde *et al.*, 2010) also reported a consistent transient increase in diastolic calcium 30 seconds into reperfusion in intact mice hearts, which they termed the Ca^{2+} bump, and the authors thought this represented the release of calcium by the sarcoplasmic reticulum.

At lower concentrations calcium accumulation may be reversible; however, as the mitochondria start to function following an ischaemic event, they expend their energy on calcium uptake to the cytosol and this severely disrupts the respiratory chain and energy production (Opie, 1989). Ongoing disruption in calcium homeostasis leads to mitochondrial calcium overload, which can trigger activation of a number of phospholipases, phosphatases, proteases and nucleases, resulting in fusion of the inner and outer membrane of the mitochondria (Dong *et al.*, 2006), mitochondrial permeability transition pore (MPTP) opening (Piper *et al.*, 1998) and cytochrome C release. If not reversed, this situation will result in irreversible cell injury and death (Crompton, 2000; Duchen, 2000; Orrenius *et al.*, 2003; Racay, 2008). See Figure 2.3.

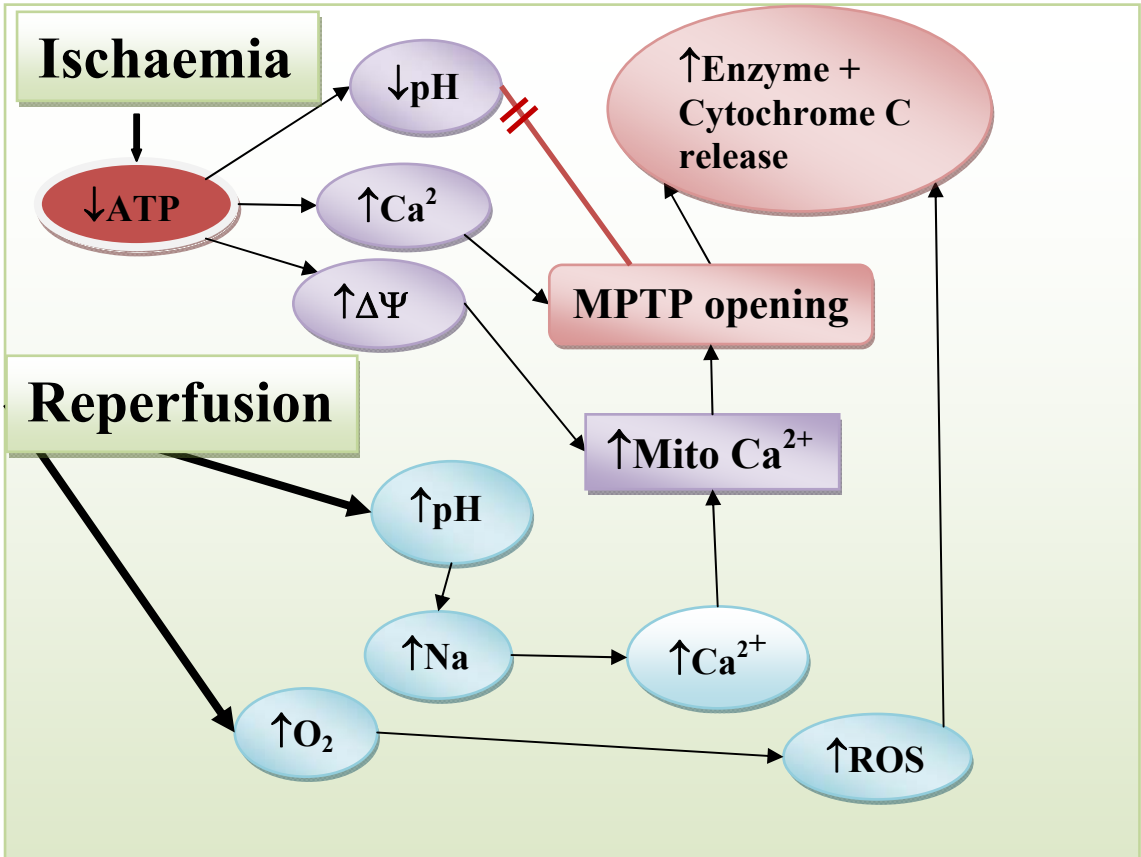

Figure 2.3: Proposed mechanism of ischaemia reperfusion injury
During ischaemia anaerobic respiration leads to an accumulation of hydrogen ions, stimulating Na^+/H^+ exchange. The rise in intracellular sodium leads to a reversal of the Na/Ca exchanger and calcium entry. Anaerobic respiration, a less efficient process than aerobic respiration and ongoing ion pump activity leads to declining ATP. An increase in both sarcolemmal and mitochondrial membrane potential ($\Delta\Psi$) leads to entry of calcium, which if on-going may cause the opening of the MPTP, during ischemia this is inhibited by the low pH.
Upon reperfusion there is an abrupt change in pH, further driving calcium entry and the opening of the MPTP. There is also a burst of ROS causing free radical damage and leading to increased enzyme activity and cytochrome C release. Adapted from Murphy and Steenbergen, 2008.

2.6.2 Calcium loading and the mitochondrial permeability transition pore: Role in myocyte injury and death

Calcium uptake into the mitochondria occurs via the mitochondrial calcium uniporter. As with the sarcolemma, calcium uptake is limited during ischaemia due in part to the mitochondrial membrane potential. However, upon reperfusion and reinstatement of the mitochondrial membrane potential elevated cytosolic calcium

drives the mitochondrial uniporter to overload the mitochondria with calcium. This rise in mitochondrial calcium has been suggested to trigger MPTP opening (Murphy and Steenbergen, 2008). The MPTP, closed under physiological conditions opens in response to high concentrations of calcium (Tandan *et al.*, 2009; Heusch *et al.*, 2010), inorganic phosphate (Di Lisa *et al.*, 2007), reactive oxygen species (Vercesi *et al.*, 1997); (Halestrap, 2009) and depolarisation of the inner mitochondrial membrane (Duchen, 2000). See Figure 2.4 below. It is now well accepted that the MPTP opening is a major factor in ischaemia reperfusion injury, however, despite extensive studies and its exact molecular structure remains unknown (Haworth and Hunter, 1979; Crompton, 2000; Hausenloy *et al.*, 2004; Dahlem *et al.*, 2006; Leshnower *et al.*, 2008; Halestrap, 2009). When open, the MPTP makes the mitochondrial inner membrane abruptly permeable to all solutes of molecular weight up to about 1500 Da (Bernardi and Forte, 2007; Lemasters *et al.*, 2009). Opening of the MPTP has been implicated in many disease processes including ischaemia reperfusion injury and is thought to signal the transition from reversible to irreversible ischaemic reperfusion injury, apoptosis and necrosis (Griffiths and Halestrap, 1995; Paradies *et al.*, 2009).

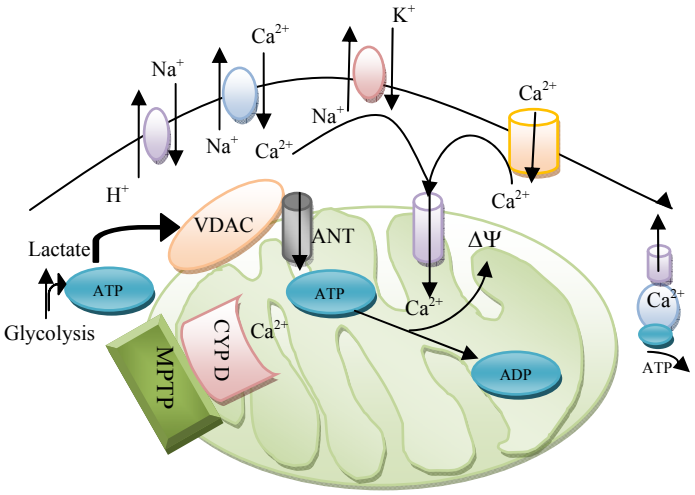

Figure 2.4: Changes in ions and metabolites leading to MPTP opening.
Following the onset of anaerobic respiration, the decline in ATP and pH, drives Na^+/H^+ and Na^+/Ca^{2+} exchange and leads to an increase in cytosolic calcium. The decline in ATP also brings about a change in the energy dependent processes maintaining the mitochondrial membrane potential ($\Delta\Psi$), allowing for entry of calcium into the mitochondria via the calcium uniporter. Increased mitochondrial calcium activates the mitochondrial permeability transition pore (MPTP). This is inhibited during ischaemia by the low intracellular pH; however, restoration of oxygen and pH upon reperfusion allows for MPTP opening. MPTP opening is the major regulator of both apoptotic and necrotic cell death. The restoration of electron transport is a major source of reactive oxygen species (ROS) generation and damage from this, along with increased mitochondrial calcium, causes activation of the MPTP. MPTP opening occurs through changes in adenine nucleotide translocase (ANT) and the voltage dependent anion channel (VDAC) which merge together to form the MPTP. Opening of the MPTP is modulated by cyclophillin D (CYPD). Sustained MPTP opening is incompatible with cell survival and the subsequent mitochondrial swelling leads to rupture and cytochrome C release. MPTP inhibitors such as CYA (binds cyclophillin D) and melatonin (prevents cardiolipin peroxidation and MPTP opening) are thought to act by reducing the MPTP's sensitivity to calcium and ROS.
Adapted from Murphy and Steenbergen, 2008.

Inhibiting the opening of the MPTP has become a therapeutic target for cardioprotection (Heusch *et al.*, 2010). Cyclosporine A (Bernardi, 1996), magnesium (Racay, 2008), nitric oxide release (Jones and Bolli, 2006), melatonin (Petrosillo *et al.*, 2009a) and pH (< 7) all inhibit or delay MPTP opening (Halestrap, 1991).

2.6.2.1 Ischaemia reperfusion injury from the inflammatory response

IRI results in an inflammatory response in the recipient upon implantation and reperfusion of the donor organ which can lead to acute and chronic rejection (de Groot and Rauen, 2007). Damage to the endothelium of the donor heart from neurohormonal and haemodynamic changes of brain death, subsequent inadequate tissue perfusion prior to harvest is further exacerbated during prolonged ischaemia in hypothermic storage and warm reperfusion upon implantation (Jamieson and Friend, 2008). During cold ischaemic storage, generation and secretion of ROS and calcium overload leads to the release of cytokines, chemokines and an up regulation of adhesion molecules. This leaves the endothelium with an adhesive, thrombogenic surface which upon reperfusion allows the attachment of inflammatory cells (Burne-Taney and Rabb, 2003; Boros and Bromberg, 2006). This inflammatory response involves innate immunity (Tsan and Gao, 2004), complement production (Pratt *et al.*, 2002) and the adaptive immune response (Linfert *et al.*, 2009). Leukocytes recruited through chemical signalling, infiltrate the post-ischaemic tissue via the enhanced permeability of the vascular tissue (Rabb, 2002; Linfert *et al.*, 2009).

2.6.3 Clinical consequences of ischaemia reperfusion injury following transplantation

IRI is an unavoidable consequence of heart transplantation due to the period of ischaemia between harvesting the donor heart and completion of implantation into the recipient. The consequences of IRI described in the recipient include microvascular dysfunction (Labarrere *et al.*, 2003), retardation of the actin and myosin filament disengagement (Cleveland *et al.*, 1996), reduction of end-diastolic compliance (Davies *et al.*, 1987) and reduced excitation-coupling upon reperfusion

(Fukumoto *et al.*, 1991). Collectively this amounts to myocardial stunning requiring increased inotrope use post transplant (Marasco *et al.*, 2007). As discussed earlier, myocardial stunning has been linked to the development of primary graft failure (Jahania *et al.*, 2000), damage to the microvasculature and endothelium which lead to an increase in the probability of developing cardiac allograft vasculopathy (Schmauss and Weis, 2008) and chronic rejection (Labarrere *et al.*, 2003). Although the pathogenesis of donor graft failure and cardiac allograft vasculopathy is complex, it appears to be linked to suboptimal preservation and inadequate protection from regional and global ischaemia during harvest, storage and reperfusion of the donor organ (Bourge *et al.*, 1993; Jahania *et al.*, 2000; Valantine, 2004a) particularly in organs from older and sicker donors (Russo *et al.*, 2007).

2.7 Can Heart Storage Times be safely Extended Beyond 4 – 6 hours?

The severity of IRI is dependent on the length of the ischaemic period (Bolli and Marban, 1999), which also becomes a major risk factor for mortality and morbidity following heart transplantation (Hosenpud *et al.*, 2001). Evidence for a link between suboptimal protection of the donor heart during the ischaemic period and poor recipient outcomes is found in the fact that prolonged ischaemic times are consistently determined an independent predictor of primary graft failure and one year mortality both in clinical studies (Lima *et al.*, 2006) and as reported by the ISHLT registry (Taylor *et al.*, 2005). See Figure 2.5 below.

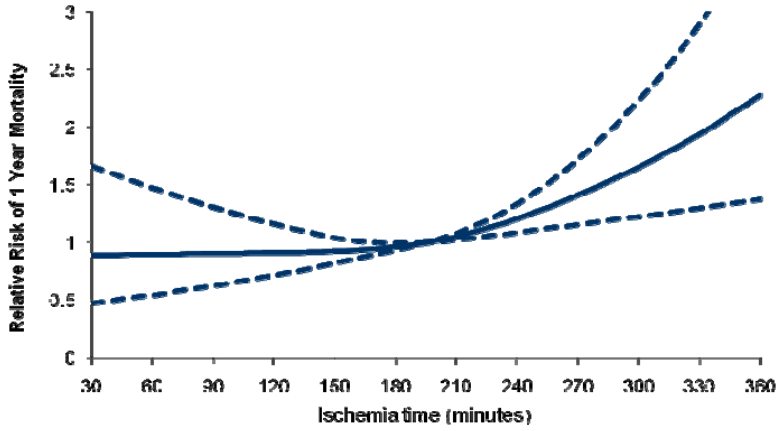

Figure 2.5: Relative risk of one-year mortality vs ischaemia time (95% confidence), This figure shows the relationship between ischaemia time and the relative risk of mortality in the first year, taken from the ISHLT Registry Statistics 2009 (ISHLT, 2009)

The ability to limit the ischaemic time is impractical due to logistical constraints such as distance between donor and recipient, particularly within Australia (McCrystal *et al.*, 2004). Therefore, in an effort to reduce mortality and morbidity following heart transplantation, reducing IRI during the entire transplantation process, becomes paramount. Despite over 30 years of research and innovation in transplantation surgery and development of preservation solutions, the safe storage times have not changed beyond 4 – 5 hours (Kirklin *et al.*, 2004; Maria Rosa *et al.*, 2010). Improved preservation of the donor heart would provide: safe extension of ischaemic times; improved utilisation of hearts from extended criteria donors and improved outcomes for transplant recipients.

2.7.1 Suboptimal preservation by current hyperkalaemic cardioplegic and preservation solutions

Preservation of the donor heart is closely related to myocardial protection during cardiac surgery. Currently most preservation solutions contain potassium >15 mmol/L. See Figure 2.6 below.

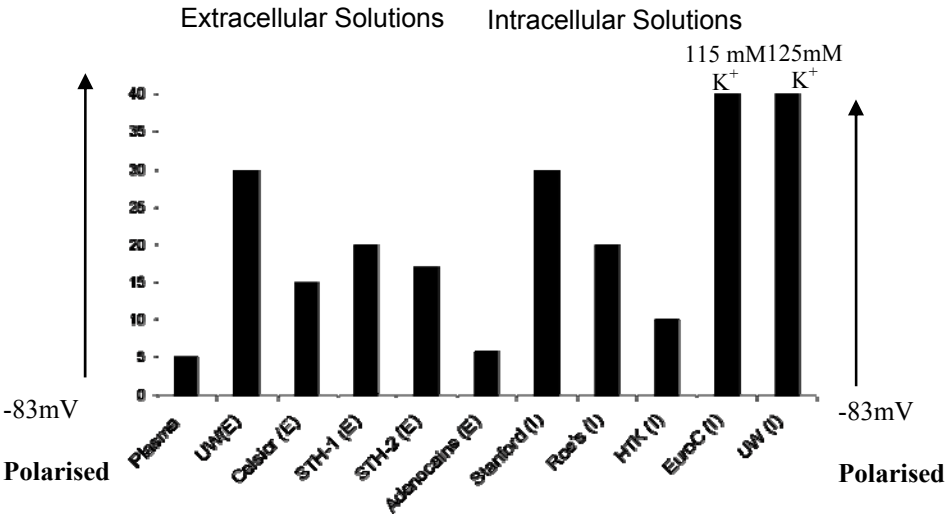

Figure 2.6: Potassium concentration of commonly used hyperkalaemic solutions (>9.0 mM K+). High potassium solutions depolarise the cell membrane of the myocyte, vascular smooth muscle and endothelial cells. Solutions depicted include University of Wisconsin solution (UW); St Thomas Hospital solutions 1 and 2 (STH-1 and STH-2) which differ in their calcium contentrations; Histadine Tryptophan and Ketoglutarate solution (HTK or Custodial); Eurocollins solution (Euro C). In brackets after the solution name is the letter I or E to indicate whether the solutions is Intracellular or Extracellular.

Strongly hyperkalaemic solutions such as UW (intracellular) arrest the heart by depolarising the cell membrane from -83 mV to -30 mV. See Figure 2.6 above. Whereas, mildly hyperkalaemic solutions such as Celsior (15 mmol/L) depolarise the membrane to around -50 mV (Chambers, 1999).

Concerns regarding hyperkalaemic solutions include unnatural membrane voltages, ion imbalances and calcium overload (Chambers, 2003); Coronary vasoconstriction during harvest, storage and 'hot shot' reanimation (Saldanha and Hearse, 1989) and damage to the coronary vascular endothelium during prolonged storage (He and Yang, 1996).

2.7.1.1 Unnatural membrane voltages and exacerbation of damage by hyperkalaemic solutions

Preservation of the donor heart for transplant is a much more demanding process than that of cardiac surgery. While, Conti and colleagues (Conti *et al.*, 1978) demonstrated that cooling the heart added to the protection afforded by hyperkalaemic cardioplegia during the short time frames of cardiac bypass surgery, prolonged cold ischaemic storage in hyperkalaemic preservation solutions exacerbates the adverse effects of global ischaemia (Kleber *et al.*, 1987; Wilde and Aksnes, 1995). In fact, a worldwide survey of heart transplant centres determined that the use of cardioplegic solutions as storage solutions resulted in a 2.5 times increase in the number of deaths when compared to cold saline alone (Wheeldon *et al.*, 1992).

At a resting membrane potential of -50 mV, although the voltage dependent Na^+ fast channels of the heart muscle are dramatically reduced to ~3.5% availability and 0.1% of maximal Na^+ conductance compared to their resting state (-80 mV), the high driving force ($\Delta G_{NA+out/in}$=-15 $KJmol^{-1}$) will lead to sodium entry via a small remaining Na^+ 'window' current (Bers *et al.*, 2003). See Figure 2.7 below. During prolonged periods of global ischaemia and hypoxia, ongoing ionic imbalances lead to further Ca^{2+} accumulation as well as the activation of anaerobic metabolism

(Snabaitis and Chambers, 1999; Fallouh and Chambers, 2008). A fall in intracellular pH leads to the activation of Na^+/H^+ exchanger and subsequent reversal of the Na^+/Ca^{2+} exchanger (3 Na^+ out 1 Ca^{2+} in) and therefore further calcium loading which can cause cell injury, arrhythmias, stunning, and cell death (necrosis and apoptosis) (Suleiman *et al.*, 2001). Correcting these ionic imbalances leads to rapid ATP depletion from energy dependent ion pumps leading to reduced myocardial recovery. Although, hyperkalaemic cardioplegic solutions remain the most popular and widely used solutions for bringing about a rapid mechanical arrest they may not provide optimal cardioprotection (Chambers, 2003).

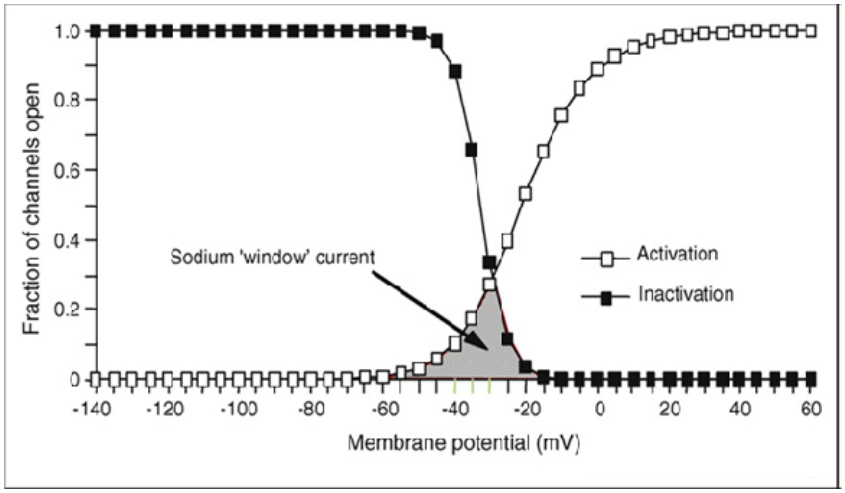

Figure 2.7: The activation and inactivation variables of the fast Na^+ channels in cardiac myocytes
This figure shows the relationship between membrane potential and the activation and inactivation status of the Na^+ channels. The shaded section represents the range of membrane potentials over which the sodium window current is thought to operate. (Fallouh *et al.*, 2009)

2.7.1.2 Hyperkalaemic damage of the coronary vasculature

2.7.1.2.1 Vasoconstriction during arrest induction and harvest of the donor heart

The relationship between hyperkalaemia and increasing coronary vasoconstriction has been known for many decades in transplant and cardiac surgery (Norton and Detar, 1972; Sellke *et al.*, 1993a). High potassium arrest and preservation solutions particularly intracellular solutions (100 – 140 mM K^+) compromise the harvesting procedure by constricting the coronary vessels and limiting distribution of the preservation solution (Leicher *et al.*, 1983; Sellke *et al.*, 1993a). Vasoconstriction also arises during post-storage wash and reanimation of the heart after implantation (Jeevanandam *et al.*, 1992; Mankad *et al.*, 1992; Parolari *et al.*, 2002; He *et al.*, 2004). Potassium-induced depolarisation of smooth muscle leads to opening of voltage-dependent Ca^{2+} channels and the increased intracellular Ca^{2+} causes vasoconstriction (He *et al.*, 2004). Thus during harvest, depolarising potassium can lead to vasoconstriction and maldistribution of cardioplegia or preservation solutions (Leicher *et al.*, 1983).

2.7.1.3 Vasoconstriction during storage and implantation of the donor heart

To some extent, hypothermia during storage mitigates hyperkalaemic vasoconstriction (Chitwood *et al.*, 1979), however, the vasoconstrictive effect still prevails at moderate hypothermia or normothermia during harvest prior to cold-storage and post-storage implantation and reanimation (Sellke *et al.*, 1996). The vasculature of more marginal donors may be more sensitive to potassium's vasoconstrictive effects as they may have multiple co morbidities with pre-existing

endothelial dysfunction (hypertension, diabetes, hyperlipidaemia, metabolic syndrome) and/or up regulation of endothelin-1 (Goodwin *et al.*, 2002).
High potassium has also been linked to coronary artery spasm. This may be an important factor, both during harvest and following hypothermic hyperkalaemic storage of the donor organ, for adult (Ruel *et al.*, 2004) and pediatric heart transplant recipients (Nomura *et al.*, 1997).

2.7.1.4 Damage to the vascular endothelium

Prolonged hypothermic storage in hyperkalaemic preservation solutions has been associated with impairment of endothelial function in numerous studies (Swanson *et al.*, 1988; Cartier *et al.*, 1993; Sellke *et al.*, 1993a; Drinkwater *et al.*, 1995; Kur *et al.*, 2009). Damage to the endothelium during the arrest and harvest phase such as neurohormonal and mechanical injury may be further exacerbated by prolonged hypothermic storage in hyperkalaemic preservation solutions (Mankad *et al.*, 1997; Parolari *et al.*, 2002) and may lead to endothelial neutrophil and platelet adhesion upon reperfusion (Vinten-Johansen, 2004).

2.7.1.5 Impairment of endothelial relaxation response during prolonged storage

Impairment of endothelial relaxation responses and vasomotor dysfunction has been shown to be dependent on both the duration and concentration of the hyperkalaemic exposure (Cartier *et al.*, 1993; Sellke *et al.*, 1993a). High potassium solutions were demonstrated in both the Langendorff rat heart model (Saldanha and Hearse, 1989) and an *in vivo* porcine model of cardiopulmonary bypass (Sellke *et al.*, 1993a; Sellke *et al.*, 1993b) to reduce endothelium dependent vasorelaxation and this was exacerbated during longer ischaemic times (Curro *et al.*, 1997; Budrikis *et al.*, 1999).

Exposure to hyperkalaemic preservation solutions has also been linked to loss of ACh-dependent relaxation, and this may be exacerbated by ischaemia and hypothermia (Tyers, 1997; Parolari *et al.*, 2002; Yang and He, 2005). In 1991, Yacoub and colleagues reported that increasing the potassium concentration in St. Thomas's solution (Plegisol) or Bretschneider's (HTK or Custodial) solution from 20 to 30 mM played a critical role in the loss of endothelium-dependent vasodilation (Mankad *et al.*, 1991). In 1993, Cartier and colleagues (1993) showed that a single dose of cold UW solution resulted in loss of endothelium-dependent vasodilation, and in 1994, Pearl and colleagues demonstrated that multiple doses of UW exacerbated the effect with complete loss of endothelial nitric oxide (NO). Endothelial dysfunction has been reported following prolonged storage in other preservation solutions (Radovits *et al.*, 2008) particularly affecting the distal artery segments from isolated rat hearts (Kevelaitis *et al.*, 1999).

2.7.1.5.1 Depolarisation of the endothelial cell membrane a key trigger for inflammatory responses

Membrane depolarisation is a key 'trigger' or signal in vascular endothelium. In 2000, Sohn and colleagues (2000) demonstrated that depolarisation of human endothelial cells with high potassium (90 mM) was directly responsible for the production of superoxide (O^{2-}). Increased production of vascular endothelial O^{2-} reduces nitric oxide (NO)-dependent relaxation, promotes leakiness, and platelet and neutrophil adhesion (Li and Shah, 2004). Depolarisation of endothelial cells may also promote platelet aggregation through increased superoxide production (O_2^-) and inactivation of endothelial ectonucleotidases (Krotz *et al.*, 2002). Neutrophil and platelet adhesion and aggregation are thought to be key events in acute vascular

thrombosis, particularly when activated platelets come into contact with the subendothelial matrix, like collagen (Siess, 1989; Matsuzaki *et al.*, 2005).

There is overwhelming experimental and clinical evidence showing that depolarising K^+ is vasoconstrictive and pro-spasmodic. This could be exacerbated by preservation induced damage and an up regulation of pro-inflammatory, pro-coagulative markers leaving the endothelium vulnerable to immune attack and consequences such as cardiac allograft vasculopathy and chronic rejection (Chomette *et al.*, 1988; Stoica *et al.*, 2002);(Denton *et al.*, 2000; Ramzy *et al.*, 2005; Schmauss and Weis, 2008). In fact, storage of hearts in UW (125mMK^+) has been linked to endothelial damage and associated with a higher incidence cardiac allograft vasculopathy in the first year post transplant when compared with extracellular type solutions (Drinkwater *et al.*, 1995).

In conclusion, there is overwhelming experimental and clinical evidence showing that depolarising K^+ included in almost all cardioplegic and preservation solutions is vasoconstrictive and pro-spasmodic. Damage to the donor heart following brain death and during harvest may be exacerbated during extended hypothermic storage (Stoica *et al.*, 2002) leading to an up regulation of pro-inflammatory, pro-coagulative markers leaving the endothelium vulnerable to immune attack and consequences such as cardiac allograft vasculopathy and chronic rejection (Chomette *et al.*, 1988; Stoica *et al.*, 2002);(Denton *et al.*, 2000; Ramzy *et al.*, 2005; Schmauss and Weis, 2008).

2.8 Alternatives to Hyperkalaemic Arrest, Preservation and Reperfusion

Alternative methods of achieving cardiac arrest have been demonstrated experimentally using compounds such as the sodium channel blockers, tetrodotoxin (TTX) (Snabaitis *et al.*, 1997a), procaine (Engelman *et al.*, 1978; Hearse *et al.*, 1981) or lignocaine (Boudoulas *et al.*, 1978b; Hearse *et al.*, 1981; Sunamori *et al.*, 2001; Watanabe *et al.*, 2002), K_{ATP} channel openers and short acting beta blockers such as esmolol (McCully, 2002; Fallouh *et al.*, 2010) and adenosine (Chambers and Hearse, 1999). These compounds maintain the membrane voltage close to resting membrane potential in a strategy termed 'polarised arrest'.

2.8.1 Potential advantages of polarised arrest

The potential advantages of polarised arrest versus depolarised arrest are that at resting membrane potentials there are fewer 'open' membrane channels, pores and exchangers compared with the depolarised state (Cohen *et al.*, 1995; Chambers and Hearse, 1999). Arresting the myocyte at or near the resting membrane potential (E_m) would theoretically reduce energy utilisation as transmembrane ionic gradients are balanced and fewer pumps and channels are activated. Sodium and calcium influx and subsequent calcium overload would be minimised as the sodium and calcium channels are not activated at this membrane potential (Chambers, 2003).

Polarised arrest has been associated with maintenance of high energy phosphates (Tyers *et al.*, 1974), reduced oxygen consumption (Sternbergh *et al.*, 1989);(Snabaitis *et al.*, 1997a), reduced ionic changes (Haigney *et al.*, 1994), activation of the Ca ATPase and prevention of calcium loading (Watanabe *et al.*,

2002) and therefore improved long term protection of donor hearts (Hoenicke *et al.*, 2000) when compared to hyperkalaemic arrest.

2.8.2 Direct blockade of sodium channel activation

2.8.2.1 Sodium channel blockers

Sodium channel blockers arrest the heart by inhibiting the initial influx of sodium in phase 0 of the action potential and therefore conduction of the action potential. Several studies have demonstrated both their effectiveness as arresting and cardioprotective agents (Snabaitis *et al.*, 1997a; Asano *et al.*, 2003; Dobson, 2004; Yamaguchi *et al.*, 2007).

2.8.2.2 Tetrodotoxin

Early studies of frog muscle fibres found that tetrodotoxin (TTX) treated fibres resisted induced action potentials (Narahashi *et al.*, 1960). In the mid 1970s Tyers and colleagues, demonstrated the cardioprotective qualities of TTX in isolated rat hearts following 60 minutes of normothermic cardioplegic arrest (Tyers *et al.*, 1974). They suggested that cardioprotection was afforded through the preservation of high energy phosphate stores during ischaemia and reduction of the calcium current. Since then TTX added to cardioplegic solutions has been shown to lower oxygen consumption during arrest (Sternbergh *et al.*, 1989), preserve high energy phosphates and produce significantly better functional recoveries following long term (5–8 hours) hypothermic arrest (7.5ºC) when compared to hearts arrested and stored in hyperkalaemic solutions (Snabaitis *et al.*, 1997a). Due to concerns over TTX toxicity, interest has turned to other drugs known to bring about polarised arrest of the myocyte (Fallouh *et al.*, 2009).

2.8.2.3 Local anaesthetics

Local anaesthetics such as lignocaine and procaine also block sodium entry at high concentrations and can interrupt action potential conduction in nerve and muscle cells. These drugs act by binding to the inner pore of voltage gated sodium channels in a 1:1 fashion and preventing the flow of sodium into the cell (McNulty *et al.*, 2007); (Cummins, 2007).

2.8.2.4 Mechanism of action of lignocaine

Lignocaine's mechanism of action has been widely investigated (Cardinal *et al.*, 1981; Colatsky, 1982; Fozzard *et al.*, 2005; McNulty *et al.*, 2007). Lignocaine is thought to bind the inner membrane of both activated and inactivated sodium channels in human atrial and ventricular cells (Makielski and Falleroni, 1991; Jia *et al.*, 1993) and prevent the flow of sodium ions through the pore (McNulty *et al.*, 2007; Hanck *et al.*, 2009). This indirectly prolongs the inactivation state of the channel (Opie, 2004b).

Lignocaine, at lower concentrations, has also been shown experimentally to have sodium channel–independent effects which may contribute to its reported dose dependent cardioprotective properties in models of regional and global ischaemia (Boudoulas *et al.*, 1978a; Hearse *et al.*, 1981; Kyo *et al.*, 1983; Tomoda *et al.*, 1990; Latocha and Bernauer, 1991; Butwell *et al.*, 1993; Hollmann *et al.*, 2001; Canyon and Dobson, 2004; Yamaguchi *et al.*, 2007). Lignocaine is well known clinically as an anti arrhythmic, particularly for reducing the incidence of ventricular arrhythmias and ventricular fibrillation following myocardial ischaemia (MacMahon *et al.*, 1988; Tosaki *et al.*, 1988; Barrett *et al.*, 1995; Alexander *et al.*, 1999). In rat

cardiomyocytes lignocaine has been reported to down regulate K_{ATP} channels (Olschewski *et al.*, 1996), preserve high energy phosphates (Schaefer *et al.*, 1994), and scavenge free radicals. Lignocaine has also been shown to possess immunosuppressive effects, in particular, the reduction of neutrophil adherence *in vitro* (Schiffer *et al.*, 1977) and *in vivo* (MacGregor *et al.*, 1980). Lignocaine has also been shown to attenuate cytokine release (de Klaver *et al.*, 2006) and suppress neutrophil function and enhance apoptosis (Kawasaki *et al.*, 2010). Lignocaine may also provide protection to vascular endothelium through the reduction of calcium entry (Kinoshita *et al.*, 2001; Shan *et al.*, 2004). Lignocaine has been shown to protect cell membranes in lung microsomes through inhibition of NADPH-dependent lipid peroxidation (Das and Misra, 1992), possibly by protecting from palmitoyle-L-carnitine induced mechanical and metabolic dysfunction (Arakawa *et al.*, 1997). As a consequence, lignocaine has been added to preservation solutions for its reported membrane stabilization benefits (Szekeres, 1986; Sultan *et al.*, 1992).

2.8.2.5 Lignocaine as an adjunct to cardioplegic and preservation solutions

Lignocaine has been used effectively as an additive to hyperkalaemic crystalloid and blood based cardioprotective solutions (Kyo *et al.*, 1983; Leicher *et al.*, 1983; Fiore *et al.*, 1990; Ebel *et al.*, 2001; Watanabe *et al.*, 2002; Yamaguchi *et al.*, 2007). For instance, Leicher and colleagues (1983) demonstrated that lignocaine as an additive to a high potassium (30 mM) solution, improved cardioplegia distribution and protected the heart over 75 minutes of arrest. In 2002 Watanabe and colleagues (2002) found in a rat heart that lignocaine containing cardioplegia prevented calcium loading following 60 minutes of global ischaemia, by significantly increasing the activity of the sarcoplasmic reticulum Ca ATPase during reperfusion.

Lignocaine may also be an effective arrest agent on its own or in combination with agents other than potassium. In 2001 Sunamori and colleagues (2001) demonstrated superior protection of guinea pig hearts following 6 and 12 hour cold static storage in a low sodium and zero potassium solution containing 1 mM lignocaine and 8 mg of magnesium. Lignocaine as an additive to hyperkalaemic cardioplegia (Okamura *et al.*, 1982; Fiore *et al.*, 1990; Dias *et al.*, 2004) or in combination with other agents such as magnesium (Sunamori and Harrison, 1979; Yamaguchi *et al.*, 2007) can enhance cardioprotection during ischaemia and reperfusion.

2.8.2.6 Limitations of lignocaine

The use of lignocaine as a primary arresting agent, however, has not seen clinical uptake due primarily to concern over its potential for arrhythmic and neurological toxicity (Waller, 1981b). Yamaguchi and colleagues (Yamaguchi *et al.*, 2007) while using a lignocaine dose of 1.4 mM in combination with 5.5 mM magnesium in a canine model of cardiac bypass surgery, found equivalent systolic left ventricular function when compared to hyperkalaemic blood cardioplegia. However, they found the plasma concentration of lignocaine reached 0.08 mM which is much greater than the therapeutic index of lignocaine in man 0.03 – 0.04 mM (Foldes *et al.*, 1960; Fallouh and Chambers, 2007).

2.9 Potassium Channel Openers and Adenosine

Polarised arrest can be induced by compounds such as the potassium channel openers (K_{ATP} openers), a chemically diverse group which includes agents such as nicorandil, aprikalim and pinacidil (Cohen *et al.*, 1993; Maskal *et al.*, 1995;

Jayawant *et al.*, 1999). Adenosine has also been shown to bring about polarised arrest of the heart (Chambers, 2003).

2.9.1 ATP-sensitive potassium channel activation

In 1983 Noma described the presence of adenosine triphosphate sensitive potassium (K_{ATP}) channel in guinea pig ventricular cells (Noma, 1983). They have since been described throughout the cardiovascular system (Lacza *et al.*, 2003b) and in other tissues including skeletal muscle (Debska *et al.*, 2002), brain (Lacza *et al.*, 2003a; Yamauchi *et al.*, 2003), kidney (Cancherini *et al.*, 2003), smooth muscle (Pluja *et al.*, 1998) and pancreatic beta cells (Yokoshiki *et al.*, 1998). These channels have been termed the "metabolically regulated channels", as they are linked to intracellular ATP. Closed under normal physiological conditions, these channels open under conditions of hypoxia or ischaemia, thus linking the metabolic state of the cell to its secretory activity or in the case of myocytes, shorten the action potential and decrease myocardial contractility (Nichols *et al.*, 1991). The opening of the $K_{ATP,}$ in both the sarcolemma and the mitochondria, is also thought to be involved in the mediation of both acute and delayed ischaemic preconditioning (IPC) (Gross and Auchampach, 1992; Garlid *et al.*, 1997; Bernardo *et al.*, 1999; Patel *et al.*, 2005). IPC is the phenomenon in which single or multiple brief periods of ischaemia have been shown to bring about cardioprotection against a subsequent more prolonged bout of ischaemia (Light, 1999; Grover and Garlid, 2000; Downey and Cohen, 2001; Gross *et al.*, 2003; Auchampach *et al.*, 2004).

2.9.2 K_{ATP} openers in cardioplegic and preservation solutions

Interest among cardiac surgeons in K_{ATP} openers to bring about a polarised arrest of the heart for clinical cardioplegia, increased during the 1990s, due in part to the problems with hyperkalaemic cardioplegia and depolarised arrest discussed earlier. It was hoped that the cardioprotective effects of the K_{ATP} openers, such as the vasoprotection, vasodilation and reduction in infarct size, seen in various experimental models of ischaemia reperfusion, would translate to cardioprotection during surgically induced global ischaemia (Chambers, 1999) and preservation of hearts for transplant (Hoenicke *et al.*, 2000). For example, Hoenicke and colleagues (Hoenicke *et al.*, 2000) found that pinacidil added to a histidine enriched lactobionate and Krebs Henseleit buffer solution protected isolated rabbit hearts during 4 hours of hypothermic (4^0C) storage with better recovery of diastolic compliance and coronary flow when compared to hearts stored UW and St Thomas solutions. Unfortunately despite promising experimental findings in isolated hearts, the K_{ATP} openers failed to translate clinically (Dirksen *et al.*, 2007). Reperfusion arrhythmias limited their use in cardioplegic and preservation solutions as the sole arresting agent (Cohen *et al.*, 1993; Jayawant and Damiano, 1998; Jayawant *et al.*, 1999; Grover and Garlid, 2000). These limitations make it unlikely that the K_{ATP} channel openers will replace hyperkalaemic based solutions.

2.9.2.1 K_{ATP} openers as additives to hyperkalaemic cardioplegic and preservation solutions

As with many alternative arrest strategies, the K_{ATP} channel openers may find utility as cardioprotective additives to traditional hyperkalaemic cardioplegic and preservation solutions rather than as the primary arresting agents (McCully and

Levitsky, 2003; Rousou *et al.*, 2004). When used as adjuncts to hyperkalaemic solutions in experimental studies the K_{ATP} openers have been shown to decrease calcium loading and preserve ventricular contractility in a number of models of ischaemia reperfusion injury *in vitro* (Dorman *et al.*, 1997a; Dorman *et al.*, 1998; Rousou *et al.*, 2004), *in vivo* (Irie, 1988) and this has also been replicated in clinical trials (Li *et al.*, 2000; Hayashi *et al.*, 2001). In preservation solutions for extended cold static storage the K_{ATP} openers have also found utility. Yang and He (Yang and He, 2005) found the addition of 0.5 mmol/L of pinacidil to HTK (Custodial) solution significantly improved functional recoveries, coronary flow and reduced troponin release in a Langendorff model. Yet, other studies have demonstrated no additional benefit (Galinanes *et al.*, 1992c; Ducko *et al.*, 2000).

2.9.3 Adenosine

In 1989 Schubert and colleagues (Schubert *et al.*, 1989) proclaimed "Adenosine is a potential cardioplegic agent by virtue of its specific inhibitory properties on nodal tissue" following their studies which found adenosine at a concentration of 10 mM could bring about a more rapid arrest and provide improved protection of the isolated rat heart in comparison with 20 mM K^+ cardioplegia. Since then there has been a great deal of interest in adenosine as a cardioprotective agent.

Adenosine's physiological effects on the heart were first described in 1928 (Drury and Szent-Gyorgyi, 1929) and its role in the heart has been studied for over 80 years. This naturally occurring purine nucleotide is present in all cells and has been termed a "retaliatory metabolite" due to enhanced local release and beneficial effects under metabolic stress (Newby *et al.*, 1983; Peart and Headrick, 2007).

2.9.3.1 Adenosine as an agent for cardiac arrest

Drury and Szent-Gyorgi (1929) observed consistent sinus rate slowing of the heart (to 50% of baseline) of anaesthetized dogs following the administration of bolus doses of adenosine (Drury and Szent-Gyorgyi, 1929). Transient sinus bradycardia and atrioiventricular (AV) conduction block following adenosine administration were also reported in normal humans by Honey and colleagues (Honey *et al.*, 1930), who were investigating the use of bolus adenosine (0.5 to 100 mg) for the treatment of patients with chronic atrial fibrillation. Although, an ineffective treatment for chronic atrial fibrillation, adenosine has been used since the 1950s to treat supraventricular tachycardia (SVT). In 1988, Belardinelli and co-workers proved adenosines ability at a concentration of 50 μM to arrest the heart in a hyperpolarized state (-12 mV) (Belardinelli *et al.*, 1988). Adenosine has since become a widely used drug in diagnostic and therapeutic cardiology.

2.9.3.2 Mechanism of cardioprotection

The underlying mechanism by which adenosine affords cardioprotection has been well studied (Wyatt *et al.*, 1989; de Jong *et al.*, 1990; Boehm *et al.*, 1991; Bolling *et al.*, 1994; Alekseev *et al.*, 1996; Cox *et al.*, 1997; Chauhan *et al.*, 2000; Fogelson *et al.*, 2000; Sato *et al.*, 2000; Headrick *et al.*, 2003; Auchampach *et al.*, 2004) and at first was thought to be as a consequence of preservation of myocardial high energy phosphate stores (Ely *et al.*, 1985; Silverman *et al.*, 1985; Boehm *et al.*, 1991; Mentzer *et al.*, 1993). However, it is now well recognized that the actions of adenosine are both receptor and non receptor mediated, with a number of post receptor activities leading to protection of the myocardium during ischaemia and

improved post ischaemic functional recovery (Peart *et al.*, 2003; Headrick and Peart, 2005).

Adenosine binds to at least four distinct receptor subtypes (A_1, A_{2A}, A_{2B} and A_3) located on myocytes, neutrophils, smooth muscle and endothelial cells (Sommerschild and Kirkeboen, 2000). These receptors are coupled via G-proteins to multiple end effectors including enzymes, channels, transporters and cytoskeletal components (Mubagwa and Flameng, 2001). In myocytes adenosine or adenosine receptor agonists have been shown to decrease glycolysis and ATP depletion (Ely *et al.*, 1985), acidosis (Lasley *et al.*, 1990; Lasley and Mentzer, 1992) and lactate accumulation (Fralix *et al.*, 1993). Adenosine activation of the A_1 receptor appears to have anti-ischaemic actions via the opening of K_{ATP} channels and stimulation of outward potassium conductance and thereby limiting cellular calcium conductance; this may be an intrinsic energy saving mechanism.

Studies involving A_1 and A_3 receptor agonists and antagonists, as well as animal models over expressing A_1 receptors, provided evidence for the role of adenosine receptor activation and subsequent opening of K_{ATP} channels in the intrinsic cardioprotection of ischaemic pre-conditioning (IPC) (Lasley *et al.*, 1990; Downey *et al.*, 1993; Matherne *et al.*, 1997; McCully *et al.*, 1998; Kitakaze, 1999; Sato *et al.*, 2000) and post conditioning (Vinten-Johansen *et al.*, 2005).

Both endogenously produced and exogenously administered adenosine show anti-inflammatory actions such as suppression of tissue factor expression (Broussas *et al.*, 2002), neutrophil super oxide generation (Cronstein *et al.*, 1986) and degranulation and adherence (Deguchi *et al.*, 1998; Vinten-Johansen *et al.*, 2003;

Hasko and Cronstein, 2004; Lappas *et al.*, 2005; Takahashi *et al.*, 2007). During ischaemia adenosine can also blunt the stimulatory effects of catecholamines (Schrader *et al.*, 1977; Tikh *et al.*, 2006) and inhibit the release of norepinephrine (Lorbar *et al.*, 2004).

2.9.4 Adenosine as a treatment during ischaemia and reperfusion

Based on encouraging results from animal based research, in both *in vivo* and *in vitro* models of cardiac regional and global ischaemia, adenosine has been utilized in a number of cardiovascular interventions. As pretreatment and maintenance of cardiac arrest, on its own or as an additive to cardioplegia and reperfusion solutions, adenosine has proven safe and effective. Clinical trials in humans have demonstrated safety of concentrations of adenosine from 0 to 50 μmol/L in blood, with hypotension only becoming a problem at higher concentrations (Fremes *et al.*, 1996). In animal models of reperfusion injury, adenosine has been shown to reduce infarct size, improve left ventricular function and improve coronary blood flow (Babbitt *et al.*, 1989; Babbitt *et al.*, 1990; Homeister *et al.*, 1990). In human reperfusion trials, treatment with both dipyridamol, to stimulate endogenous adenosine production and exogenous adenosine have been shown to improve tolerance to ischaemia (Heidland *et al.*, 2000).

Adenosine has also been trialed as an intracoronary infusion pretreatment prior to Percutaneous Coronary Interventions (PTCI) (Marzilli *et al.*, 2000) and following myocardial infarction as a treatment for reperfusion injury. In all cases adenosine was found to reduce infarct size, improve functional recoveries (Mahaffey *et al.*, 1999) and prevent ventricular arrhythmias (Heidland *et al.*, 2000). However, while a

recent multicentre randomized placebo controlled trial the Acute Myocardial Infarction Study of Adenosine or AMISTAD which tested the use of intravenous adenosine administered at the time of thrombolytic therapy following myocardial infarction, showed an encouraging trend towards a reduction in infarct size particularly in anterior infarction, there was also a trend towards more adverse events in adenosine treated patients (Mahaffey *et al.*, 1999).

2.9.5 Adenosine as an adjunct to hyperkalaemic cardioplegic and preservation solutions

Adenosine's combined cytoprotective, immunosuppressive and anti-inflammatory effects make it a useful additive to preservation solutions for cardiac transplantation. In 1976 Hearse and colleagues (Hearse *et al.*, 1976) reported that adenosine alone or in combination with hyperkalaemic cardioplegia improved post ischaemic cardiac function. Adenosine was first introduced as an additive to hyperkalaemic cardioplegic solutions in 1980 by Foker and colleagues (Foker *et al.*, 1980). Since then, adenosine has been used successfully as an adjunct to hyperkalaemic cardioplegic solutions (Katayama *et al.*, 1997a) and during surgical procedures requiring global surgical ischaemia (Thornton *et al.*, 1992; Toombs *et al.*, 1992; Vinten-Johansen and Nakanishi, 1993; Yao and Gross, 1993) both in large animal models and in clinical trials (Mentzer *et al.*, 1999; Thourani *et al.*, 1999; Vinten-Johansen *et al.*, 1999).

When added to hyperkalaemic cardioplegic solutions adenosine can also improve time to arrest and post ischaemic functional and coronary flow recoveries of hyperkalaemic solutions (de Jong *et al.*, 1990);(Alekseev *et al.*, 1996);(Katayama *et*

al., 1997a); (Jovanovic *et al.*, 1997; Fogelson *et al.*, 2000). Adenosine has also been reported to slow the rate of depolarisation induced by 16 mmol/L potassium (Alekseev *et al.*, 1996), reduce calcium loading (Alekseev *et al.*, 1996) through a protein kinase C (PKC) dependent mechanism (Jovanovic *et al.*, 1997), improve metabolism during arrest and myofilament co-operation post arrest (Fogelson *et al.*, 2000) and modulate the activity of post receptor protein kinases A and C known to phosphorylate cardiac troponin I (Law *et al.*, 2005). In a clinical model of baboons Boehm and colleagues were able to demonstrate equal if not better cardioprotection than St Thomas no.2 solution (Plegisol) (Boehm *et al.*, 1990). The addition of adenosine (100 μmol/L to 2 mmol/L) to cold blood cardioplegia was found to be safe and the number of adverse events reduced following surgery (Mentzer *et al.*, 1996; Mentzer *et al.*, 1997).

Despite numerous encouraging experimental studies showing enhanced surgical cardioprotection, clinical results have been equivocal (Mentzer *et al.*, 1997). The benefits of adenosine as an adjunct appear to be temperature dependent. Cohen and colleagues (Cohen *et al.*, 1998) during the Cardiac Arterial Bypass Graft (CABG) trial found no haemodynamic or metabolic benefit with the addition of lower doses (15, 50 or 100 μmol/L) of adenosine to warm blood cardioplegia, although left ventricular ATP levels were preserved and there was higher lactate release in the adenosine supplemented groups. This may be due to its rapid degradation by adenosine deaminase which it appears may be overcome by the use of hypothermia.

Adenosine has also proven a useful pre treatment and additive to cardiac preservation solutions for transplantation. Fremes and colleagues (Fremes *et al.*,

1995) pre-treated isolated rat hearts for 10 minutes with 50 μmol/L adenosine before 8 hours of storage in either University of Wisconsin or St Thomas Hospital No. 2 solution (Plegisol) and found that pretreated hearts had better preserved ATP stores prior to storage and better recoveries of developed pressure accompanied with reduced CK and LDH release following reperfusion. Belzer and Southard reportedly added 5 mmol/L of adenosine to UW to provide a substrate for the regeneration of ATP following storage (Southard *et al.*, 1990; Corps *et al.*, 2009).

2.9.6 Adenosine and lignocaine (AL) cardioplegia

The ability of a novel combination of a sodium fast channel blocker lignocaine (500 μM) and K_{ATP} channel opener adenosine (200 μM), to bring about a polarised arrest of the heart was first reported by Dobson and Jones (2004). Adenosine was chosen because of its negative chronotropic, dromotropic, and ionotropic properties as well as its ability to open ATP–sensitive potassium (K_{ATP}) channels via an A_1 receptor mediated mechanism. Lignocaine a local anaesthetic for its voltage sensitive sodium channel properties (Cardinal *et al.*, 1981; Colatsky, 1982; Xiao *et al.*, 2004) and non-sodium channel dependent effects described earlier (Tomoda *et al.*, 1990; Hollmann *et al.*, 2001). By targeting the adenosine receptors, the SA node, the sodium fast channels and opening K_{ATP} channels at the same time, this cardioplegic formulation containing adenosine and lignocaine can effectively arrest the heart at or near resting membrane potential by preventing the rapid sodium induced depolarisation of the action potential, and shortening the action potential by increasing potassium efflux (Dobson, 2004). Studies estimating the heart membrane potential from tissue potassium measurements found the E_m to be -83 ± 2 mV for control hearts and -83 ±2 mV for AL and -75 ± 2 mV for AL arrested hearts, this

was in stark contrast to hearts arrested in St Thomas solution (Plegisol) which were determined to be -43 ± 3 mV (Dobson, 2004; Sloots and Dobson, 2010) but in good accord with other studies recording membrane potentials both measured and calculated (Masuda *et al.*, 1990; Snabaitis *et al.*, 1997a); (Kleber, 1983). This group were able to demonstrate 70-80% recovery of cardiac output, 85-100% recovery of heart rate, systolic pressure and rate pressure product and 70-80% of base line coronary flows. Only 14% of the hearts arrested in a similar fashion using hyperkalaemic St Thomas Hospital Solution (Plegisol) survived after 4 hours.

In a clinically relevant model of canine cardiac bypass, Corvera and colleagues (Corvera *et al.*, 2005) demonstrated superior cardioprotection during warm arrest and recovery and equivalent protection in cold crystalloid blood environments. When compared to hyperkalaemic St Thomas Hospital cardioplegic solution (Plegisol) the adenosine and lignocaine cardioplegia improved functional recovery, was anti-arrhythmic, prevented calcium loading, cell swelling and preserved high energy phosphates. An interesting finding was that the combination seemed to improve endothelial function with a reduction in coronary endothelial resistance during ischaemia. In direct contrast to the studies of Dobson and Jones (2004), in which AL demonstrated more rapid arrest times in rat hearts when compared to St Thomas (Plegisol), Corvera and colleagues (2005) found arrest times were increased at both hypothermic and normothermic temperatures. In fact at hypothermic temperatures the hearts did not arrest fully. This may be dosage or temperature related, as lignocaine has been shown to become inactive at cooler temperatures (Asano *et al.*, 2003) and there have been concerns over the rapid break down of

adenosine by adenosine deaminase in blood cardioplegia at warmer temperatures (Cohen *et al.*, 1998).

Recently, studies from our laboratory have shown the importance of keeping potassium within normokalaemic limits for optimal protection at 32-33°C, a factor that may be difficult in blood cardioplegia. These studies showed higher (depolarising) or lower (hyperpolarising) potassium concentrations resulted in significantly higher coronary vascular resistance during arrest, slower times to first beat (stunning) and lower cardiac outputs with lower contractility in isolated rat hearts (Dobson, 2010; Sloots and Dobson, 2010). Recently the AL arrest combination has been used clinically in both adult and paediatric cardioplegia (Jin *et al.*, 2008; O'Rullian *et al.*, 2008).

2.10 Potential Additives to Adenosine and Lignocaine Solution to enhance Cardiprotection for Long Term Preservation

2.10.1 Mycardial Fuels

The rate of metabolism and ATP utilization by the normal healthy heart is phenomenal even at rest, with the myocardial ATP pool turning over approximately every 12 seconds (Stanley, 2004). Normally oxidation of fatty acids and pyruvate in the mitochondria provide the main energy source (97%) for myocardial metabolism and the oxidation of glucose and lactate (3%) in roughly equal proportions. When the blood supply is normal the choice of fuels is largely controlled by their availability and the demand of the heart, carbohydrates in the fed state and fatty acids during fasting. During ischaemia, there is a decrease in the arterial delivery of both oxygen and fuels, oxidative metabolism decreases and glycolysis is activated. Glycolysis can provide limited amounts of energy even in the absence of oxygen.

However, in severe ischaemia when the delivery of glucose and oxygen is compromised other metabolic fuels are utilized from lipolysis and gluconeogenesis. Normal oxidative phosphorylation is blocked by an increase in the concentration of non-esterified fatty acids from lipid breakdown and no energy is produced. There is a subsequent rise in the cytoplasmic concentration of NADH and the NADH/NAD ratio increases. Anaerobic respiration leads to an increase in cytoplasmic lactate and protons, this increase in lactate in-turn further inhibits glycolysis. It has been argued that glucose metabolism and glycolysis protect the heart during periods of ischaemia whilst fatty acid metabolism leads to harmful consequences, therefore, the use of myocardial fuels has the potential for use in therapeutic intervention (Opie, 2004a).

Pyruvate is not normally considered a myocardial fuel source due to lower plasma concentrations (0.1- 0.2 mM), yet the heart is responsive to the addition of endogenous pyruvate (Mallet *et al.*, 2005). At supraphysiological levels (2 – 10mM) pyruvate has been shown to enhance cardiac performance without a subsequent increase in heart rate (Bunger *et al.*, 1989), preserve high energy phosphate without a subsequent increase in myocardial oxygen consumption (Ochiai *et al.*, 2001) and improve intracellular calcium homeostasis (McCormack and Denton, 1989). Studies have also indicated that in addition to supplying energy, pyruvate is able through its alpha-keto carboxylate structure, to neutralize non-enzymatically peroxides and peroxy-nitrate and help detoxify harmful reactive oxygen species and increase endogenous antioxidant stores (Mallet and Sun, 2003). Also the accumulation of citrate related to pyruvate addition to cardioplegic solutions enhances NADPH production which helps to maintain the glutathione: glutathione disulfide (GSH/GSSG) redox potential.

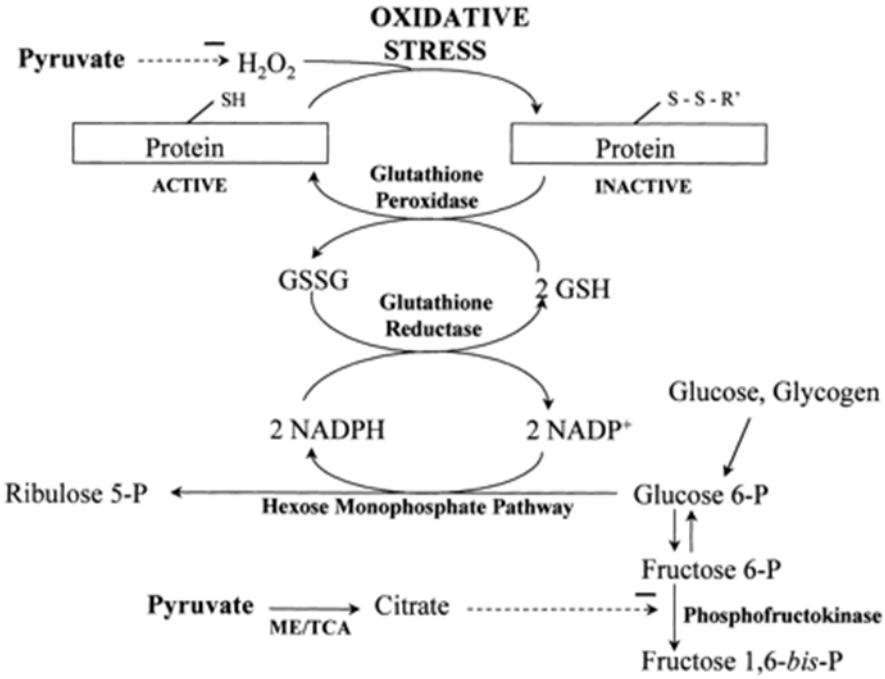

Figure 2.8: Antioxidant mechanisms of pyruvate.
Oxidative stress inactivates proteins by oxidizing sulfhydryl moieties. Pyruvate prevents this oxidation by neutralizing hydrogen peroxide. Pyruvate could also indirectly reverse sulfhydryl oxidation by increasing NADPH production by the hexose monophosphate shunt. Flux through this pathway is increased when glycolytic flux is restrained by citrate formed by pyruvate carboxylation. NADPH supplies reducing equivalents to regenerate reduced glutathione (GSH) from glutathione disulfide (GSSG); GSH restores protein sulfhydryls (Mallet *et al.*, 2005).

Augmenting cardioplegic and cardiac preservation solutions with exogenous pyruvate may help to improve cardioprotection during elective global ischaemia through its dual action of energy yielding and antioxidant capabilities. A recent clinical trial of pyruvate fortified cardioplegia by Olivencia-Yurvati et al (Olivencia-Yurvati *et al.*, 2003) provided promising results with improved recoveries when compared to lactate fortified cardioplegia.

2.10.2 Insulin

The cardioprotective effects of insulin are well documented (Carvalho *et al.*, 2011; Ng *et al.*, 2012) particularly during reperfusion (Jonassen *et al.*, 2000). The cardioprotective effects of insulin is reported to be due to insulin's ability to

facilitate myocardial glucose uptake during cold exposure (Orme and Kelly, 1977) and its other multiple cardioprotective properties such as stimulation of NO production via ischemia-induced protein kinase C-dependent phosphatidylinositol 3'- kinase-Akt-dependent signalling and inhibition of superoxide anion (O_2^-) (Fischer-Rasokat and Doenst, 2003);(Ji *et al.*, 2010). As an additive to cardioplegic and cardioprotective solutions insulin has been associated with improved ventricular recovery, lower lactate release, incidence of arrhythmias and lower inotropic support (Rao *et al.*, 1998). However, a clinical trial failed to deomstrate any added benefit of insulin fortified cardioplegia in emergency CABG (Lazar, 2002; Rao *et al.*, 2002). The addition of insulin to cardiopreservation solutions for donor hearts for transplanr may provide protection during long term preservation.

2.10.3 MPTP Inhibitors

It has become clear that the MPTP plays a central role during necrotic cell death and as such has become a popular target for cardioprotection. Current cardioprotective strategies involve pharmacologically targeting the loss of mitochondrial membrane integrity through agents which inhibit MPTP opening. This includes drugs such as sanglifehrin A, cyclosporin A (CYA) (Bernardi, 1996), non-immunosuppressive analogues of CYA and melatonin (Petrosillo *et al.*, 2009a).

2.10.3.1 Cyclosporin A

Several groups have reported that Cyclosporin A (CYA) can inhibit MPTP opening after ischaemia reperfusion in models such as isolated hearts (Griffiths and Halestrap, 1993; Halestrap *et al.*, 1997), *in vivo* animal experiments (Leshnower *et al.*, 2008) and during percutaneous coronary intervention (PCI) in patients presenting

with myocardial infarction (Piot *et al.*, 2008). It is thought that CYA blocks interaction of the matrix cyclophillin D with the pore and therefore inhibits opening of the MPTP (Woodfield *et al.*, 1998). Augmenting cardioplegic and cardiac preservation solutions with low dose CYA may help to improve cardioprotection during elective global ischaemia through inhibition of MPTP opening. Investigations of the use of CYA as an additive to currently available cardioplegic solutions have reported improved outcomes following augmentation of cardioplegia with CYA (Duan *et al.*, 2011; Pritzwald-Stegmann *et al.*, 2011). In an isolated rabbit heart model of ischaemia reperfusion Pritzwald-Stegmann et al. reported improved recovery of ventricular pressures with reduced oxygen consumption during the first 10 minutes of reperfusion while ATP levels were maintained (Pritzwald-Stegmann *et al.*, 2011) when hearts were stored for 90 minutes in HTK solution augmented with 1umol/L CYA.

2.10.3.2 Melatonin

Melatonin, the chief secretory product of the pineal gland, has been shown to protect the heart against ischaemia reperfusion injury (Lagneux *et al.*, 2000; Albarran *et al.*, 2001; Reiter *et al.*, 2001b; Sahna *et al.*, 2005).This cardioprotection has been attributed to melatonin's ability to directly neutralise a number of toxic reactants and indirectly stimulate anti-oxidative enzymes and following this a cascade of free radical scavenger metabolites (Okatani *et al.*, 2001; Reiter *et al.*, 2001a). Melatonin also stimulates gene expression and sctivities of superoxide dismutase (Antolin *et al.*, 1996; Albarran *et al.*, 2001), catalase and glutathione peroxidise (Okatani *et al.*, 2001). However, more recently melatonin has also been shown to inhibit MPTP opening (Petrosillo *et al.*, 2009b).

The discovery of melatonin's ability to protect the heart from ischaemia reperfusion injury has prompted several investigators to evaluate it as a component of cardioplegia and cardiac preservation solutions. Gao et al. demonstrated superior recoveries in a working rat heart model with greater than 80% recovery of cardiac output following 12 hours of cold static storage in St Thomas with the addition of 0.1mmol/L melatonin (Gao *et al.*, 2003). Petriosillo et al. investigated the mechanism of melatonin cardioprotection by measuring mitochondrial oxygen consumption, complex I and complex III activity, hydrogen peroxide production, lipid peroxidation as well as cardiolipin content and oxidation in isolated mitochondria derived from isolated rat hearts following 30 minutes of global ischemia treated with and without exogenous melatonin (Petrosillo *et al.*, 2006). They demonstrated that melatonin at a physiological concentrations strongly protected the oxidation of integral proteins involved in mitochondrial oxidative metabolism. In further studies the same group were able to demonstrate that melatonin prevented mitochondrial NAD^+ release, cytochrome c release and cardiolipin oxidation in an isolated rat heart model of ischemia reperfusion injury (Petrosillo *et al.*, 2009a). Due to its reported antioxidant, MPTP inhibition activities as well as its small size and amphiphilic nature allowing it to reach all compartments of the myocyte quickly, the addition of melatonin to a cardioprotective solution may enhance recoveries of cardiac function following long term arrest of the donor heart.

2.11 Conclusion

Despite its success, heart transplantation is in trouble, with a reliance on older and sicker donors and the recurring problems of primary graft failure and cardiac allograft vasculopathy limiting its long term success. This may be linked to

suboptimal preservation of the donor organ throughout the entire transplantation process, which for the past forty years has relied on hyperkalaemic solutions for arresting, storing and reanimating the donor heart. There is an unmet need to extend safe ischaemic times and improve the current outcomes for heart transplant recipients; perhaps arresting, storing and reanimating the donor heart with a solution which maintains more natural resting membrane potentials could provide superior protection and lead to better outcomes both in the short and longer term.

CHAPTER 3: MATERIALS AND METHODS

3.1 Introduction

The isolated working heart perfusion system is the model used in all studies presented here. The experimental protocol including a detailed explanation of the surgical preparation of this model, drugs used in anaesthesia, buffer preparation, study endpoints and choice of statistical methods is given. Details of the composition of all cardioplegic, preservation and reperfusion solutions can be found in Appendix A.

3.2 Materials

3.2.1 Drugs used for anaesthesia

The drugs used to induce anaesthesia during surgical removal of the rat hearts were sodium pentobarbital (Nembutal) and thiopentone sodium (Thiobarb). Sodium pentobarbital was used until it became unavailable in Australia, due to concerns over its use in euthanasia, and was then substituted with Thiobarb (thiopentone sodium). An indepth study by Brammer and colleagues (Brammer *et al.*, 1993) investigating the affects of a number of anasthetics on rodent models for cardiovascular research, found that thiobarbital did not appear to adversely affect heart rate, blood pressure. Mullenheim and colleagues (2001) also demonstrated in an isolated rat heart model that thiopentone sodium at a similar dosage to that used in the current studies, had no effects on the functional recoveries of rat hearts treated with ischaemic pre-conditioning (IPC) (Mullenheim *et al.*, 2001). Other studies have reported negative ionotropic effects of thiopentone sodium in both isolated rat hearts (Suzer *et al.*, 1998) and humans (Gelissen *et al.*, 1996). The anaesthetic doses used in these

studies are similar to those used in the current studies, and negative effects were controlled by the use of exclusion criteria that eliminated hearts with heart rates below 200 bpm and coronary flows of less than 10 mls/min.

3.2.2 Perfusion buffer, cardioplegic solutions and reperfusion solutions

All solutions were made fresh daily. The cardioplegic (arrest and storage) and reperfusion solutions were filtered using 0.2 μm filters (Pall, Australia). Perfusion buffer and cardioplegic solutions were maintained at 37°C. The same solution was then used for storage of hearts and following heart immersion the solution is cooled to 4°C. The cardioplegic (arrest and storage) solutions were not actively bubbled with 95% O_2/5% CO_2; hence they had a higher pH. The average pO_2 of the cardioplegic solutions were between 140 – 146 mmHg and the pCO_2 was 5-10 mmHg. The reperfusion solutions were the same solutions as the cardioplegic solutions described above (See Appendix A) except that they were actively bubbled with 95% O_2/5% CO_2 to achieve a pO_2 greater than 600 mmHg. This was to facilitate slow warming and oxygenation during early reperfusion of the hearts. Components used in the preparation of the perfusion buffer and cardioplegic solutions are listed in Appendix A.

3.2.2.1 Krebs-Henseleit solution

The composition of the Krebs Henseleit (KH) solution is listed in Appendix A: Table A2. KH solution is used in the preparation of perfusion buffers and cardioplegic solutions throughout this thesis. The perfusion buffer was filtered using a one micron (1 μM) membrane in an in-line filter and then bubbled vigorously with

95%O_2/5%CO_2 to achieve a pO_2 greater than 600 mmHg. The perfusion buffer was not recirculated.

3.2.2.2 Adenosine and lignocaine (AL) cardioplegic solutions

The composition of the adenosine and lignocaine (AL) cardioplegic solutions are listed in Appendix A. All AL solutions were made fresh daily and were filtered using 0.2 μm filter. The AL solutions were not actively bubbled with 95% O_2/5% CO_2 to mimic the clinical situation; hence the higher pH.

3.2.2.3 AL (Low Ca^{2+}/High Mg^{2+}) + CYA

The composition of the AL+cyclosporin solution (AL (LowCa^{2+}:HighMg^{2+}) + CYA) is listed in Appendix A: Table A9. The concentration of 0.2 μM of cyclosporin was chosen on the basis of other papers showing cardioprotective benefits at similar concentrations in isolated rat hearts (Griffiths and Halestrap, 1993; Hausenloy *et al.*, 2002).

3.2.2.4 2xAL + melatonin and insulin solution (ALMI)

The composition of the 2xAL+melatonin and insulin solution (ALMI) is listed in Appendix A: Table A8. The solution is the same as the AL (LowCa^{2+}:HighMg^{2+}) solution (above) with the following inclusions: the concentration of adenosine (200 μM) and lignocaine (500 μM) was doubled to adenosine (400 μM) and lignocaine (1000 μM) respectively, addition of 100 μM of melatonin and 0.01 IU/ml of insulin. Higher adenosine and lignocaine concentrations were chosen due to reports indicating lignocaine's reduced ability to effectively block sodium fast channels at lower temperatures (Makielski and Falleroni, 1991) and adenosine's

reported reduction in cardioprotective properties at lower temperatures (Katayama *et al.*, 1997a). Melatonin was chosen because of its reported activity as a potent free radical scavenger, powerful anti-oxidant (Albarran *et al.*, 2001) and cardioprotective agent (Petrosillo *et al.*, 2009a). Insulin was chosen because of its anti-oxidant and cardioprotective properties ((Ji *et al.*, 2010)). See discussion at the end of Chapter 7.

3.2.2.5 Celsior solution

The composition of the Celsior solution is listed in Appendix A: Table A10. The arrest solution was not actively bubbled with 95% O_2/5% CO_2 to mimic the clinical situation. Due to an inability to obtain lactobionate from Sigma Aldrich, due to quarantine concerns, the Celsior solution used in the final chapter of this thesis was purchased as a premade solution from Clifford Hallam Healthcare Australia (agent for Genzyme) and used as supplied with no modification.

3.2.2.6 Histadine tryptophan ketoglutarate solution (HTK or Custodial)

The composition of the histadine tryptophan ketoglutarate solution (HTK, Bretschneider Solution or Custodial) solution is listed in Table A12; Appendix A. The HTK (Custodial) solution was made fresh daily and was filtered using 0.2 μm filters and maintained at 37°C. The arrest solution was not actively bubbled with 95% O_2/5% CO_2 to mimic the clinical situation.

3.3 Ethical Conduct of Experimental Studies

The research presented and reported in these studies was conducted within the guidelines for research and ethics outlined in the in the Joint NHMRC/AVCC Statement and Guidelines on Research Practice (1997), the Australian code for the

Conduct of Responsible research (2007), the James Cook University Policy on Experimentation Ethics Standard Practices and Guidelines (2001), and the James Cook University Statement and Guidelines on Research practice (2001). Research methodology reported in this thesis received clearance from the James Cook University Experimentation Ethics Review, animal ethics numbers A781, A1084 and A1515.

3.4 Animals

3.4.1 Housing and monitoring

Male Sprague-Dawley rats weighing approximately 300-450 g were obtained from James Cook University's breeding colony. Animals were fed water and standard pellets *ad libitum* and housed in a 10/14 hour light/dark cycle. The cage was lined with sawdust and environmental enhancers (objects for rats to interact with) were provided. On the day of the experiment, the required number of rats was transferred about 500m to the laboratory in a standard rat cage with a minimum of delay to keep stress to a minimum.

3.5 Isolated Perfused Rat Heart Methodology

First described by Oscar Langendorff in 1895 (Mankad *et al.*, 1991; Bell *et al.*, 2011), the isolated rat heart perfusion method, involves an artificial circuit which enables the study of small mammalian hearts. Originally applied to the study of heart biology by physiologists, biochemists and morphologists, it has been used extensively by pharmacologists and a variety of cardiovascular researchers in many ways to investigate the cardiovascular and pharmacological effects of drugs, therapies and gene alterations (Skrzypiec-Spring *et al.*, 2007). In Langendorff's

model, the aorta is cannulated to allow retrograde perfusion of the coronary arteries by a perfusion buffer usually Krebs Henseleit. In 1967, this model was modified by Neely and colleagues to allow for the placement of a second cannula into the left atrium, thus allowing for the heart to generate antegrade flow or cardiac output and perfuse its own coronary arteries in a working heart model (Neely *et al.*, 1967). Since this time, the isolated working heart model has been used extensively by researchers studying cardiac metabolism, physiology, electrophysiology and pharmacology, particularly in the development of cardioplegic solutions. Benefits of this model include its low cost, high throughput and reproducibility, and clinical relevance. The system used in the present study is based on these systems, incorporating the modifications of Neely and colleagues, which allow for the direction of perfusate to be switched rapidly from retrograde non-working or Langendorff mode to working mode where the heart ejects perfusate from the aorta to produce cardiac output (Neely *et al.*, 1967).

3.5.1 The Langendorff (non-working) isolated heart preparation

In Langendorff perfusion flow through the aorta is retrograde to physiologic aortic flow with no cardiac output. The buffer perfuses the coronary circulation at a constant flow rate and at a constant hydrostatic pressure of 90 cmH_2O (68mmHg). The flow forces the aortic valve shut and perfusion fluid is directed into the coronary ostia, thereby perfusing the ventricles and draining into the coronary sinus (Sutherland and Hearse, 2000). See Figure 3.1 below.

The experiments described in this thesis all employ the Langendorff mode whilst the heart is attached to the cannula and during the tying off of the vessels before

switching to working mode. The Langendorff mode is also used for the introduction of the cardioplegic or preservation solutions.

3.5.2 The working isolated heart preparation

The working heart preparation mimics normal cardiac physiology with left ventricular filling via cannulation of the left atria and ejection of fluid through the aorta in the normal direction. Coronary perfusion is established under normal cardiac motion and can be sampled via a cannula placed in the coronary sinus. This model has the advantage of allowing the measurement of pump function against an after-load (Sutherland and Hearse, 2000).

3.6 Advantages and disadvantages of the isolated perfused rat heart model

The isolated rat heart perfusion model in working mode with its wide range of biochemical, morphological and functional measurements is ideally suited for investigating the different conditions and preservation solutions for heart transplantation in particular the assessment of ischaemia reperfusion injury following prolonged storage. With minor alterations to the design of the model, all modes of heart preservation can be studied from cold static storage (CSS), cold continuous perfusion and warm intermittent as well as blood perfused making this circuit versatile and appropriate for the study of improved strategies for heart transplantation (Skrzypiec-Spring *et al.*, 2007).

Disadvantages of the isolated perfused rat heart model include the model's inability to exactly mimic normal human cardiac physiology. The heart, when removed from the body is no longer stimulated by the autonomic nervous or hormonal systems

which play a large role in cardiac function *in vivo*. While both rat and human hearts are empty and non-working in the physical sense and are perfused via the coronary vessels following storage, the rat heart in working mode has preload and afterload that are pre-set during normothermic reperfusion while in cardiac bypass surgery the preload and afterload of the human donor heart are dynamic variables. Additionally the model suffers from the unnatural use of crystalloid buffers; clinically whole blood or blood additives would be used, and the lack of components with oncotic properties results in cell swelling and fluid retention. There is also an increased risk of ischaemic pre-conditioning during surgery and attachment of the heart; this may lead to altered function following surgery the impact of this can be easily limited by the use of carefully selected exclusion criteria.

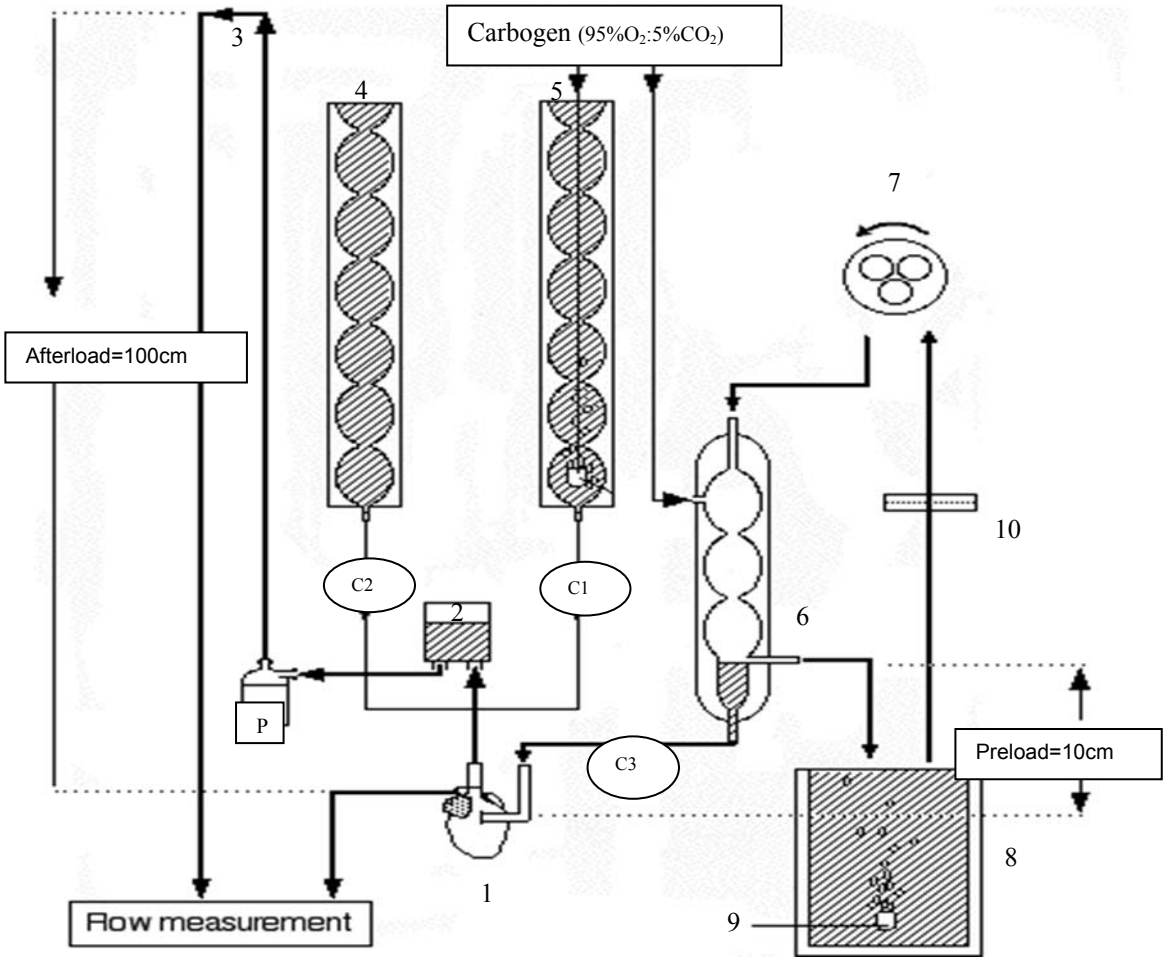

Figure 3.1: Isolated Perfused Rat Heart - Langendorff and Working Heart Model
A schematic representation of the perfusion circuit used in the current studies.

1. Isolated heart: Heart is cannulated through the aorta and left atrium.
2. Compliance chamber and air trap
3. Afterload chamber
4. Cardioplegia column
5. Langendorff column (warmed and oxygenated)
6. Working heart heat exchanger /pre load chamber
7. Working heart perfusion pump
8. Working heart buffer reservoir (warmed and oxygenated)
9. Oxygenator
10. In-line filter

C1 – C3 clamps 1, 2 and 3
P – Pressure transducer
— Tygon® Tubing

3.6.1.1 Isolated rat heart

Figure 3.2: Isolated rat heart

The isolated rat heart is attached to the apparatus by sliding the aorta over a grooved stainless steel cannula (3 mm wide) and once attached, the aorta is sutured in place. The heart is left open to the air and not enclosed in a warming chamber, to mimic the conditions of open heart surgery. The aortic cannula is connected by Tygon® tubing to the Langendorff column which contains warmed and oxygenated Krebs Henseleit buffer. The aortic cannula is also connected to the compliance chamber and air trap and beyond this by Tygon® tubing and pressure transducer to the afterload.

To facilitate working mode the left atria is cannulated via one of the orifices of the pulmonary arteries and securely tied off to avoid leaks. Following cannulation, the tubing from the Langendorff column may be clamped at C1 to stop the Langendorff flow and the apparatus is then switched to working mode by opening the clamp C3 and allowing fluid to flow into the left atrium. See Figure 3.1 above.

3.6.1.2 Compliance chamber and air trap

The compliance chamber is a water-jacketed chamber located 10 cm above the heart and is joined to the aortic cannula and the after load. The air chamber is partially filled with 2 ml of air with an air-fluid level positioned above the inlets and outlets of the chamber. During Langendorff or retrograde perfusion, it traps any air bubbles that may come from the Langendorff chamber and prevents air entering the coronary

circulation. In working heart mode the trapped air provides some elasticity to the pressure generated by the left ventricle.

Figure 3.3:
After-load chamber

3.6.1.3 Afterload chamber

The afterload chamber (Figure 3.3) is a plastic chamber located 100 cm above the heart and connected to the heart via the compliance chamber and pressure transducer by Tygon® tubing. It is open to the air and ensures that the heart is exposed to 100 cm H_2O (76mmHg) after-load when the apparatus is in working mode.

3.6.1.4 Langendorff and cardioplegia columns

Figure 3.4:
Langendorff column

The Langendorff and cardioplegia columns are water jacketed columns, the tops of which are located 90 cm above the heart. These columns allow for oxygenation, via a bubbler, of the Langendorff perfusion fluids, either Krebs Henseleit or the cardioplegia solutions. When operating in Langendorff mode, buffer flows out of the stoppered outlet governed by a plastic clamp (C1) into the aortic cannula at a pressure governed by the height of the column. This is shown in Figure 3.4 above.

3.6.1.5 Working heart buffer reservoir and perfusion pump

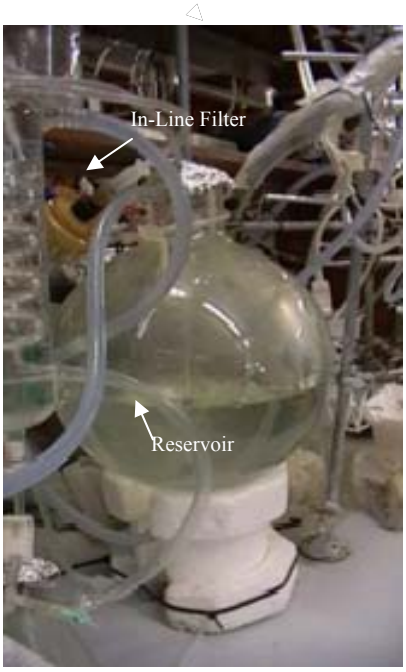

Figure 3.5:
Perfusion buffer reservoir

The perfusion buffer reservoir pictured in Figure 3.5, is a water-jacketed glass flask filled with Krebs Henseleit buffer warmed to 37ºC and oxygenated, via a bubbler, which sits in the reservoir. A peristaltic pump attached by a length of tygon tubing draws the fluid from the tank through an in-line filter containing a 1 μM glass fibre filter (Pall, Australia) and into a heat exchanger and preload chamber. The outlet of this chamber is situated 10cm above the heart and the fluid enters the heart via a cannula sutured into the left atria.

3.7 Surgical Procedures

3.7.1 Anaesthesia

Once the apparatus has been warmed and primed the rat is anaesthetised by an intraperitoneal injection of thiopentone sodium (80 mg/Kg) (Brammer *et al.*, 1993). To determine if anaesthesia was adequate pedal withdrawal reflexes were observed; if inadequate then another incremental dose of 0.5 ml of anaesthetic is given and the procedure for determining adequacy of anaesthesia is repeated.

3.7.2 Surgical removal of the heart

Figure 3.6:
Transverse mediastinal incision

Once anaesthesia was established the rat was placed in a supine position in preparation for surgery. The abdominal cavity was opened by making a transverse mediastinal incision with scissors. The diaphragm was transected and lateral incisions were made on both sides of the rib cage. See Figure 3.6. Following this the anterior chest wall was folded back and the entire heart and lung block excised with a single cut to the great vessels leaving the top of the heart. This is shown in Figure 3.7.

Figure 3.7:
Anterior chest wall folded back

The heart was then placed in a dish containing approximately 40 ml of ice cold previously oxygenated Krebs Henseleit solution, as shown in Figure 3.8, to which 0.2 mls of heparin (5000IU/ml) had been added. Contractions stopped immediately. The oesophagus was then pulled away from the trachea, thymus, pericardium and other excess tissue was pulled away from the heart using a sharp pair of forceps.

Figure 3.8:
Heart in 40ml cooled KH

3.7.3 Cannulation of aorta and left atrium for Langendorff perfusion

Using fine tipped forceps the heart was held by the aorta and slipped about 3mm onto a grooved cannula on a standard Langendorff apparatus (Figure 3.9a) and held in place by a haemostat until sutured (Figure 3.9b). The heart was perfused in a retrograde fashion with a perfusion pressure of 90 cm H_2O (68 mmHg). The lungs were removed rapidly one lobe at a time to ensure pulmonary veins were left intact. This is shown in Figure 3.9b and 3.9c below.

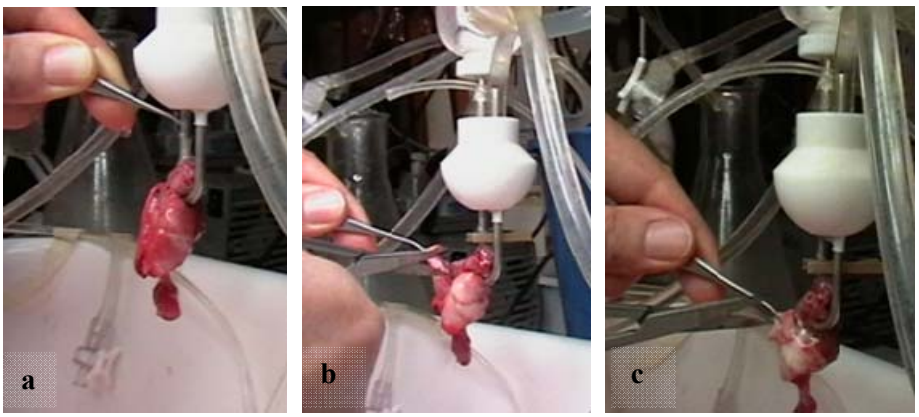

Figure 3.9: Steps 1-3 for attachment of heart to Langendorff apparatus
a. Cannulation of heart onto Langendorff apparatus; b. Removal of lung tissue and c. Removal of second and subsequent lobes of lung

The aorta was then sutured onto the cannula, shown in Figure 3.10a, and the heart turned so that the atria could be cannulated for working mode operation. A suture was placed around the pulmonary artery in preparation for cannulation for collection of coronary venous effluent. See Figure 3.10b below. For cold static storage experiments the pulmonary artery is not cannulated prior to the heart being arrested and detached for storage to avoid vessel wall damage. The artery is cannulated during warm intermittent studies in Chapter 4 and following reattachment of the heart following cold static storage in all studies. The superior and inferior vena cava,

shown in Figure 3.10c and Figure 3.10d, and pulmonary veins, shown in Figure 3.10e, were tied off to minimise leaks so that they were < 1 ml/min. This period of attachment allows for the washout of the coronary vessels and for equilibration with the perfusion fluid providing time for recovery from the brief hypoxic period (<1 minute) encountered during excision.

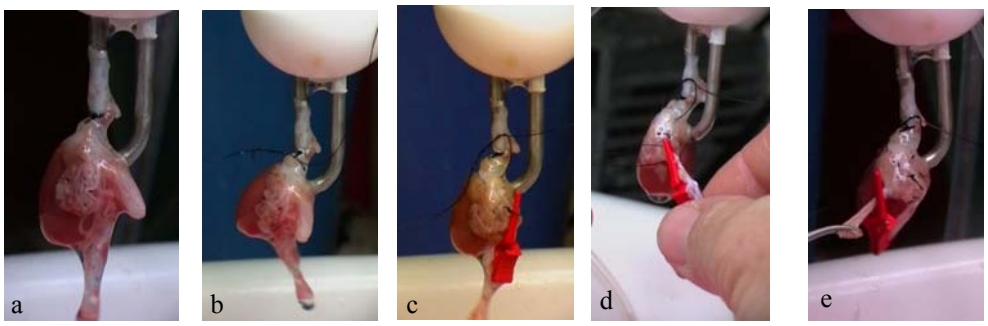

Figure 3.10: Steps 4-8 for attachment of heart to Langendorff apparatus
a. Aorta is sutured onto cannula; b. Placement of suture for coronary sinus cannulation; c.Clamping and tying off of Superior vena cava; d. Clamping and tying off of inferior vena cava and e.Clamping and tying off of pulmonary veins

3.8 Working Heart Perfusion

Figure 3.11: Insertion and tying off of left atrial cannula

The atrial cannula was inserted into a small incision made in the left atrial appendage. This was tied off, to enable working mode operation. See Figure 3.11. The preparation was then switched to the working mode by switching the supply of perfusate from the aorta to the left atrial cannula at a hydrostatic pressure of 10 cm H_2O (preload) and an afterload of 100 cmH_2O (76 mmHg). The heart was not placed in a thermostated jacket so that moderate decreases in temperature during arrest would mimic the clinical drift

in myocardial temperature. Hearts were stabilised for 15 minutes before converting to Langendorff non-working mode prior to arrest induction.

3.8.1 Functional and metabolic measurements

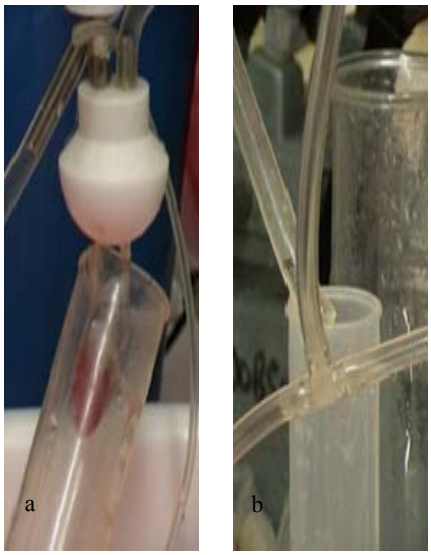

Figure 3.12:
Functional measurements
a: Collection of coronary flow
b: Collection of Aortic Flow

Aortic flow (AF), coronary flow (CF), heart rate (HR), systolic pressure (SP) and diastolic pressure (DP) were measured before, during and following arrest. Aortic pressures were measured continuously using a pressure transducer coupled to a MacLab 2e (ADInstruments, Australia). SP, DP and HR were calculated from the pressure trace using MacLab software. CF and AF were measured in volumetric cylinders as shown in Figures 3.12a and 3.12b. From these measurements the Cardiac output (CO) and rate pressure product (RPP) were calculated.

Arterial and venous perfusate pO_2 and pCO_2, pH and ion concentrations (Ca^{2+}, Cl^-, and Na^+) were measured using a Bayer 865 blood gas machine (Siemens Diagnostics, Aust). Lactate and troponin levels were measured from the venous effluent. No pacing or cardiac massage was employed during the reanimation phase in the working mode in order to mimic donor heart variability. While this potentially made comparisons difficult between groups, it allowed for measurement of spontaneous electrical recovery of hearts.

Figure 3.13:
Surface temperature check

The surface temperature of the heart was measured using a Cole-Palmer thermistor as shown in Figure 3.13. Previously we have shown that placement in the left heart chamber showed similar profiles as sub-auricular placement (Dobson, 2004). The perfusion apparatus was flushed weekly using 5 L of 3% hydrogen peroxide and 1% glacial acetic acid solution followed by a thorough rinse with 10 L 18 megaohm water.

3.8.2 Exclusion criteria

Criteria for exclusion of working hearts during the 15 mins equilibration period was a heart rate less than 200 beats/min, a systolic pressure less than 100 mmHg and coronary flow less than 10 ml/min. On average less than 1% of hearts were excluded for each study.

3.9 Intermittent Cardioplegic Delivery at Warmer Arrest Temperatures

The method of intermittent cardioplegia delivery in the isolated rat heart has been previously described by Dobson and Jones (Dobson, 2004). Arrest was induced in the Langendorff mode by a 5 min infusion of cardioplegic solution (50-100 ml) via the aorta at 37ºC and at constant pressure of 90 cm H_2O (68mmHg). During this infusion time taken to achieve full electrical and mechanical arrest was noted. Following cardioplegic infusion and complete mechanical and electrical arrest, the aorta was cross-clamped at the completion of infusion using a plastic non-traumatic aortic clip. Cardioplegia was replenished every 18 mins with a 2 mins infusion, after

which the cross-clamp was reapplied. After arrest using intermittent cardioplegia delivery, the heart was switched immediately to working mode and reperfused with oxygenated glucose-containing Krebs-Henseleit buffer at 37°C. The heart temperature during intermittent arrest ranged from 35°C during delivery to 25°C prior to the next delivery. Average heart temperature during the arrest period was 28 to 30°C.

3.10 Cold storage

The cold storage method employed in these studies was modified from that of Macdonald's group (Gao *et al.*, 2005). Baseline data were obtained for hearts in working mode at 37ºC. Arrest was induced by a 5 mins infusion of cardioplegia solution (50-100 ml) via the aorta at a constant pressure of 90 cm H_2O (68mmHg) in the Langendorff mode at normothermic temperatures (37ºC). Hearts were then gently removed from the aortic cannula and placed in 50 ml centrifuge tube containing their respective preservation solutions and immersed in the water bath set at 4°C for a period of 6 or 8 hours. After storage, the hearts were returned to the perfusion apparatus and freshly prepared and filtered arrest solutions were perfused in Langendorff mode. During reattachment to the aortic cannular hearts were slowly rewarmed for 5 mins before reanimation and reperfusion in working mode for 60 mins at 37°C using oxygenated KH (Chapter 4) or the same cardioplegia solution in which they had been stored (all other studies). AF, CF, HR, SP and DP were measured at predetermined time points before arrest and during the 60 minute reperfusion period, and were then compared with the baseline data for each heart. The CO and RPP were calculated from these parameters and also compared to pre-arrest data.

3.11 Determination of Coronary Vascular Resistance during Warmer Intermittent Delivery

Coronary vascular resistance (CVR) in megadyne sec cm^{-5} was calculated during each 2 mins cardioplegia delivery by dividing delivery pressure by flow (volume/sec) using the following equation:

$$CVR = \frac{1333 \text{ x mm Hg}}{(ml / \text{sec})} \text{ x } 10^{-6}$$

where 1mmHg = 1333 dynes cm^{-2} and 10^{-6} is a conversion factor from dynes to megadynes.

3.12 Calculation of Percent Total Tissue Water

Following the completion of the study hearts were freeze-clamped and stored at -80ºC until analysed for total tissue water and this was expressed as percent total tissue water (%TTW). Powdered tissue was obtained from all hearts following 60 min reperfusion and was dried to a constant weight at 85° C for up to 48 hours. Percent total tissue water (%TTW) was then determined by calculating the difference in wet weight and dry weight divided by wet weight and multiplied by 100. This method was validated by Ceislar et al, 1998 (Cieslar *et al.*, 1998).

3.13 Calculation of Myocardial Oxygen Consumption (MVO_2)

Myocardial oxygen consumption (MVO_2 μmole O_2/min/g dry wt heart) was calculated using the equation below (Dobson, 2004):

$$MVO_2 = \frac{(p_aO_2 - p_vO_2)}{(Bp - Vp)} \times \frac{\alpha O_2}{22.40} \times \frac{\text{Coronary Flow}}{\text{gm dry wt}} \times 1000$$

$$= \frac{\text{mmHg}}{\text{mmHg}} \times \frac{\text{ml/ml}}{\text{ml/mmol}} \times \frac{\text{ml/min}}{\text{gm dry wt}} \times 1000$$

where P_aO_2 and P_vO_2 are the partial pressures of oxygen (mmHg) in the arterial and venous perfusion lines. B_p is the barometric pressure (760mmHg) and V_p is the water vapour pressure at 37°C = 47.1 mmHg. The molar volume for oxygen at standard temperature and pressure (STP) was 22.40 ml/mM (Dobson, 2004). The αO_2 is the Bunsen solubility coefficient defined as that volume of oxygen gas dissolved in one ml of solution at a specified temperature reduced to STP (0°C, 760 mmHg). Coronary venous effluent was measured in ml/min and heart weight expressed as g dry wt. Percent total tissue water (%TTW) was determined by the difference in wet weight and dry weight divided by wet weight and multiplied by 100.

3.14 Measurement of Lactate, ATP and Troponin T Release during the Final Minute of the Warm Reperfusion

Coronary effluent (1.0 – 1.5 ml) was collected in 1.5 ml Eppendorf tubes during the last minute of the rewarming period and stored at -20°C until analysis. Lactates were analysed on Cobas Integra 800 using a colorimetric kit (Roche Diagnostics, Australia). Output results were in mmol/L and converted to µmol/min/g dry wt using coronary effluent flow (CF) and heart weight as in the equation below:

Lactate (µmol/min/g dry wt) = Lactate (µmol/ml) x CF (ml/min)

x 1/ heart weight (g dry wt)$^{-1}$

3.15 Calculation of Total ATP Production during Warm Reperfusion

Total ATP production was calculated using the following equation:

$$\text{Total ATP (}\mu\text{mol/g dry weight heart)} = ATP_{aerobic} + ATP_{anaerobic}$$

ATP (aerobic) was defined as the total ATP produced via mitochondrial oxidative phosphorylation assuming 1.0 µmol O_2 forms 6.0 µmol ATP for a P:O ratio of 3.0 according to the equation below:

$$\text{ATP (aerobic)} = MVO_2\ (\mu\text{mol } O_2\text{/min/gm dry wt}) \times 6\ (6\ \mu\text{mol ATP/}\mu\text{mol } O_2)$$

ATP (anaerobic) was defined as the ATP produced from anaerobic glycogenolytic metabolism where 1 µmole glucosyl unit from glycogen forms 2 µmoles of lactate with the formation of 3 µmoles ATP. ATP (anaerobic) was calculated from lactate output using the equation below:

$$\text{ATP (anaerobic)} = \text{lactate (}\mu\text{mol/min/g dry wt)} \times 3/2\ (3\ \mu\text{mol ATP/2 }\mu\text{mol lactate})$$

This equation assumes that ATP generated anaerobically was principally derived from endogenous heart glycogen. The lactate and O_2 consumption measurements were taken during the last minute of the 5 mins rewarming period with the different oxygenated reperfusion solutions.

3.16 Troponin T release at 5 min Warm Reperfusion

Troponin T was measured on fresh effluent using the Roche Cardiac T Quantitative test on the Roche Cardiac Reader System (Roche Diagnostics Australia). The lower detection limit for this assay is 0.03 μg/L. Interpretation of troponin T results is based on the following reference ranges: (0.03 - 0.05 μg/L) no myocardial damage; (0.05-0.1 μg/L) myocardial damage possible; (>0.1 μg/L) myocardial damage detected (Collinson *et al.*, 2001).

3.17 Statistical Analysis

All results are expressed as mean ± standard error of the mean (SEM). Statistics were performed using SPSS software. One-way Analysis of Variance (ANOVA) was used to compare rat weights, arrest times and time to first beat, heart rate, developed pressures, rate-pressure product, aortic flow, coronary flow and cardiac output at separate time points. Two-way ANOVA with repeated measures was used to compare functional variables during 60 min recovery over multiple time points for the different treatment groups. Post hoc analysis was performed using Bonferroni and Dunnett (2-sided) post hoc tests. The alpha level of significance for all experiments was set at $p<0.05$.

CHAPTER 4: FROM AL CARDIOPLEGIA TO PRESERVATION OF THE DONOR HEART

4.1 Introduction

As discussed in the introduction the composition of current cardiac harvest and preservation solutions is based on 3 principles;

1. rapid electrical and mechanical arrest of the heart;
2. provision of a physical and biochemical environment to maintain the viability of the structural components of the tissue
3. minimisation of reperfusion injury.

These solutions rely almost exclusively on hyperkalaemia for rapid arrest and hypothermic storage (4°C) to halt metabolism (Jahania *et al.*, 1999; Maathuis *et al.*, 2007). Despite these protective strategies, safe adult donor heart preservation times from harvest to reanimation remain limited to about 4-5 hours of cold-ischaemic storage (2° to 4°C) (Jahania *et al.*, 1999). Longer ischaemic storage times have been linked to primary graft failure and cardiac allograft vasculopathy (Bourge *et al.*, 1993; Schmauss and Weis, 2008).

To date a non-depolarising, normokalaemic AL solution developed in our laboratory for use as a warm cardioplegic solution in cardiovascular surgery has not been tested at the colder temperatures and longer time frames required for organ preservation (Dobson, 2004). The aim of this chapter was to compare in isolated rat hearts the cardioprotection provided by AL cardioplegia using two modes of delivery, cold storage (4°C) and warm with intermittent flushes (28-30 °C). Functional recoveries

of hearts stored in AL were compared with those of hearts stored in the current gold standard, Celsior preservation solution for 2 hours arrest and 6 hours arrest. The longer 6 hour arrest is representative of longer storage and transport times encountered during heart transplantation.

The effect of adding 1 and 5 mM L-pyruvate to the AL solution at the warmer arrest temperature was also investigated. As an additive to AL cardioplegia at warmer temperatures L-pyruvate may improve post ischaemic recoveries of isolated rat hearts. Previous studies have demonstrated that supraphysiological concentrations of L-pyruvate (2-10 mmol/L) can enhance post ischaemic performance of hearts undergoing ischaemia reperfusion (Bunger *et al.*, 1989; Dobsak *et al.*, 1999; Knott *et al.*, 2005; Mallet *et al.*, 2005).

4.2 Experimental Design

4.2.1 Experimental groups

Rat hearts were randomly assigned to one of 12 cardioplegia/preservation groups:

1) AL alone for 2 hours of cold (4°C) storage (n=8);

2) AL alone for 6 hours of cold (4°C) storage (n=8);

3) AL alone for 2 hours arrest with warmer (28-30°C) intermittent infusions (n=8);

4) AL alone for 6 hours arrest with warmer (28-30°C) intermittent infusions (n =8);

5) AL + 1 mM L-pyruvate for 2 hours arrest with warmer (28-30°C) intermittent infusions (n =8);

6) AL + 1 mM L-pyruvate for 6 hours of warmer (28-30°C) arrest with intermittent infusions (n =8);

7) AL + 5 mM L-pyruvate for 2 hours of warmer (28-30°C) arrest with intermittent infusions (n =8);

8) AL + 5 mM L-pyruvate for 6 hours arrest with warmer (28-30°C) intermittent infusions (n =8);

9) Celsior for 2 hours of cold (4°C) storage (n=6);

10) Celsior for 6 hours of cold (4°C) storage (n=5);

11) Celsior for 2 hours of warmer (28-30°C) arrest with intermittent infusions (n=6);

12) Celsior for 6 hours of warmer (28-30°C) arrest with intermittent infusions (n=7).

While Celsior is an extracellular-type solution and not originally designed for warmer temperatures, treatment groups 11 and 12 were included for comparison with AL cardioplegia, as Celsior was designed in part to reduce injury to the heart in the transition from cold-to-warm reperfusion (Menasche *et al.*, 1994). The number of

Celsior hearts included in both the 2 and 6 hour arrest groups was less than that of the AL hearts due to both time and financial constraints. Glutathione an important component of Celsior solution as it is a potent anti-oxidant, appears to be unstable in solution (Gnaiger *et al.*, 2000); when the Celsior data was analysed a number of hearts had to be excluded due to poor performance, this could possibly be attributed to the fact that the glutathione had auto-oxidised. The groups were repeated with fresh glutathione however, the components of the Celsior solution are expensive (particularly the lactobionate) and therefore as a consequence, numbers were limited by cost and time constraints.

4.2.2 Study design

Hearts were rapidly removed from anaesthetised rats and placed in ice-cold heparinised modified KH buffer (Appendix A) as described in Chapter 3: Materials and Methods section 3.4. Briefly, hearts were attached to a Langendorff apparatus and perfused at a pressure head of 90 cm H_2O (68 mmHg). The hearts were allowed to equilibrate in working mode for 15 minutes prior to taking baseline measurements. See Figure 4.1 below. Hearts were then switched to Langendorff mode and arrested with a 5 minute normothermic (37ºC) induction dose of the cardioplegic solution (AL+ 0, 1 or 5 mM L-pyruvate (groups 1-8) or Celsior (groups 9-12)). Groups (3-8, 11 and 12) in the warm intermittent study were left on the apparatus and cardioplegia was replenished every 18 mins with a 2 minute infusion for the arrest period (2 or 6 hours). Coronary flows were measured during the 2 minutes flushes so that coronary vascular resistance (CVR) could be determined. After arrest using intermittent cardioplegia delivery, the heart was switched

immediately to the working mode and reperfused with oxygenated glucose-containing Krebs-Henseleit (KH) buffer at 37°C.

Those hearts in the cold storage groups (1, 2, 9 and 10) were removed after the induction dose, placed in the same solution for storage in a refrigerated water bath for 2 or 6 hours. Following storage in the various preservation solutions, hearts were then reattached in Langendorff mode using the same but freshly prepared oxygenated arrest solution as the perfusate (5 mins). Once canulated, hearts were then switched to working mode and the functional measurements taken at 10, 15, 30, 45 and 60 mins of reperfusion. See Figure 4.1 below. The primary endpoints (as discussed in section 3.1) HR, AF, CF, SP, DP, CO and RPP were compared to pre-arrest values and expressed as a percentage recovery of pre-arrest values. Hearts were freeze-clamped and stored at -80ºC until analysed for total tissue water and this was expressed as percent total tissue water (%TTW).

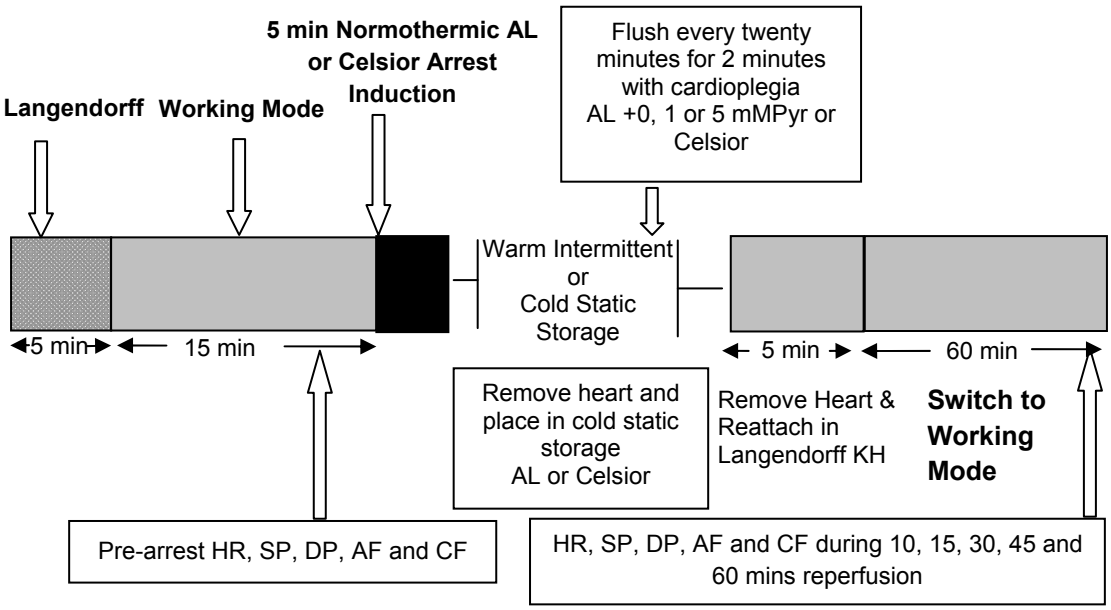

Figure 4.1: Experimental protocol
Isolated rat hearts (n=88) were arrested in AL Cardioplegia + 0, 1 or 5 mM Pyr or Celsior. The warm intermittent group was left on the apparatus and perfused every 18 minutes with 2 minute flushes for 2 or 6 hours. The hearts in the cold static storage groups were removed from the apparatus and stored in 50ml of the same solution at 4ºC for 2 or 6 hours. Each group was rewarmed in Langendorff mode for 5 min with their respective oxygenated arrest solution. (HR) Heart rate; (SP) systolic pressure; (DP) diastolic pressure; (AF) aortic flow and (CF) coronary flow.

4.3 Results

4.3.1 Arrest times

Full normothermic electrochemical arrest in AL without added L-pyruvate was achieved in 16 ± 2 sec (n=32) and this was not significantly different in the presence of 1 mM L-pyruvate (25 ± 8 sec, n=16) or 5 mM L-pyruvate (18 ± 4 sec, n=16). Celsior hearts took a longer time to arrest (39 ± 4 sec, n=24; $p<0.05$); this was significantly different from the AL arrest groups.

4.3.2 Cold storage (4°C): Functional recoveries following 2 hour arrest

The functional data for the AL and Celsior groups undergoing cold static storage for 2 hours are shown in Tables 4.1 and 4.2. After 2 hrs, there were no functional differences between the AL and Celsior groups ($p>0.05$). At 60 min reperfusion in working mode, return of AF was between 65 - 70%, CF was between 82 - 86%, CO was between 69 - 72%, HR was between 94 - 103%, SP was between 92 - 93%, DP was between 99 - 106%, RPP was between 92 - 98%, of pre-arrest values See Tables 4.1 and 4.2 below.

4.3.3 Warmer arrest with intermittent flushes (28-30°C): Functional recoveries following 2 hour arrest

Recovery of functional parameters in hearts arrested at warmer temperatures for 2 hours, are shown in Tables 4.1 and 4.2 below. After 2 hours arrest with AL cardioplegia, recovery of pre-arrest AF was 75 ± 3%, CF was 94 ± 5% and HR was 106 ± 5% (Tables 4.1 and 4.2). The addition of 1 mM L-pyruvate increased recovery of aortic flow from 75 ± 3% to 83 ± 3%; however, this improvement was not significant ($p>0.05$). Similarly the addition of 1 mM L-pyruvate led to no significant changes in HR (106 ± 4%) or CF (100 ± 3%) compared with AL alone (Tables 4.1 and 4.2). The addition of 5 mM L-pyruvate to AL led to decreases in HR, AF and CF of around 10-14%, however; these changes were not significantly different from AL supplemented with 0 or 1 mM L-pyruvate. RPP for AL with 0, 1 and 5 mM L-pyruvate were 90-99% of pre-arrest values and not significantly different. CO was 80 ± 3, 86 ± 2 and 79 ± 3% at 60 min reperfusion following 2 hours arrest for the three AL groups, and not significantly different. After a 2 hour arrest with Celsior cardioplegia, recovery of pre-arrest AF was 26 ± 7%, CF was 82 ± 18% and HR was

82% of pre-arrest values (Tables 4.1 and 4.2). SP and DP were 74 and 85% of pre-arrest values, RPP was 73% and CO was 56% of pre-arrest values (Tables 4.1 and 4.2). Despite all three groups of AL hearts having a 65 to 80% higher AF and 40-65% higher CO than Celsior hearts, the differences were not significant, due in part to the higher standard errors in the Celsior group after 2 hrs of arrest at the warmer temperatures (Tables 4.1 and 4.2).

Table 4.1: Functional parameters of isolated working rat hearts during pre-arrest and reperfusion following 2-hour arrest (AF, CF and CO).
Recovery of aortic flow (AF), coronary flow (CF) and cardiac output (CO) with adenosine-lignocaine (AL) cardioplegia and Celsior following 2 hours arrest adenosine - lignocaine cardioplegia and Celsior during cold storage (4°C) and AL cardioplegia with 0 mM, 1mM and 5mM L-pyruvate, and Celsior intermittently perfused every 20 min for 2 min at 68 mmHg and 37°C. Data presented as means ± SEM (n=44).

Time during pre-arrest and recovery	Treatment	n	Aortic Flow (ml/min)	Coronary Flow (ml/min)	Cardiac Output (ml/min)
15 mins Pre-Arrest	AL cold (4°C)	8	62 ± 1	21 ± 1	83 ± 2
	Celsior (4°C)	6	60 ± 4	20 ± 2	80 ± 5
	AL (28-30°C)	8	48 ± 2	16 ± 1	64 ± 2
	+ 1mM Pyr	8	46 ± 2	16 ± 1	62 ± 3
	+ 5mM Pyr	8	51 ± 3	18 ± 1	70 ± 4
	Celsior (28-30°C)	6	56 ± 1	17 ± 1	73 ± 2
10 mins Recovery	AL cold (4°C)	8	30 ± 5	14 ± 2	44 ± 7
	Celsior (4°C)	6	19 ± 9	8 ± 4	27 ± 12
	AL (28-30°C)	8	32 ± 4	16 ± 1	48 ± 4
	+ 1mM Pyr	8	32 ± 4	18 ± 1	49 ± 4
	+ 5mM Pyr	8	29 ± 1	18 ± 1	46 ± 2
	Celsior (28-30°C)	6	9 ± 8	8 ± 5	17 ± 12
15 mins Recovery	AL cold (4°C)	8	42 ± 5	15 ± 1	56 ± 6
	Celsior (4°C)	6	34 ± 5	13 ± 1	47 ± 5
	AL (28-30°C)	8	32 ± 3	15 ± 1	47 ± 3
	+ 1mM Pyr	8	31 ± 4	16 ± 1	46 ± 3
	+ 5mM Pyr	8	28 ± 3	17 ± 1	44 ± 5
	Celsior (28-30°C)	6	23 ± 8	14 ± 3	38 ±10
30 mins Recovery	AL cold (4°C)	8	46 ± 3	15 ± 1	61 ± 2
	Celsior (4°C)	6	40 ± 3	14 ± 1	54 ± 3
	AL (28-30°C)	8	37 ± 2	16 ± 1	52 ± 3
	+ 1mM Pyr	8	37 ± 2	16 ± 1	54 ± 2
	+ 5mM Pyr	8	41 ± 3	16 ± 1	58 ± 4
	Celsior (28-30°C)	6	26 ± 7	13 ± 3	40 ± 8
45 mins Recovery	AL cold (4°C)	8	43 ± 3	17 ± 1	58 ± 2
	Celsior (4°C)	6	38 ± 2	15 ± 1	53 ± 2
	AL (28-30°C)	8	36 ± 2	16 ± 1	52 ± 3
	+ 1mM Pyr	8	40 ± 2	16 ± 1	56 ± 2
	+ 5mM Pyr	8	40 ± 3	16 ± 1	56 ± 4
	Celsior (28-30°C)	6	27 ± 6	15 ± 3	41 ± 9
60 mins Recovery	AL cold (4°C)	8	42 ± 3	18 ± 1	60 ± 2
	Celsior (4°C)	6	38 ± 1	16 ± 1	54 ± 1
	AL (28-30°C)	8	36 ± 3	15 ± 1	51 ± 3
	+ 1mM Pyr	8	38 ± 2	16 ± 1	53 ± 2
	+ 5mM Pyr	8	39 ± 3	15 ± 1	55 ± 4
	Celsior (28-30°C)	6	26 ± 7	14 ± 3	41 ± 9

Table 4.2: Functional parameters of isolated working rat hearts during pre-arrest and reperfusion following 2-hour arrest (HR, SP, DP and RPP).
Recovery of heart rate (HR), systolic pressure (SP), diastolic pressure (DP) and rate pressure product (RPP) following 2 hour arrest with adenosine-lignocaine (AL) cardioplegia and Celsior during cold storage (4°C) and AL cardioplegia with 0 mM, 1mM and 5mM L-pyruvate, and Celsior intermittently perfused every 20 min for 2 min at 68 mmHg and 37°C. Data presented as means ± SEM (n=44).

Time during pre-arrest and recovery	Treatment	n	Heart Rate (bpm)	Systolic Pressure (mmHg)	Diastolic Pressure (mmHg)	Rate Pressure Product (mmHg/min)
15 mins Pre-Arrest	AL cold (4°C)	8	288 ± 4	121 ± 2	60 ± 1	34696 ± 664
	Celsior (4°C)	6	285 ± 9	121 ± 1	60 ± 0	34406 ± 1153
	AL (28-30°C)	8	268 ± 8	121 ± 2	60 ± 0	32299 ± 920
	+ 1mM Pyr	8	270 ± 8	121 ± 1	59 ± 1	32688 ± 738
	+ 5mM Pyr	8	274 ± 9	119 ± 1	56 ± 2	32499 ±1098
	Celsior (28-30°C)	6	281 ± 6	123 ± 2	60 ± 0	34397 ± 682
10 mins Recovery	AL cold (4°C)	8	211 ± 31	108 ± 15	53 ± 6	25372 ± 3748
	Celsior (4°C)	6	146 ± 58	66 ± 25	35 ± 11	16695 ± 7389
	AL (28-30°C)	8	234 ± 15	126 ± 4	60 ± 0	29515 ± 2306
	+ 1mM Pyr	8	210 ± 11	126 ± 3	58 ± 3	26266 ± 993
	+ 5mM Pyr	8	206 ± 15	129 ± 4	51 ± 3	26149 ± 1061
	Celsior (28-30°C)	6	82 ± 52	46 ± 24	26 ± 12	10067 ± 6576
15 mins Recovery	AL cold (4°C)	8	284 ± 11	113 ± 4	59 ± 1	31944 ± 979
	Celsior (4°C)	6	263 ± 21	108 ± 3	63 ± 2	28085 ± 1991
	AL (28-30°C)	8	238 ± 8	113 ± 4	60 ± 0	26833 ± 1312
	+ 1mM Pyr	8	240 ± 10	116 ± 2	65 ± 3	27874 ± 959
	+ 5mM Pyr	8	230 ± 14	115 ± 3	59 ± 1	26223 ± 1124
	Celsior (28-30°C)	6	203 ± 45	95 ± 20	52 ± 11	23320 ± 5424
30 mins Recovery	AL cold (4°C)	8	293 ± 14	115 ± 4	59 ± 1	33359 ± 694
	Celsior (4°C)	6	269 ± 14	113 ± 3	64 ± 2	30228 ± 1595
	AL (28-30°C)	8	268 ± 12	111 ± 4	60 ± 0	29633 ± 1173
	+ 1mM Pyr	8	265 ± 9	113 ± 3	64 ± 2	29679 ± 735
	+ 5mM Pyr	8	249 ± 7	116 ± 2	59 ± 1	28924 ± 616
	Celsior (28-30°C)	6	226 ± 46	89 ± 19	52 ± 10	24152 ± 5038
45 mins Recovery	AL cold (4°C)	8	298 ± 13	115 ± 5	59 ± 0	33905 ±1008
	Celsior (4°C)	6	276 ± 14	113 ±3	63 ± 2	30888 ± 1406
	AL (28-30°C)	8	275 ± 10	111 ± 4	60 ± 0	30485 ± 1147
	+ 1mM Pyr	8	285 ± 9	113 ± 3	65 ± 3	31965 ± 920
	+ 5mM Pyr	8	250 ± 6	116 ± 2	60 ± 0	28869 ± 401
	Celsior (28-30°C)	6	235 ± 47	90 ± 18	51 ± 10	25433 ± 5209
60 mins Recovery	AL cold (4°C)	8	303 ± 14	113 ± 3	59 ± 1	33991 ± 751
	Celsior (4°C)	6	267 ± 10	111 ± 3	64 ± 2	31773 ± 1010
	AL (28-30°C)	8	285 ± 15	110 ± 4	60 ± 0	31233 ± 1451
	+ 1mM Pyr	8	287 ± 14	113 ± 3	65 ± 3	32336 ± 1629
	+ 5mM Pyr	8	256 ± 9	114 ± 3	59 ± 1	28963 ± 431
	Celsior (28-30°C)	6	230 ± 47	91 ± 19	51 ± 10	25043 ± 5167

4.3.4 Cold storage (4°C): Functional recoveries following 6 hours of arrest

The functional data for the AL and Celsior groups undergoing cold storage for 6 hours are shown in Tables 4.3 and 4.4. After 6 hours of cold storage (4°C), the AL hearts spontaneously returned 61% of AF, 80% of CF, 68% of CO, 105% of HR, 90% of SP, 105% of DP and 94% of RPP, of their pre-arrest values. As shown in Tables 4.3, 4.4 and Figure 4.2. In contrast, the Celsior hearts returned significantly lower functional parameters over 60 min of reperfusion ($p<0.05$). Celsior hearts returned 46% of AF, 50% of CF and 47% of CO, 81% of HR, 69% of SP, 93% of DP and 63% of RPP, at 60 min reperfusion after 6 hours storage arrest compared to their pre-arrest values. This is shown in Tables 4.3 and 4.4 and Figure 4.2. Despite the same protocol of re-attachment and rewarming of hearts (5 min) after cold storage, Celsior hearts took 5 mins longer to reanimate in working mode than AL hearts (Figure 4.2), and an increased number of early reperfusion arrhythmias were observed in Celsior hearts when compared with AL hearts. Although these were not recorded as ECG's were not measured during the reperfusion protocol. AL hearts returned a significantly higher cardiac output (45% higher) than Celsior hearts over 60 mins of reperfusion after 6 hrs arrest. See Figure 4.2 below.

Table 4.3: Functional parameters of isolated working rat hearts during pre-arrest and reperfusion following 6 hour arrest (AF, CF and CO)
Recovery of aortic flow (AF), coronary flow (CF) and cardiac output (CO) with adenosine-lignocaine (AL) cardioplegia and Celsior following 2 hours arrest adenosine - lignocaine cardioplegia and Celsior during cold storage (4°C) and AL cardioplegia with 0 mM, 1mM and 5mM L-pyruvate, and Celsior intermittently perfused every 20 min for 2 min at 68 mmHg and 37°C. Data presented as means ± SEM (n=44).

Time during pre-arrest and recovery	Treatment	n	Aortic Flow (ml/min)	Coronary Flow (ml/min)	Cardiac Output (ml/min)
15 mins Pre-Arrest	AL cold (4°C)	8	61 ± 2	20 ± 2	81 ± 3
	Celsior (4°C)	5	54 ± 4	22 ± 2	76 ± 5
	AL (28-30°C)	8	48 ± 2	17 ± 1	65 ± 3
	+ 1mM Pyr	8	50 ± 3	16 ± 1	65 ± 4
	+ 5mM Pyr	8	49 ± 2	17 ± 1	65 ± 2
	Celsior (28-30°C)	7	54 ± 1	18 ± 1	72 ± 1
10 mins Recovery	AL cold (4°C)	8	16 ± 5	9 ± 3	25 ± 9
	Celsior (4°C)	5	0 ± 0	0 ± 0	0 ± 0
	AL (28-30°C)	8	10 ± 3	10 ± 2	26 ± 3
	+ 1mM Pyr	8	24 ± 3*	15 ± 1	37 ± 4*
	+ 5mM Pyr	8	17 ± 3	14 ± 1	31 ± 3
	Celsior (28-30°C)	7	0 ± 0	1 ± 1	1 ± 1
15 mins Recovery	AL cold (4°C)	8	30 ± 4*	15 ± 1*	45 ± 4*
	Celsior (4°C)	5	17 ± 7	10 ± 3	27 ± 10
	AL (28-30°C)	8	19 ± 3	12 ± 1	31 ± 4
	+ 1mM Pyr	8	26 ± 2*	14 ± 1	41 ± 3*
	+ 5mM Pyr	8	15 ± 2	14 ± 1	29 ± 2
	Celsior (28-30°C)	7	0 ± 0	1 ± 1	1 ± 1
30 mins Recovery	AL cold (4°C)	6	39 ± 3*	16 ± 2*	53 ± 3*
	Celsior (4°C)	5	22 ± 7	12 ± 2	34 ± 9
	AL (28-30°C)	8	21 ± 2	12 ± 1	33 ± 3
	+ 1mM Pyr	8	32 ± 3*	13 ± 1	47 ± 4*
	+ 5mM Pyr	8	24 ± 3	13 ± 1	37 ± 3
	Celsior (28-30°C)	7	0 ± 0	1 ± 1	1 ± 1
45 mins Recovery	AL cold (4°C)	6	39 ± 3	16 ± 2	55 ± 3
	Celsior (4°C)	5	24 ± 7	12 ± 2	36 ± 10
	AL (28-30°C)	8	24 ± 2	13 ± 1	37 ± 2
	+ 1mM Pyr	8	35 ± 2*	13 ± 1	49 ± 3*
	+ 5mM Pyr	8	25 ± 3	13 ± 1	38 ± 4
	Celsior (28-30°C)	7	0 ± 0	1 ± 1	1 ± 1
60 mins Recovery	AL cold (4°C)	8	37 ± 3*	16 ± 2*	55 ± 3*
	Celsior (4°C)	5	25 ± 7	11 ± 2	36 ± 10
	AL (28-30°C)	8	24 ± 2	13 ± 1	36 ± 3
	+ 1mM Pyr	8	35 ± 3*	13 ± 1	49 ± 4*
	+ 5mM Pyr	8	24 ± 3	13 ± 1	37 ± 4
	Celsior (28-30°C)	7	0 ± 0	1 ± 1	1 ± 1

***denotes significance between treatment groups p<0.05.**
Celsior (28-30°C) group was excluded from statistical analysis as they were non-viable (dead) after 6 hour storage period.

Table 4.4: Functional parameters of isolated working rat hearts during pre-arrest and reperfusion following 6 hour arrest (HR, SP, DP and RPP)
Recovery of heart rate (HR), systolic pressure (SP), diastolic pressure (DP) and rate pressure product (RPP) following 2 hour arrest with adenosine-lignocaine (AL) cardioplegia and Celsior during cold storage (4°C) and AL cardioplegia with 0 mM, 1mM and 5mM L-pyruvate, and Celsior intermittently perfused every 20 min for 2 min at 68 mmHg and 37°C. Data presented as means ± SEM (n=44).

Time during pre-arrest and recovery	Treatment	n	Heart Rate (bpm)	Systolic Pressure (mmHg)	Diastolic Pressure (mmHg)	Rate Pressure Product (mmHg/min)
15 mins Pre-Arrest	AL cold (4°C)	8	279 ± 9	123 ± 3	60 ± 0	34296 ± 895
	Celsior (4°C)	5	295 ± 19	121 ± 4	60 ± 0	35529 ± 1744
	AL (28-30°C)	8	297 ± 10	124 ± 3	60 ± 0	36989 ± 1386
	+ 1mM Pyr	8	271 ± 7	122 ± 2	60 ± 0	33253 ± 1245
	+ 5mM Pyr	8	264 ± 10	120 ± 0	56 ± 2	31695 ± 1151
	Celsior (28-30°C)	7	259 ± 5	127 ± 2	60 ± 0	32920 ± 306
10 mins Recovery	AL cold (4°C)	8	129 ± 44	74 ± 24	39 ± 12	14861 ± 5206
	Celsior (4°C)	5	8 ± 8	7 ± 3	6 ± 3	96 ± 96
	AL (28-30°C)	8	153 ± 35	85 ± 19	46 ± 10	23073 ± 1603
	+ 1mM Pyr	8	201 ± 8	128 ± 4	63 ± 3	25628 ± 1246
	+ 5mM Pyr	8	216 ± 8	111 ± 5	56 ± 3	23850 ± 822
	Celsior (28-30°C)	7	7 ± 6	8 ± 6	5 ± 5	309 ± 289
15 mins Recovery	AL cold (4°C)	8	253 ± 14*	108 ± 4*	63 ± 2*	27144 ± 1653*
	Celsior (4°C)	5	189 ± 46	75 ± 24	44 ± 12	16649 ± 6141
	AL (28-30°C)	8	230 ± 16*	111 ± 2*	61 ± 1*	25548 ± 1793*
	+ 1mM Pyr	8	256 ± 10*	114 ± 2*	65 ± 3*	29110 ± 990*
	+ 5mM Pyr	8	238 ± 7*	101 ± 1*	60 ± 0*	24084 ± 678*
	Celsior (28-30°C)	7	0 ± 0	6 ± 3	4 ± 3	0 ± 0
30 mins Recovery	AL cold (4°C)	8	289 ± 13*	109 ± 3*	61 ± 1*	31476 ± 932*
	Celsior (4°C)	5	237 ± 47	88 ± 19	56 ± 12	24303 ± 6080
	AL (28-30°C)	8	259 ± 12*	111 ± 3*	61 ± 4*	28615 ± 1131*
	+ 1mM Pyr	8	269 ± 13*	113 ± 3*	63 ± 2*	30224 ± 1043*
	+ 5mM Pyr	8	269 ± 11*	103 ± 2*	60 ± 0*	27579 ± 1093*
	Celsior (28-30°C)	7	0 ± 0	4 ± 2	3 ± 2	0 ± 0
45 mins Recovery	AL cold (4°C)	8	291 ± 12*	111 ± 3*	63 ± 2*	32003 ± 883*
	Celsior (4°C)	5	247 ± 54	87 ± 19	54 ± 11	24966 ± 6144
	AL (28-30°C)	8	278 ± 11*	109 ± 2*	64 ± 2*	30128 ± 1086*
	+ 1mM Pyr	8	277 ± 15*	111 ± 3*	64 ± 2*	30634 ± 1290*
	+ 5mM Pyr	8	277 ± 10*	101 ± 1*	60 ± 0*	28083 ± 1198*
	Celsior (28-30°C)	7	0 ± 0	4 ± 2	1 ± 1	0 ± 0
60 mins Recovery	AL cold (4°C)	8	292 ± 12*	111 ± 3*	63 ± 2*	32392 ± 837*
	Celsior (4°C)	5	240 ± 61	84 ± 19	56 ± 12	22257 ± 6248
	AL (28-30°C)	8	281 ± 11*	109 ± 2*	65 ± 2*	30470 ± 1085*
	+ 1mM Pyr	8	286 ± 14*	113 ± 3*	63 ± 2*	32060 ± 982*
	+ 5mM Pyr	8	280 ± 11*	101 ± 1*	60 ± 0*	28353 ± 1299*
	Celsior (28-30°C)	7	0 ± 0	4 ± 2	1 ± 1	0 ± 0

***denotes significance between treatment groups p<0.05.**
Celsior (28-30°C) group was excluded from statistical analysis as they were non-viable (dead) after 6 hour storage period.

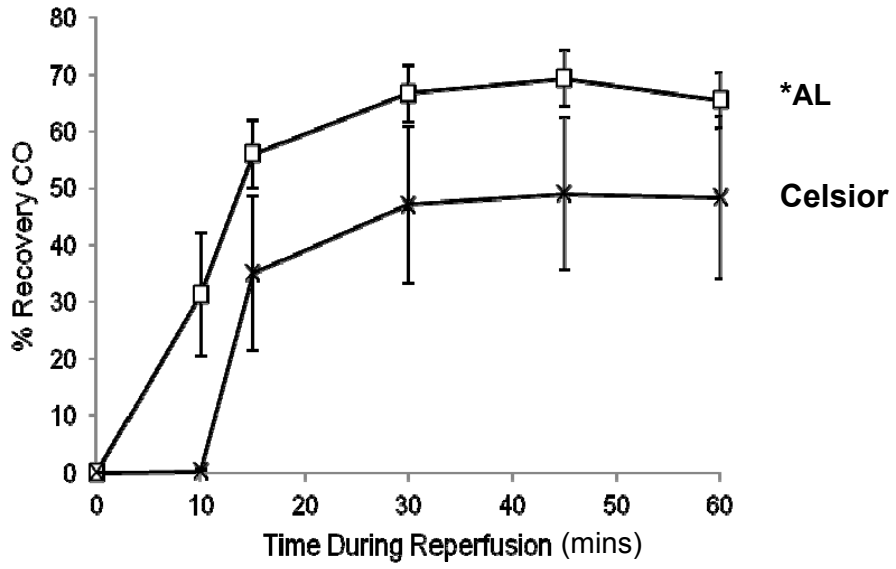

Figure 4.2: Recovery of cardiac output (%CO) following 6 hours cold (4°C) storage.
Percentage recovery of cardiac output during 60 minute reperfusion following 6 hours of cold storage with AL cardioplegia alone (□) (n=8) and Celsior (X) (n=5). Cardiac output is expressed as percentage of pre-arrest values, which were taken at 5 min before arrest. Data presented as means ± SEM (n=13)
* shows significant difference between the AL and Celsior ($p < 0.05$)

4.3.5 Warmer arrest with intermittent flushes (28-30°C): Functional recoveries following 6 hour arrest

Recovery of functional parameters for hearts arrested at warmer temperatures for 6 hours are shown in Tables 4.3 and 4.4. After 6 hours of intermittent cardioplegia, AL recovery of pre-arrest HR was 95 ± 3%, AF was 50 ± 3% and CF was 76 ± 4% at 60 minutes of reperfusion (Table 4.3). The addition of 1 mM L-pyruvate to AL cardioplegia significantly increased recovery of AF over the 60 min reperfusion period ($p<0.05$); however, not HR or CF (Tables 4.3 and 4.4). The increase in AF in the presence of 1 mM L-pyruvate was apparent at all reperfusion times (Table 4.3). CO was also significantly higher in hearts with AL in the presence of 1 mM L-pyruvate (49 ± 4 ml min^{-1}) compared with 0 mM L-pyruvate (36 ± 3 ml min^{-1}) or

with 5 mM L-pyruvate (37 ± 4 ml min^{-1}) (Table 4.3, Figure 4.3). RPP for AL with 0, 1 and 5 mM L-pyruvate were 82%, 96% and 90% of pre-arrest values; however, they were not significantly different. Under identical 6 hour arrest conditions, hearts perfused with Celsior failed to return a HR, developed pressures (4 mmHg), AF and CF (and therefore had zero CO) during the 60 min reperfusion (Figure 4.3, Tables 4.3 and 4.4). In direct contrast to the AL hearts, the Celsior hearts were visibly small, hard, distorted and discoloured.

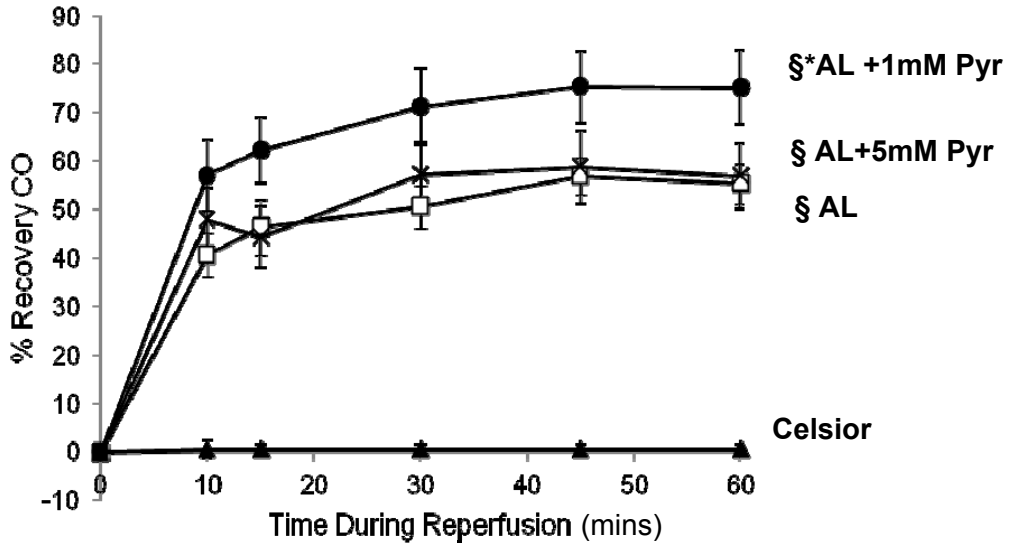

Figure 4.3: Recovery of cardiac output (%CO) following 6 hours warm (28 – 30 °C) arrest.
Treatment groups: AL cardioplegia supplemented with 0mM L-pyruvate (□) (n=8); 1 mM L-pyruvate (•) (n=8); 5 mM L-pyruvate (**X**) (n=8) and Celsior (▲) (n=7). Data presented as means ± SEM (n=31)
§ All AL groups had significantly higher CO than Celior ($p<0.0001$).
* AL with 1 mM L-pyruvate had significantly higher CO than AL with 0 or 5 mM L-pyruvate ($p<0.05$). See Chapter 3 Materials and Methods for details.

4.3.6 Warmer arrest with intermittent flushes (28-30°C): CVR and %TTW recoveries following 2 and 6 hours arrest

The coronary vascular resistance (CVR) prior to cardioplegic arrest for AL solution with 0, 1 and 5 mM L-pyruvate and Celsior hearts were 0.32 ± 0.02, 0.35 ± 0.01, 0.33 ± 0.01, and 0.31 ± 0.02 megadyne sec cm^{-5} respectively (Figure 4.4). The pre-arrest CVRs were not significantly different. During 2 min intermittent infusions; CVR in the three AL groups increased by 1.3 times at 3 hours to 1.7 and 1.9 times at 6 hours, however, these differences were not significant ($p>0.05$). The CVR in Celsior hearts increased 1.8 times (0.56 ± 0.08) at 3 hours, and 2.8 times (0.86 ± 0.17) at 6 hours respectively. At 3 hours, the CVR values for the Celsior group were significantly higher than AL with 5 mM L-pyruvate (0.56 vs 0.36) and AL solution alone (0.56 vs 0.42) ($p<0.05$). However, despite Celsior hearts having 30 to 76% higher CVR at the 6 hour reperfusion time point compared to the AL groups, the differences failed to reach statistical significance, in part due to the large standard errors of the Celsior group See Figure 4.4 below.

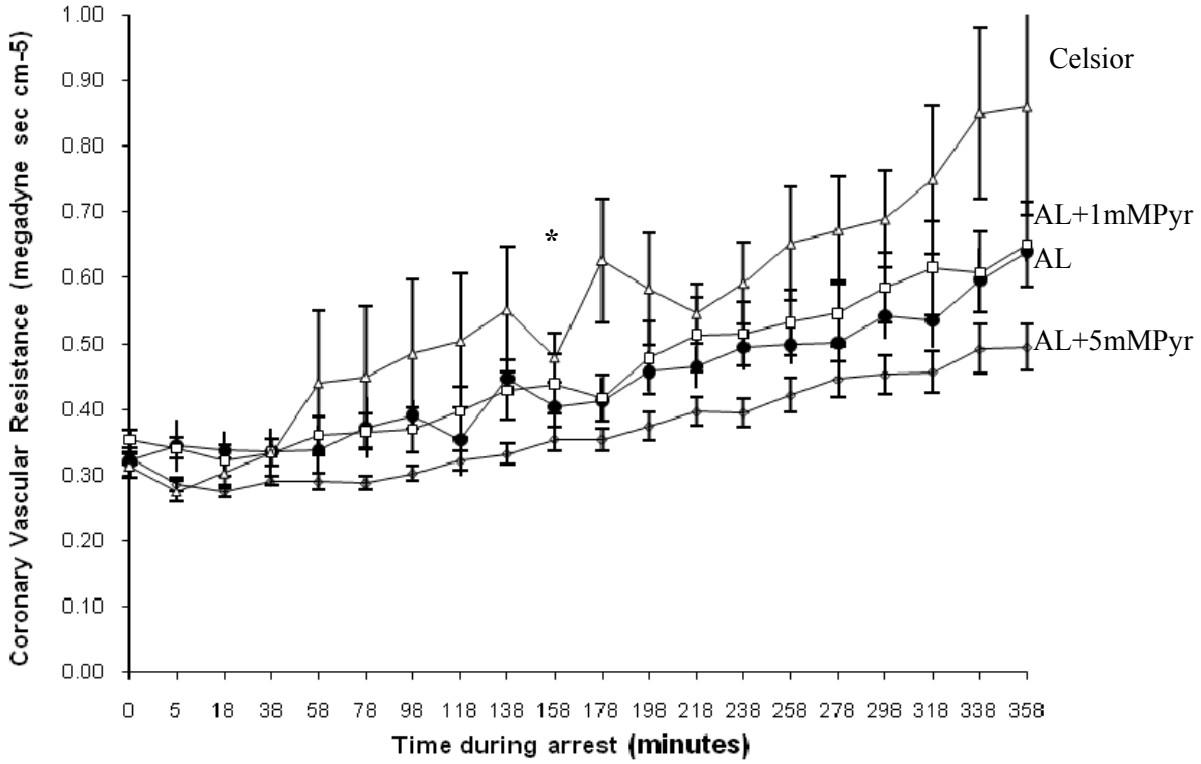

Figure 4.4: Coronary vascular resistance (CVR) during the 6 hr arrest period.
Coronary vascular resistance measured at each 2minute flush throughout the 6 hour arrest for hearts stored in AL cardioplegia supplemented with 0 mM L-pyruvate (●), 1 mM L-pyruvate (□) or 5 mM L-pyruvate (◊), and Celsior (Δ). CVR was calculated during the 2 min cardioplegia delivery times every 18 min over the total arrest time.
Data presented as means ± SEM (n=44). No significant differences were found between any groups at pre-arrest. There were no significant differences in the first two hours between the 2 and 6 hr arrest protocols for three AL groups tested.
* AL plus 5 mM Pyr and AL alone had significantly lower CVR than Celsior at 178 min. ($P<0.05$).

Values for percent total tissue water (%TTW) after 2 and 6 hours cold storage and 60 min reperfusion for AL was 84 ± 1% and 86 ± 1% respectively. For Celsior hearts, the %TTW was 82 ± 2% and 84 ± 1% for 2 and 6 hours cold storage respectively. Following warm intermittent arrest and 60 mins reperfusion, the %TTW were 87 ± 1%, 88 ± 1% and 87 ± 1% for AL with 0, 1 and 5 mM L-pyruvate respectively (Not significantly different, $p>0.05$). The total tissue water for Celsior hearts was 84 ± 4% and this was not significantly different from the AL groups.

4.4 Discussion

The current study identifies a non-depolarising, normokalaemic AL solution for preservation which is versatile at both 4°C and 28 to 30°C for up to 6 hours. The addition of 1 mM L-pyruvate to the warmer cardioplegia significantly improved the recovery of CO by 36% and returned 106% of HR, 93 to 105% of developed pressures, 70% of AF, and 81% of CF when compared with pre-arrest values working against a pressure head of 76 mmHg (100 cm H_2O). In addition, the coronary vascular resistance (CVR) in AL hearts increased less than twofold over the 6 hour arrest period at the warmer arrest temperatures. While AL cardioplegia showed functional equivalency to Celsior after 2 hours of cold and warm arrest, AL hearts showed significantly improved outcomes after 6 hours recovering 55% to 75% of cardiac output. After 6 hours of intermittent delivery at 28 to 30°C Celsior hearts failed to recover.

4.4.1 Cold storage for 2 and 6 hours (4°C)

2 Hours Cold Storage: As discussed in the introduction, cold storage is the most widely used form of preservation in heart transplant practices (Maathuis *et al.*, 2007). This study has demonstrated functional equivalence between AL cardioplegia and Celsior after 2 hours of cold storage with 70% return of CO (Table 4.1). However, after 6 hours of cold storage, the AL group had significantly higher functional recoveries with a 22% higher HR, 32% higher SP, 46% higher RPP, 48% higher AF and 45% higher CF and this translated into a 45% higher CO (Tables 4.3 and 4.4, Figure 4.2). Lower functional recoveries following long term storage in Celsior is in agreement with other studies. For example, the working rat heart study of the Macdonald group, reported a return of CO of 22 ± 9% (15% AF and 31% CF) at 30 mins reperfusion after 6 hours of storage in Celsior at 2-3°C (Gao *et al.*, 2005). They also reported that the cardiac output increased to about 70% with the addition of cariporide and glyceryltrinitrate to Celsior (Gao *et al.*, 2005). Unfortunately, there are few isolated working heart studies in the literature comparing Celsior with other preservation solutions. Most Celsior studies involve Langendorff hearts which perform no physical work. The working rat heart study of Nickless and colleagues showed 80% recovery of cardiac output (71% AF and 93% CF) after 5 hours cold (2°C) immersion in University of Wisconsin (UW) solution (Nickless *et al.*, 1998). Unfortunately, this group did not report functional recovery data beyond 15 min reperfusion, and therefore precludes any direct comparison with AL hearts over 60 min of reperfusion.

While functional recovery of AL arrested hearts was superior to Celsior after 6 hours cold storage, further studies are required to optimise the AL solution at these

hypothermic temperatures. A potentially clinically relevant feature of AL cardioplegia in the cold or warm is the spontaneous return of function of hearts without the need for defibrillation (Dobson, 2004; Sloots *et al.*, 2007). Non-depolarising, normokalaemic AL in a physiological glucose-containing KH solution spontaneously returned good functional recoveries at both cold and warm temperatures.

4.4.2 Intermittent flushes at warmer arrest temperatures (28 to 30°C)

This study has also shown that AL cardioplegia administered intermittently (2 min flushes every 18 min) at 28-30°C spontaneously returned 80% of CO after 2 hours (Table 4.1) and 55% CO after 6 hours (Tables 4.3 and 4.4). In contrast, under identical conditions, extracellular Celsior storage solution failed to protect the heart after 6 hours at 28-30°C (Table 4.3 and 4.4), with no cardiac output over the 60 mins of reperfusion (Table 4.3, Figure 4.4). This work supports the earlier study of Dobson and Jones (2004) which showed that St Thomas solution (Plegisol) performed poorly under identical conditions with only 14% of hearts recovering function after 4 hours of arrest at 28-30°C (Dobson, 2004). Ou and colleagues (Ou *et al.*, 1999) also reported that the hyperkalaemic University of Wisconsin (UW) solution is not protective and may be deleterious at warmer temperatures. To be fair, Celsior was not designed for warmer arrest and storage temperatures; it was originally designed as a perfusion fluid during initial donor arrest, post-storage graft implantation and early reperfusion (Menasche *et al.*, 1994).

4.4.2.1 Warm Intermittent Arrest was improved by the addition of pyruvate

When AL cardioplegia was supplemented with 1 mM L-pyruvate, the CO return increased from 55% to 75% after 60 min reperfusion ($p<0.05$) without an increase in heart rate or developed pressure (Tables 4.3 and 4.4). Enhanced postischaemic contractile performance and cytosolic phosphorylation potential has been reported by others in various models of ischaemia and reperfusion, including isolated rat hearts (Bunger *et al.*, 1989)) and clinical trials of cardiopulmonary bypass in both pigs (Knott *et al.*, 2005) and humans (Olivencia-Yurvati *et al.*, 2003). As in this study, the addition of pyruvate improved contractile force without a similar increase in heart rate. Pyruvate is a potent oxidisable myocardial fuel, and has been shown to decrease the cytosolic lactate/pyruvate ratio (lower $NADH/NAD^+$ ratio), enhance cytosolic $ATP/ADP/P_i$ ratio, improve sarcoplasmic reticular calcium transport and suppress hydroxyl free radical production and reduce oxidative stress (Mallet and Sun, 1999; Bassenge *et al.*, 2000; Hasenfuss *et al.*, 2002; Mallet *et al.*, 2003). These beneficial effects of pyruvate fortification have also been shown in stunned or injured hearts; however, benefits were only seen while the pyruvate was in contact with the myocytes and then only if the pyruvate fortified cardioplegia contained glucose (Mentzer *et al.*, 1989). In this study the pyruvate is washed out at the beginning of the reperfusion period, therefore the functional improvements seen here are due to pyruvate's effects during the intermittent ischaemic periods.

Possible reasons for why the addition of 1 mM L-pyruvate improved aortic flow and cardiac output compared to 0 or 5 mM L-pyruvate is difficult to reconcile without metabolic analysis. Although the precise mechanism is still unknown, higher pyruvate concentrations have been associated with a transient drop in contractile

performance (Hermann *et al.*, 2000). Pyruvate has also been reported in an NMR study of myocardial metabolism to increase the phosphcreatine/P_i ratio (Zweier and Jacobus, 1987), therefore the phosphate concentration of the AL solution may alter the effectiveness of increasing concentrations of L-pyruvate. Furthermore, a study by Zima and colleagues (2003) in isolated terminal *ci*sternal vesicles raised concerns over pyruvate's pro-arrhythmic effects; which may be concentration dependent. The study showed that 4 or10 mM pyruvate reduced the frequency of spontaneous calcium sparks through inhibition of calcium channels which could potentially become pro-arrythmic (Blatter *et al.*, 2003).

4.4.2.2 Instability of L-pyruvate in solution

Pyruvate used to enhance cardioprotective solutions is added as a sodium salt, Na-pyruvate. This can significantly increase the sodium burden in some solutions; not the case here, however, as the addition of 1 and 5 mM respectively to AL solution increased the sodium concentration from 145 – 150 mM. What was also found in the literature during my study was that pyruvate in alkaline solution maybe unstable, forming a non-metabolisable metabolite, para-pyruvate, which inhibits a key Krebs cycle enzyme (Woo *et al.*, 2004; Mallet *et al.*, 2005). This may have limited the use of pyruvate as an additive to cardioplegia for organ preservation. Therefore, L-pyruvate was not included in further studies as this would limit clinical translation of the AL preservation solution.

4.4.3 Possible reasons for the functional versatility at cold and warm temperatures

The versatility of normokalaemic AL preservation solution at both the cold and warm intermittent flush modalities appears to be related to arresting the heart at or

near the resting membrane potential for the myocyte known as polarised arrest (Dobson, 2010; Sloots and Dobson, 2010). In past studies hyperkalaemic solutions have been linked to unnatural depolarised membrane voltages (Kleber, 1983), ionic imbalances (Jovanovic *et al.*, 1997), and calcium loading (Cyran *et al.*, 1993), post implantation arrhythmias (Ellis *et al.*, 1980; Manning and Hearse, 1984) and myocardial stunning (Vinten-Johansen and Nakanishi, 1993).

Arresting the myocyte of the donor heart at more natural polarised membrane potentials has been demonstrated experimentally by Chamber's group in the rat and canine heart (Snabaitis *et al.*, 1997a; Sunamori *et al.*, 2001; Jilkina *et al.*, 2003). The basic electrophysiology behind polarised versus depolarised arrest is that at resting diastolic membrane potentials there are fewer open membrane channels, pores and exchangers compared with depolarised states (Snabaitis *et al.*, 1997b; Snabaitis *et al.*, 1997a; Chambers, 2001; Dobson, 2004; Sloots and Dobson, 2010). Maintaining a polarised membrane also appears to bring about vasorelaxation (O'Rourke, 1996) and promote anti-inflammatory properties which may prove to be important during implantation or whole blood storage methods employed in low pressure continuous perfusion systems (Ward *et al.*, 2006; Zhang *et al.*, 2010).

The polarised arrest concept is not new; during the 1960s tetrodotoxin (TTX) was shown to arrest the heart at more polarised potentials and in the 1990s many studies demonstrated that ATP dependent potassium channel openers such as nicorandil and pinacidil could be used as cardioprotective agents, both on their own as arresting agents and as additives to commonly used hyperkalaemic preservation solutions (Behling and Malone, 1995; Dorman *et al.*, 1997b; Jilkina *et al.*, 2003). However,

these failed to reach clinical translation because of reanimation ventricular instability and arrhythmias of the heart (Loubani and Galinanes, 2002) (Fallouh and Chambers, 2008; Fallouh *et al.*, 2009). Snabaitis and colleagues (Snabaitis *et al.*, 1997a) extended the use of TTX as polarising agent for use in donor hearts, however, this has not been pursued clinically due to toxicity concerns.

4.4.4 Clinical considerations: Cold storage, intermittent flushes or continuous perfusion?

The optimal perfusion dynamics, ionic and metabolic composition for arrest, storage and reanimation of human donor hearts remain controversial (Jahania *et al.*, 1999; Bethea *et al.*, 2003). While intermittent flush techniques similar to the method employed in the present study (or continuous low-pressure infusion delivery during cold-storage) are not widely practiced today, their superiority has been experimentally demonstrated over the past 20 years and this has been reviewed in Nickless and colleagues (Nickless *et al.*, 1998), and Jahania and colleagues (Jahania *et al.*, 1999). The major clinical objections to intermittent flush or continuous perfusion methods are largely logistical, although myocardial extracellular oedema and associated decreased ventricular compliance have been a concern with constant infusion storage techniques (Bethea *et al.*, 2003; Nameki *et al.*, 2006). Some of these limitations have been partially solved by Hassanein and colleagues (1998) and others who have developed portable perfusion apparatus for use in donor human beating heart preservation (Hassanein *et al.*, 1998). The apparatus incorporates a low-pressure infusion of a warm oxygenated nutrient solution to the human donor heart, and is currently undergoing clinical trials in the UK and Germany. AL preservation solution may be applicable for use with these apparatuses.

4.5 Conclusion

This chapter demonstrates the versatility of AL solution for use in cold and warmer arrest and with different preservation modalities for up to 6 hours. Currently there is no preservation solution that can extend the human heart safely beyond 4 to 5 hours. AL's versatility may allow for warmer harvest, preservation and reanimation temperatures; this may reduce endothelial dysfunction and reduce the incidence of post ischaemic arrhythmias and left ventricular stunning (Ozeki *et al.*, 2007).

CHAPTER 5: EARLY REPERFUSION WITH AL ARREST/REPERFUSION STRATEGY FOLLOWING 6 HOURS OF COLD STORAGE

"The cardiac surgeon is in a unique position to protect the heart before ischaemia is induced and to avoid further damage during the reperfusion period."

Beyersdorf, F. 2009.

5.1 Introduction

In heart transplantation longer global ischaemic times in often pre-injured organs can potentially lead to severe ischaemia reperfusion injury (IRI). During storage the ischaemic endothelium develops an adhesive, thrombogenic surface, which allows the attachment of inflammatory cells (Gueler *et al.*, 2004), generation and secretion of ROS, cytokines, chemokines (Boros and Bromberg, 2006) and adhesion molecules (Burne-Taney and Rabb, 2003) thus augmenting an inflammatory response upon reperfusion (Boros and Bromberg, 2006). IRI remains an important cause of organ dysfunction and allograft rejection following heart transplantation (Foley and Chari, 2007).

The aim of this Chapter was to investigate the possible benefits of using AL cardioplegia for reperfusion immediately following cold storage and prior to normothermic spontaneous reanimation. Specifically the study investigated the effects of a warm oxygenated AL arrest/reperfusion strategy following 6 hours of cold storage in the isolated rat heart. Hearts preserved for 6 hours at 4°C in AL cardioplegia or Celsior were reperfused and rewarmed for 5 mins with either KH

(polarising non-arrest), AL (polarising arrest) or Celsior (depolarising arrest) solutions before being switched to working mode to compare their functional recoveries.

5.2 Experimental Design

5.2.1 Arrest solutions for 5 min warm reperfusion following cold storage

The composition of the AL and Celsior arrest solutions and non-arrest KH used to reperfuse the heart immediately after cold storage in the 5 minute rewarm period are described in Appendix A. The difference between these solutions and the arrest and preservations solutions used for storage is that the reperfusion solutions were actively bubbled with 95% O_2/5% CO_2 to achieve a pO_2 greater than 600 mmHg and not recirculated. This was to facilitate slow warming and oxygenation during the early reperfusion of ischaemic hearts.

5.2.2 Experimental groups

Rat hearts were randomly assigned to one of 5 arrest/preservation and rewarm groups (n = 8 each group):

1) AL cold (4°C) storage plus 5 mins of rewarming with KH reperfusion;

2) AL cold (4°C) storage plus 5 mins of rewarming with oxygenated AL arrest/reperfusion;

3) Celsior cold (4°C) storage plus 5 mins of rewarming with Celsior arrest reperfusion;

4) Celsior cold (4°C) storage plus 5 mins of rewarming with KH and

5) Celsior cold (4°C) storage plus 5 mins of rewarming with oxygenated AL arrest/reperfusion.

Celsior reperfusion following Celsior storage was chosen because it was originally designed to provide a single solution for use throughout all stages of heart transplantation, particularly during the last two phases of implantation and reperfusion (Menasche *et al.*, 1994). Celsior rewarm was not studied with AL cold storage, as Celsior solution was not designed as a *normothermic* reperfusion solution (Menasche *et al.*, 1994).

5.2.3 Study design

Hearts (n=40) were rapidly removed from anaesthetised rats and placed in ice-cold heparinised modified KH buffer (Appendix A). Details of heart preparation, attachment and perfusion are described in Chapter 3: Materials and Methods section 3.4. Briefly, hearts were attached to a Langendorff apparatus and perfused at a pressure head of 90 cm H_2O (68 mmHg). The pulmonary artery was not cannulated prior to arrest and detaching the heart for cold storage to prevent vessel wall damage. Therefore, CF before cold storage was measured not directly from the pulmonary artery but from pulmonary artery drainage using a volumetric flask placed below the heart. The artery was cannulated during reattachment of the heart following cold storage for collection of coronary venous effluent and O_2 consumption measurements (see Figure 5.1 below). Functional data (AF, CF, HR, SP, DP) were measured after 15 minutes equilibration immediately before arrest and at 5, 10, 15, 30, 45 and 60 minute time points during the 60 min reperfusion. This data was compared with the baseline (pre-arrest) data for each group (see Figure 5.1).

The method of cold storage for rat hearts has been described in Chapter 3: Materials and Methods. Briefly, hearts were arrested at a constant pressure head of 90 cm H_2O

(68 mmHg) using AL cardioplegia or Celsior solution for 5 mins (50-100 ml) at 37°C for their respective groups. Hearts were gently removed from the apparatus and placed in 50 ml centrifuge tubes containing their respective air-equilibrated preservation solutions, and the sealed tubes immersed in the water bath set at 4°C for a period of 6 hours. See Figure 5.1. Following 6 hours of cold static storage, the hearts were immediately reattached to the perfusion apparatus and fresh, warm oxygenated solutions (AL, KH or Celsior) were used to reperfuse the heart in non-working Langendorff mode at a pressure head of 90 cm H_2O (68 mmHg) for 5 mins (Figure 5.1). The warm reperfusion period of 5 minutes was chosen because the first few minutes appear to the most critical for optimal cardioprotection following ischaemia (Piper *et al.*, 2004). Effluent collected during the last minute of this 5-minute rewarming period was assayed for pH, troponin T and lactate release. Hearts were then switched to working mode and reperfused for 60 minutes at 37°C using oxygenated KH. Hearts were allowed to spontaneously return function during reperfusion and were not electrically assisted if function did not return.

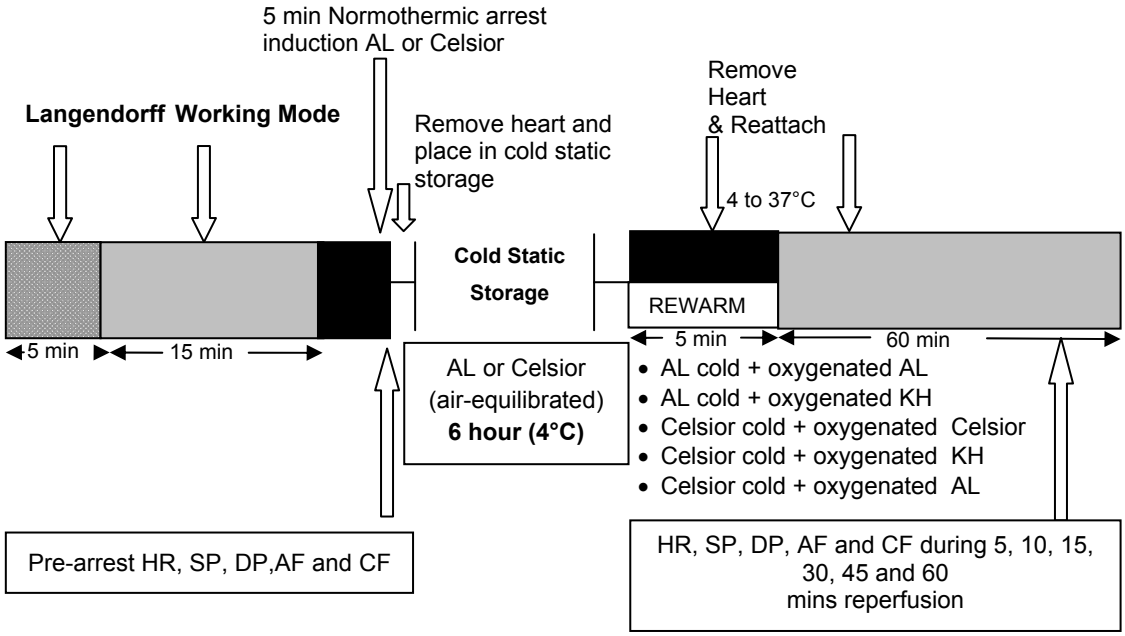

Figure 5.1: Experimental protocol
Isolated rat hearts (n=40) were placed in cold static storage for 6 hours in 1) AL polarising cardioplegia or 2) Celsior depolarising preservation solutions, and continuously rewarmed in Langendorff mode for 5 min with different oxygenated reperfusion strategies (see Chapter 3:Materials and Methods for details). (HR) Heart rate; (SP) systolic pressure; (DP) diastolic pressure; (AF) aortic flow and (CF) coronary flow.

5.3 Results

Functional properties prior to and following cold storage are shown in Tables 5.1 and 5.2 and Figures 5.2, 5.3, 5.4 and 5.5 below. During pre-arrest, there were no significant differences between the five groups in aortic flow, coronary flow, cardiac output, HR, developed pressures or rate-pressure product (Tables 5.1 and 5.2).

Table 5.1: Functional parameters of isolated working rat hearts during different rewarm reperfusion strategies following 6 hours cold (4°C) storage.
Recovery of aortic flow (AF), coronary flow (CF) and cardiac output (CO) during 60 mins reperfusion following 6 hour cold storage in AL or Celsior solutions and rewarming with different reperfusion strategies. Data presented as means ± SEM (n=40).

	Cold Arrest + Rewarm Treatment	n	Aortic Flow (ml/min)	Coronary Flow (ml/min)	Cardiac Output (ml/min)
15 mins Pre-Arrest	AL 6 hr arrest				
	+Krebs Henseleit	8	62 ± 5	24 ± 2	86 ± 4
	+AL cardioplegia	8	65 ± 2	22 ± 2	87 ± 3
	Celsior 6 hr arrest				
	+Celsior rewarm	8	57 ± 3	22 ± 2	79 ± 5
	+ Krebs Henseleit	8	67 ± 2	22 ± 1	90 ± 2
	+ AL cardioplegia	8	59 ± 2	21 ± 1	80 ± 2
5 mins Recovery	AL 6 hr arrest				
	+Krebs Henseleit	8	8 ± 2	14 ± 1	23 ± 3
	+AL cardioplegia	8	16 ± 5*	8 ± 3	24 ± 7*
	Celsior 6 hr arrest				
	+Celsior rewarm	8	1 ± 1§	2 ± 2§#	3 ± 3§
	+Krebs Henseleit	8	1 ± 1	12 ± 1	13 ± 2
	+AL cardioplegia	8	18 ± 4‡	16 ± 1	34 ± 5
10 mins Recovery	AL 6 hr arrest				
	+Krebs Henseleit	8	13 ± 3	14 ± 1	27 ± 3
	+AL cardioplegia	8	27 ± 3*	17 ± 1*	44 ± 3*†
	Celsior 6 hr arrest				
	+Celsior rewarm	8	8 ± 6	9 ± 4	17 ± 9
	+Krebs Henseleit	8	6 ± 3	12 ± 1	18 ± 4
	+AL cardioplegia	8	16 ± 3‡	15 ± 1§	31 ± 4§
15 mins Recovery	AL 6 hr arrest				
	+Krebs Henseleit	8	19 ± 2	14 ± 1	33 ± 3
	+AL cardioplegia	8	30 ± 4	15 ± 1	46 ± 4
	Celsior 6 hr arrest				
	+Celsior rewarm	8	12 ± 5	11 ± 2	23 ± 7
	+Krebs Henseleit	8	13 ± 3	12 ± 1	24 ± 4
	+AL cardioplegia	8	21 ± 3	15 ± 1	36 ± 4
30 mins Recovery	AL 6 hr arrest				
	+Krebs Henseleit	8	25 ± 1	14 ± 1	38 ± 2
	+AL cardioplegia	8	39 ± 2*†	16 ± 1*	55 ± 3*
	Celsior 6 hr arrest				
	+Celsior rewarm	8	18 ± 7	11 ± 2	29 ± 8
	+Krebs Henseleit	8	23 ± 4	13 ± 1	36 ± 5
	+AL cardioplegia	8	25 ± 3‡§	14 ± 1	39 ± 3
45 min Recovery	AL 6 hr arrest				
	+Krebs Henseleit	8	26 ± 1	13 ± 1	39 ± 1
	+AL cardioplegia	8	41 ± 3*†	16 ± 1*	57 ± 3*†
	Celsior 6 hr arrest				
	+Celsior rewarm	8	20 ± 5	11 ± 2	31 ± 7
	+Krebs Henseleit	8	27 ± 4	13 ± 1	40 ± 5
	+AL cardioplegia	8	26 ± 3	14 ± 1	41 ± 3
60 mins Recovery	AL 6 hr arrest				
	+Krebs Henseleit	8	25 ± 1	14 ± 1	39 ± 1
	+AL cardioplegia	8	41 ± 2*†	17 ± 1*	57± 2*†
	Celsior 6 hr arrest				
	+Celsior rewarm	8	20 ± 7	11 ± 2	31 ± 9
	+Krebs Henseleit	8	26 ± 5	12 ± 1	38 ± 6
	+AL cardioplegia	8	26 ± 3	14 ± 1	40 ± 3

*AL (cold storage) with AL (rewarm) vs Celsior (cold storage) with Celsior (rewarm) $p<0.05$
† AL (cold storage) with AL (rewarm) vs AL (cold storage) with KH (rewarm) $p<0.05$
§ Celsior (cold storage) AL (rewarm) vs Celsior (cold storage) with Celsior (rewarm) $p<0.05$
# Celsior (cold storage) with Celsior (rewarm) and Celsior (cold storage) KH (rewarm) $p<0.05$
‡ Celsior (cold storage) with AL (rewarm) and Celsior (cold storage) KH (rewarm) $p<0.05$

Table 5.2: Functional parameters of isolated working rat hearts during different rewarm reperfusion strategies following 6 hour cold (4°C) storage.
Recovery of heart rate (HR), systolic pressure (SP), diastolic pressure (DP) and rate pressure product (RPP) during reperfusion following 6 hour cold storage in AL or Celsior solutions and rewarming with different reperfusion strategies. Data presented are means ± SEM (n=40).

	Cold Arrest + Rewarm Treatment	n	Heart Rate (bpm)	Systolic Pressure (mmHg)	Diastolic Pressure (mmHg)	Rate Pressure Product beats.mmHg/min
15 mins Pre-Arrest	AL 6 hr arrest					
	+ KrebsHenseleit	8	285 ± 12	127 ± 2	60 ± 0	36216 ± 1692
	+AL cardioplegia	8	282 ± 7	124 ± 2	60 ± 0	35064 ± 1018
	Celsior 6 hr arrest					
	+Celsior rewarm	8	282 ± 17	124 ± 4	60 ± 0	34880 ± 1511
	+ Krebs Henseleit	8	284 ± 6	130 ± 0	60 ± 0	36969 ± 810
	+ AL cardioplegia	8	280 ± 10	128 ± 1	60 ± 0	35613 ± 1038
5 mins Recovery	AL 6 hr arrest					
	+Krebs Henseleit	8	228 ± 8	98 ± 5	66 ± 4	22410 ± 1579
	+AL cardioplegia	8	109 ± 32†	71 ± 20*	36 ± 10†	9706 ± 3905*†
	Celsior 6 hr arrest					
	+Celsior rewarm	8	38 ± 26$^{\#}$	26 ±18$^{\#}$	15 ± 9$^{\#}$	3051 ± 3216
	+Krebs Henseleit	8	178 ± 17	87 ± 4	62 ± 4	15863 ± 2106
	+AL cardioplegia	8	197 ± 19§	126 ± 5§	60 ± 1‡§	24774 ± 2382§
10 mins Recovery	AL 6 hr arrest					
	+Krebs Henseleit	8	242 ± 15	98 ± 4	66 ± 4	23844 ± 1890
	+AL cardioplegia	8	239 ± 7	118 ± 2*	62 ± 1*	28038 ± 821*
	Celsior 6 hr arrest					
	+Celsior rewarm	8	157 ± 71	60 ± 23	38 ± 14	15378 ± 7221
	+Krebs Henseleit	8	195 ± 15	96 ± 4	67 ± 2	18858 ± 1857
	+AL cardioplegia	8	217 ± 14	109 ± 4§	66 ± 1‡§	23598 ± 1413
15 mins Recovery	AL 6 hr arrest					
	+Krebs Henseleit	8	259 ± 13	103 ± 2	69 ± 1	26450 ± 1140
	+AL cardioplegia	8	256 ± 10	111 ± 3	64 ± 2	28163 ± 1005
	Celsior 6 hr arrest					
	+Celsior rewarm	8	161 ± 37	80 ± 15	53 ± 8	16585 ± 4175
	+Krebs Henseleit	8	207 ± 8	103 ± 3	70 ± 0	21350 ± 1070
	+AL cardioplegia	8	234 ± 22	112 ± 4	65 ± 1	25784 ± 1950
30 mins Recovery	AL 6 hr arrest					
	+Krebs Henseleit	8	286 ± 15	108 ± 2	68 ± 1	30663 ± 1438
	+AL cardioplegia	8	277 ± 9	114 ± 3*	63 ± 2	31498 ± 531
	Celsior 6 hr arrest					
	+Celsior rewarm	8	228 ± 43	89 ± 15	59 ± 9	23153 ± 4970
	+Krebs Henseleit	8	243 ± 14	109 ± 4	68 ± 1	26401 ± 1257
	+AL cardioplegia	8	261 ± 16	113 ± 3	64 ± 2	29039 ± 1072
45 mins Recovery	AL 6 hr arrest					
	+Krebs Henseleit	8	273 ± 21	106 ± 3	68 ± 1	28887 ± 2138
	+AL cardioplegia	8	279 ± 7	114 ± 3*	62 ± 1	31669 ± 789*
	Celsior 6 hr arrest					
	+Celsior rewarm	8	238 ± 37	89 ± 12	59 ± 7	23760 ± 3867
	+Krebs Henseleit	8	268 ± 9	109 ± 2	68 ± 2	29096 ± 996
	+AL cardioplegia	8	260 ± 14	112 ± 3§	65 ± 1	28825 ± 960
60 mins Recovery	AL 6 hr arrest					
	+Krebs Henseleit	8	290 ± 13	105 ± 3	68 ± 1	30265 ± 1018
	+AL cardioplegia	8	284 ± 8	114 ± 3*	62 ± 1	32661 ± 955*
	Celsior 6 hr arrest					
	+Celsior rewarm	8	238 ± 46	88 ± 15	60 ± 9	22147 ± 4874
	+Krebs Henseleit	8	274 ± 9	106 ± 3	67 ± 2	28581 ± 986
	+AL cardioplegia	8	272 ± 14	113 ± 3§	64 ± 2	30021 ± 991

*AL (cold storage) with AL (rewarm) and Celsior (cold storage) with Celsior (rewarm) $p<0.05$
† AL (cold storage) with AL (rewarm) and AL (cold storage) with KH (rewarm) $p<0.05$
§ Celsior (cold storage) AL (rewarm) vs Celsior (cold storage) with Celsior (rewarm) $p<0.05$
‡ Celsior (cold storage) with AL (rewarm) and Celsior (cold storage) KH (rewarm) $p<0.05$
$^{\#}$ Celsior (cold storage) with Celsior (rewarm) and Celsior (cold storage) KH (rewarm) $p<0.05$

5.3.1 Recovery of aortic flow (AF)

At 5 mins reperfusion in working mode, the AL cold group rewarmed with KH and the oxygenated AL arrest/reperfusion strategy recovered 13 and 25% of their pre-arrest AF values respectively, and these increased to 21% and 42% at 10 mins (Table 5.1, Figure 5.2). In contrast, hearts preserved in Celsior cold and rewarmed with Celsior or KH each generated only 2% of pre-arrest AF at 5 min respectively. However, when rewarmed with the oxygenated AL arrest/reperfusion strategy, the AF increased 18-fold at 5 mins (Table 5.1 Figure 5.2). At 10 mins, AF in the cold Celsior with Celsior and KH rewarm groups, increased to 14 and 9% of pre-arrest values respectively; however, these recoveries were still one-third to one-half of the value if rewarmed with the oxygenated AL arrest/reperfusion strategy. At 30 mins, the cold AL group rewarmed with KH, increased AF by 1.9 fold and the AL rewarm group by 1.4 fold, after which time the AF remained relatively stable (Table 5.1, Figure 5.2).

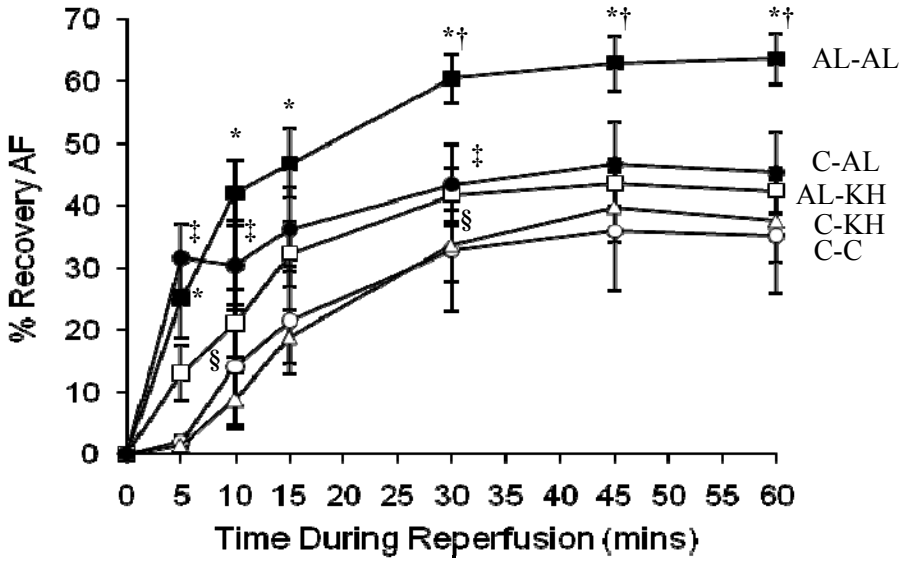

Figure 5.2: Recovery of aortic flow (%AF) with different rewarm reperfusion strategies
Percentage recovery of AF following 6 hours of cold storage and rewarm; the five groups were: 1) AL (cold) with KH rewarm (□), 2) AL (cold) with AL rewarm (■), 3) Celsior (cold) with Celsior rewarm (○), 4) Celsior (cold) with KH rewarm (Δ), and 5) Celsior (cold) with AL rewarm (●). Data presented as means ± SEM (n=40).
***** AL (cold storage) with AL (arrest/reperfusion strategy) and Celsior (cold storage) with Celsior (rewarm) $p<0.05$
† AL (cold storage) with AL (arrest/reperfusion strategy) and AL (cold storage) with KH (rewarm) $p<0.05$
§ Celsior (cold storage) with Celsior (rewarm) and Celsior (cold storage) AL (arrest/reperfusion strategy) $p<0.05$
‡ Celsior (cold storage) with AL (rewarm) and Celsior (cold storage) KH (rewarm) $p<0.05$

5.3.2 Recovery of coronary flow (CF)

There was a rapid spontaneous return of CF in the cold AL groups rewarmed with KH and the oxygenated AL arrest/reperfusion strategy with 58 and 36% of pre-arrest values recovered at 5 mins (Table 5.1 and Figure 5.3). However, the KH rewarm group did not increase CF further at 10, 30 and 60 mins reperfusion. The cold AL with the oxygenated AL arrest/reperfusion strategy increased CF over twofold at 10 mins compared with 5 mins, and stabilised at 30 and 60 mins reperfusion (Figure 5.3). In direct contrast to all other groups at 5 mins, the cold Celsior with Celsior

rewarm recovered only 9% of pre-arrest CF. However, rewarming cold Celsior with KH or the oxygenated AL arrest/reperfusion strategy led to significantly higher, 55% and 76% recovery of AF at 5 mins (Table 5.1). At 10 mins, the cold Celsior with Celsior rewarm increased coronary flow from 2 to 9 ml/min (41% baseline) and, as with KH rewarm or oxygenated AL arrest/reperfusion strategy, it did not increase further over the 60 mins reperfusion period (Table 5.1, Figure 5.3).

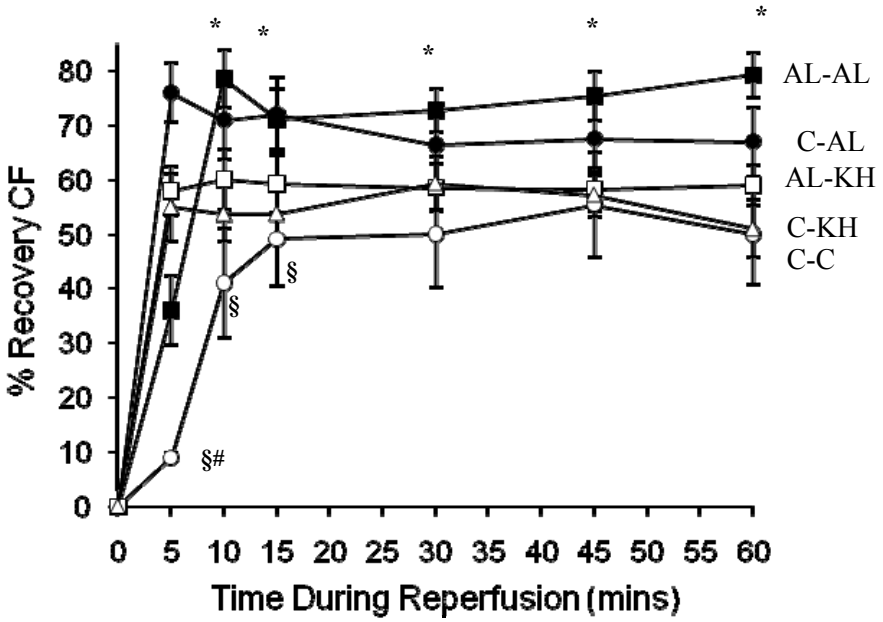

Figure 5.3: Recovery of coronary flow (%CF) with different rewarm reperfusion strategies
Percentage recovery of CF following 6 hours of cold storage and rewarm; the five groups were: 1) AL (cold) with KH rewarm (□), 2) AL (cold) with AL rewarm (■), 3) Celsior (cold) with Celsior rewarm (○), 4) Celsior (cold) with KH rewarm (Δ), and 5) Celsior (cold) with AL rewarm (●).Data presented as means ± SEM (n=40).
* AL (cold storage) with AL (arrest/reperfusion strategy) and Celsior (cold storage) with Celsior (rewarm) $p<0.05$
§ Celsior (cold storage) with Celsior (rewarm) and Celsior (cold storage) AL (rewarm) $p<0.05$
# Celsior (cold storage) with Celsior (rewarm) and Celsior (cold storage) KH (rewarm) $p<0.05$

5.3.3 Recovery of cardiac output (CO)

The cold AL group rewarmed with KH and the oxygenated AL arrest/reperfusion strategy returned 27 and 28% of pre-arrest CO respectively at 5 mins. The KH group did not increase further at 10 mins, whereas by this time, hearts rewarmed with the oxygenated AL arrest/reperfusion strategy increased 1.8-fold (51% pre-arrest). At 30 and 60 mins the AL cold with KH rewarm increased CO to 44% and 45% pre-arrest respectively, whereas the oxygenated AL arrest/reperfusion strategy increased from 51% (10 min) to 63 and 66% of pre-arrest CO at 30 and 60 mins reperfusion. Since in the isolated working rat heart preparation CO is the sum of AF and CF, the cardiac output reflects the changes in AF and CF. One point of emphasis is that cold Celsior hearts rewarmed with Celsior returned only 4% and 22% CO at 5 and 10 mins reperfusion. At 30 min, the CO for this group increased twofold (37% return), and did not increase further at 60 mins (39% return). In contrast, if cold Celsior hearts were rewarmed with the oxygenated AL arrest/reperfusion strategy, the return of CO was more rapid and significantly higher at 5 mins compared to Celsior and KH rewarm (Table 5.1, Figure 5.4). At 10 mins, the Celsior cold hearts with oxygenated AL arrest/reperfusion strategy returned a CO of 39%, at 30 min 49% and at 60 mins reperfusion 50% of pre-arrest value (Table 5.1, Figure 5.4).

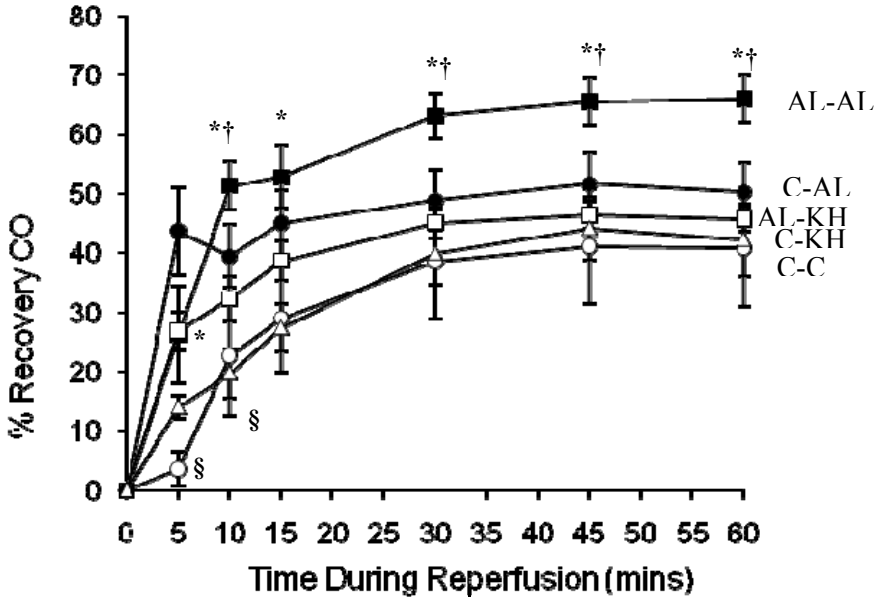

Figure 5.4: Recovery of cardiac output (%CO) with different rewarm reperfusion strategies
Percentage recovery of CO following 6 hours of cold storage and rewarm; the five groups were:The five groups were: 1) AL (cold) with KH rewarm (□), 2) AL (cold) with AL rewarm (■), 3) Celsior (cold) with Celsior rewarm (○), 4) Celsior (cold) with KH rewarm (Δ), and 5) Celsior (cold) with AL rewarm (●).Data presented as means ± SEM (n=40).
* AL (cold storage) with AL (arrest/reperfusion strategy) and Celsior (cold storage) with Celsior (rewarm) $p<0.05$
† AL (cold storage) with AL (arrest/reperfusion strategy) and AL (cold storage) with KH (rewarm) $p<0.05$
§ Celsior (cold storage) with Celsior (rewarm) and Celsior (cold storage) AL (arrest/reperfusion strategy) $p<0.05$

5.3.4 Recovery of heart rate (HR)

At 5 mins after switching from Langendorff rewarm to working mode, the AL cold group with KH rewarm and the oxygenated AL arrest/reperfusion strategy spontaneously returned a significantly higher HR (Table 5.2, Figure 5.5) and was due to the fact that these hearts begin beating in Langendorff mode; they were not arrested as ther was no cardioplegia present. However, by 10 mins, there was no significant difference between the KH or AL rewarming strategies in their return of HR. By 30 mins both rewarm groups had recovered their peak HR and this did not

change greatly at 60 mins. There were no significant differences between these two groups from 10 to 60 mins reperfusion (Table 5.2, Figure 5.5). In contrast, the heart rate for the cold Celsior hearts rewarmed with Celsior was 13% of pre-arrest value at 5 mins. This poor return was dramatically improved if cold Celsior hearts were rewarmed with KH or the oxygenated AL arrest/reperfusion strategy; the heart rate rapidly and spontaneously increased from 38 beats min^{-1} to 178 to 197 beats min^{-1} for KH and the oxygenated AL arrest/reperfusion strategy respectively (63 to 70% of baseline). As with AL cold group, there were no significant differences between rewarming with KH or oxygenated AL arrest/reperfusion strategy in return of heart rate for the cold Celsior group, although the oxygenated AL arrest/reperfusion strategy group had 7-12% higher values at 5, 10 and 30 mins (Table 5.2, Figure 5.5).

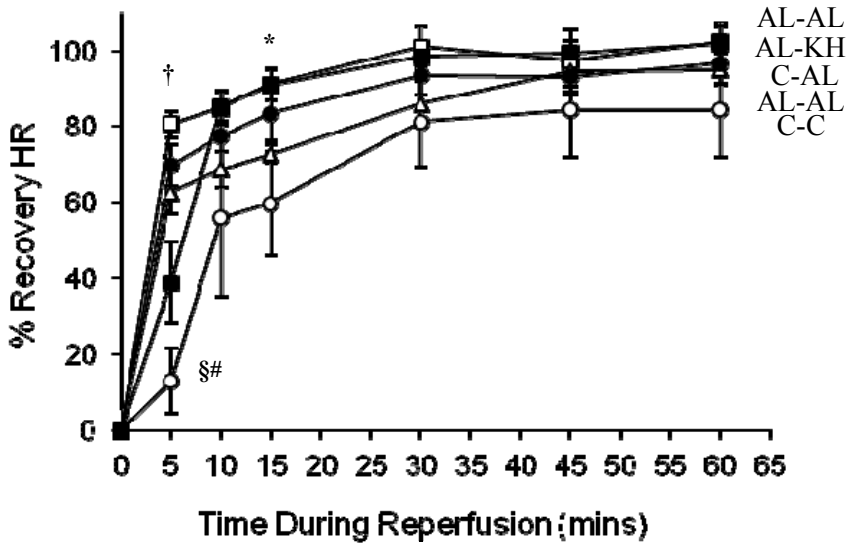

Figure 5.5: Recovery of heart rate (%HR) with different rewarm reperfusion strategies
Percentage recovery of HR following 6 hours of cold storage and rewarm; the five groups were: 1) AL (cold) with KH rewarm (□), 2) AL (cold) with AL rewarm (■), 3) Celsior (cold) with Celsior rewarm (○), 4) Celsior (cold) with KH rewarm (Δ), and 5) Celsior (cold) with AL rewarm (●).Data presented as means ± SEM (n=40).
* AL (cold storage) with AL (arrest/reperfusion strategy) and Celsior (cold storage) with Celsior (rewarm) $p<0.05$
† AL (cold storage) with AL (arrest/reperfusion strategy) and AL (cold storage) with KH (rewarm) $p<0.05$
§ Celsior (cold storage) with Celsior (rewarm) and Celsior (cold storage) AL (arrest/reperfusion strategy) $p<0.05$
# Celsior (cold storage) with Celsior (rewarm) and Celsior (cold storage) KH (rewarm) $p<0.05$

5.3.5 Recovery of systolic, diastolic pressures and rate pressure products

The developed pressures for the different groups followed similar patterns to those described for aortic flows. The cold Celsior group rewarmed with Celsior failed to generate pressures high enough to bring about recovery of function at 5 or 10 mins compared with all other groups, and these were reflected in lower rate-pressure products at the different reperfusion times (Table 5.2). At 10 mins, the Celsior cold with Celsior rewarm could only generate an average systolic/diastolic pressure of 60/38 mmHg. At 30 mins, the systolic/diastolic pressures were 89/59 mmHg

respectively, and at 60 mins were 88/60 mmHg (Table 5.2). Interestingly, a full return of diastolic pressure occurred for this Celsior group at 30 mins reperfusion; however, the highest return of systolic pressure was only 70% of pre-arrest value (Table 5.2). The lower developed pressures for the Celsior cold with Celsior rewarm were also reflected in lower rate-pressure products at the different reperfusion times (Table 5.2). The rate-pressure product is often used as a predictor of myocardial oxygen consumption under different contractile states in experimental and clinical studies.

Percent total tissue water (%TTW) for AL cold with KH and with AL rewarm were 88 ± 0.5% and 89 ± 1% respectively. Total tissue water for Celsior cold with Celsior rewarm was 86 ± 2%, Celsior with KH was 88 ± 0. 4%, and Celsior with AL was 87 ± 0.4% although this was not significant ($p>0.05$).

5.3.6 Myocardial oxygen consumption (MVO_2)

*5.3.6.1 **Myocardial oxygen consumption during 5 minute rewarm***

The mean value for the pre-arrest MVO_2 in working mode was 37.6 ± 0.8 µmol O_2/min/g dry wt (n=48). The pre-arrest MVO_2 value was a mean from 48 hearts taken from Rudd and Dobson (Rudd and Dobson, 2009). At the end of rewarming phase in the current study, the MVO_2 for cold AL preservation groups in Langendorff mode with KH or AL arrest were 23.9 ± 2.5 and 18.6 ± 1.0 µmol O_2/min/g dry wt respectively as shown in Figure 5.6. The MVO_2 for the cold Celsior group rewarmed with Celsior or AL arrest were 11.7 ± 1.2 and 13.4 ± 1.5 µmol O_2/min/g dry wt respectively (Figure 5.6). The MVO_2 during rewarming with KH was 20.9 ± 2.7 and this was significantly higher then both the Celsior groups

rewarmed with Celsior or AL ($p<0.05$). The coronary effluent flows in the final minute for AL cold (KH and oxygenated AL arrest/reperfusion strategy) groups were 12.8 ± 0.9 and 13.6 ± 0.4 ml respectively, and for Celsior cold (Celsior, KH and oxygenated AL arrest/reperfusion strategy) groups were 11.0 ± 0.8, 12.2 ± 0.6 and 11.2 ± 0.9 ml respectively.

5.3.6.2 ***Myocardial oxygen consumption during reperfusion***

During reperfusion in working mode at 10 and 60 mins, the MVO_2 for the cold AL group reperfused with KH was 27.1 ± 1.9 and 28.7 ± 1.8, and for cold AL group with AL rewarm was slightly higher at 10 mins (32.7 ± 4) and 1.6 times higher at 60 min (46.6 ± 4.9) this was significant ($p<0.05$)(Figure 5.6). The MVO_2 for the cold Celsior group with Celsior rewarm at 10 and 60 mins working mode were 17 ± 6.0 and 33.1 ± 6.0 respectively, for cold Celsior + KH rewarm were 19.0 ± 2.3 and 21.9 ± 2.8 μmol O_2/min/g dry wt, and for cold Celsior + AL rewarm were 27.1 ± 2.4 and 27.3 ± 2.7 μmol O_2/min/g dry wt respectively (Figure 5.6).

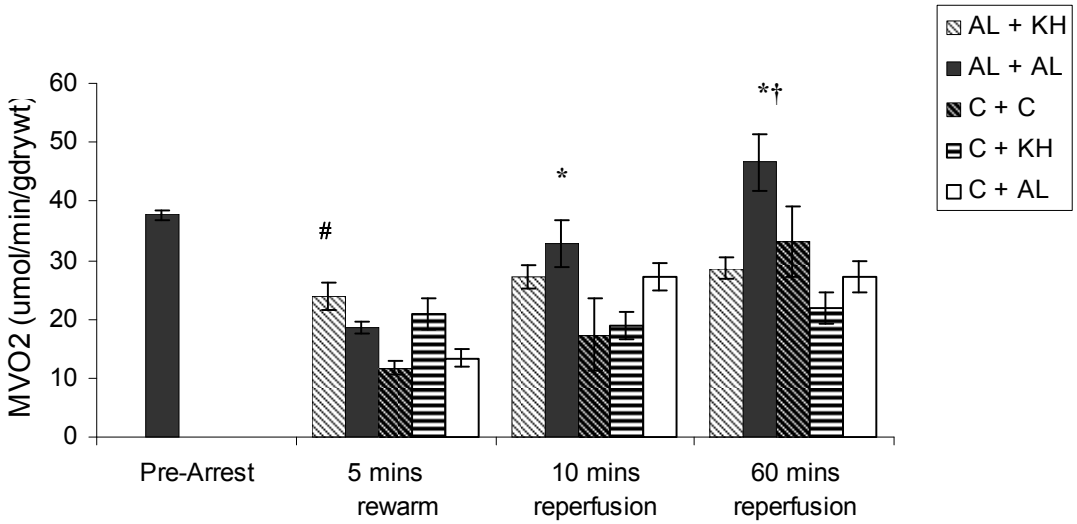

Figure 5.6: Myocardial oxygen consumption (MVO_2) for the different reperfusion strategies during 5 min rewarming period and at 10 and 60 mins reperfusion in working mode
Data presented as means ± SEM (n=40). The pre-arrest MVO_2 value was a mean from 48 hearts taken from Rudd and Dobson (Rudd and Dobson, 2009) (see Materials and Methods for details).
***** Celsior (cold) with Celsior (rewarm) vs AL (cold) with AL (rewarm) and $p<0.05$;
† AL (cold) with AL (rewarm) vs AL (cold) with KH (rewarm) $p<0.05$;
# AL (cold) with KH (rewarm) vs Celsior (cold) with Celsior (rewarm) and Celsior (cold) with AL rewarm) $p<0.05$.

5.4 Lactate output and ATP production during rewarming

The lactate outputs for the cold AL group with KH or AL rewarm were 1.09 ± 0.43 and 1.13 ± 0.51 µmol lactate/min/g dry wt (Figure 5.7). In contrast, lactate production in the cold Celsior group with Celsior rewarm was an order of magnitude higher with 9.79 ± 3.7 µmol lactate/min/g dry wt, however when rewarmed with KH or AL effluent lactates were 0.98 ± 0.64 and 1.12 ± 0.31 µmol lactate/min/g dry wt respectively, and not significantly different from the AL groups.

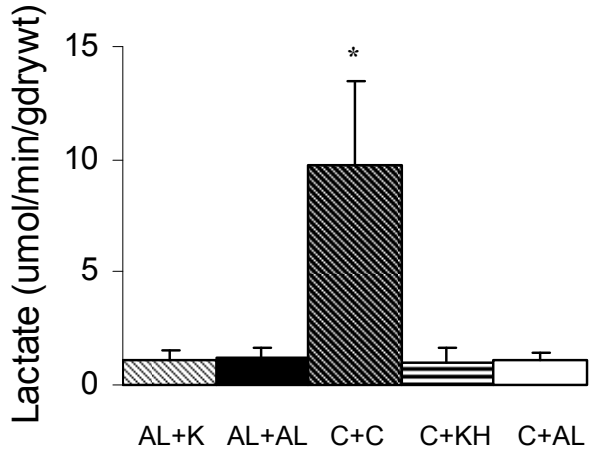

Figure 5.7: Lactate output during the last minute of the 5-minute rewarming period
Lactate output (in micromoles per minute per gram of dry weight heart) measured in the last minute of the 5-minute rewarming period in different groups immediately before switching the heart from Langendorff mode to working mode. Data presented as means ± SEM (n=40).
***** Celsior (cold) with Celsior (rewarm) significantly different from any other group ($p<0.05$).

Effluent pH values measured during the last minute of the 5 min rewarm period are shown in Figure 5.8. The pH in four of the five groups was between 7.37 and 7.43; the exception was the cold Celsior group with Celsior rewarm which had showed a significant drop in pH from 7.4 to 6.5 ± 0.16. This represented a 7.5-fold increase in hydrogen ion concentration.

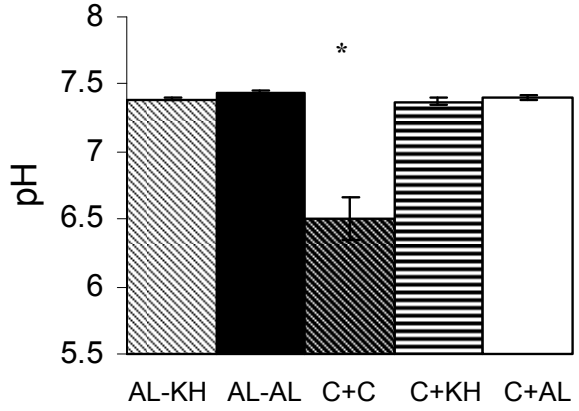

Figure 5.8: Effluent pH during the last minute of the 5-minute rewarming period
Effluent pH measured in the last minute of the 5-minute rewarming period in different groups immediately before switching the heart from Langendorff mode to working mode. Data presented as means ± SEM (n=40).
***** Celsior (cold) with Celsior (rewarm) significantly different from any other group ($p<0.05$).

The total ATP produced (aerobic and anaerobic) in the last minute of 5 min rewarm period is shown in Figure 5.9. Values for the cold AL group with KH or AL rewarm were 136 ± 10 and 118 ± 5 μmol ATP/min/gm dry wt respectively, and for the cold Celsior group with Celsior, KH or the oxygenated AL arrest/reperfusion strategy the values were 98 ± 12 and 111 ± 21 and 95 ± 3 μmol ATP /min/gm dry wt respectively (Figure 5.9). Of the total ATP produced, the anaerobic component for cold AL group with KH or AL rewarm were 1.5% and 1.7%. For the cold Celsior group with Celsior, KH or AL rewarm groups the percentages were 19.4%, 0.9% and 2.1% of the total ATP produced. The anaerobic production of ATP was significantly higher in the cold Celsior with Celsior rewarm than any other group, and over 10-fold higher than cold Celsior reanimated with AL arrest solution.

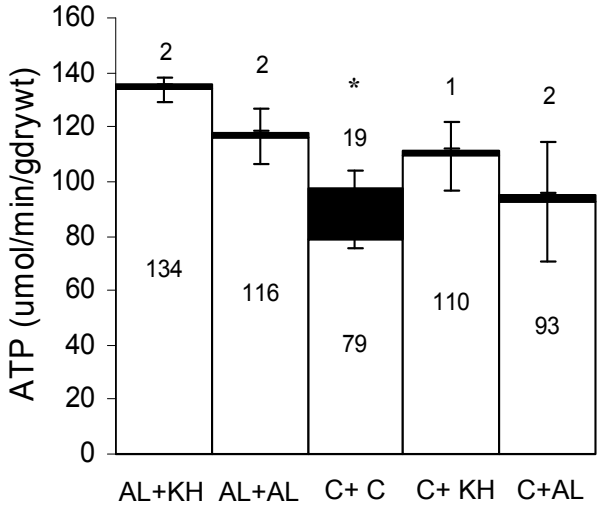

Figure 5.9: ATP output during the last minute of the 5-minute rewarming period

Adenosine triphosphate (ATP) production in the last minute of the 5-minute rewarming period calculated from oxygen consumption and lactate production (see Materials and Methods for equations).
The white vertical bars (actual concentration included in bar) represent the aerobic component to total ATP generation/replenishment, and the dark sections (actual concentration above bar) represent the anaerobic component in micromoles of ATP per minute per gram dry weight heart.
Data presented as means ± SEM (n=40).
***** Celsior (cold) with Celsior (rewarm) significantly different from any other group ($p<0.05$).

5.5 Discussion

Transferring the donor heart from a cold, depolarising storage environment to a warm environment for arousal and implantation predisposes the myocardium, vascular endothelium and electrical conduction system to ischaemic reperfusion injury, which can contribute to graft injury and possible rejection (Jahania *et al.*, 1999; Stoica *et al.*, 2001; Parolari *et al.*, 2002). This Chapter focused on the effect of rewarming isolated rat hearts using a warm oxygenated AL arrest/reperfusion strategy following 6 hours of cold storage. Rewarming the heart with the oxygenated AL arrest/reperfusion strategy significantly increased aortic and coronary flows and developed pressures when compared to KH rewarm alone. In addition, rewarming

cold depolarising Celsior stored hearts with the same oxygenated AL arrest/reperfusion strategy significantly increased reliance on aerobic versus anaerobic metabolism, with lower effluent lactate levels, no acidosis and a more rapid spontaneous return to higher flows when compared to rewarming with Celsior solution. AL cardioplegia may find clinical utility as a reperfusion strategy for reducing myocardial stunning in hearts following cold-to-warm transitions in heart transplantation and cardiac surgery.

5.5.1 Rewarming with polarising AL solution following cold storage

Hearts stored for 6 hours at 4°C in cold AL arrest solution and rewarmed with the oxygenated AL arrest/reperfusion strategy, recovered 1.6 times the AF, 1.2 times CF and 1.5 times CO when compared to hearts rewarmed with KH alone (AL vehicle) at 60 min reperfusion (Table 5.1). By contrast, the cold Celsior group rewarmed with Celsior showed suboptimal recoveries, their slow, poor recovery being partially reversed with KH or AL rewarm strategies (Figures 5.2, 5.3, 5.4, and 5.5). Therefore, rewarming hearts following depolarised or polarised arrest (4°C) in AL oxygenated, polarised arrest solution resulted in less ischaemia-reperfusion injury (Table 5.1 and 5.2, Figures 5.2, 5.3, 5.4 and 5.5). Interestingly, recovery of heart rate from cold hearts was not significantly different between AL rewarm or KH rewarm (Table 5.2), indicating that polarising normokalaemic conditions *per se* without AL provided equivalent protection of the cardiac pacemaker and possibly conduction system (Table 5.2, Figure 5.5).

5.5.2 Effect of 5 mins rewarming with the oxygenated AL arrest/reperfusion strategy on myocardial oxygen consumption (MVO_2)

In addition to improved working left ventricular and coronary function, rewarming cold AL hearts in non-working Langendorff mode with the AL arrest/reperfusion strategy resulted in a relatively higher MVO_2 during the rewarm period. This high MVO_2 was associated with a 1.6 fold higher 'maintenance' metabolism than that seen for Celsior cold hearts rewarmed with either Celsior or AL (Figure 5.6). Since MVO_2 in the cold Celsior group rewarmed with Celsior or AL was not significantly different (11.72 vs 13.39 μmol O_2/min/g dry wt), the higher MVO_2 in the AL cold hearts rewarmed with AL is likely to be the result of the antecedent polarised AL arrest in cold storage. A normokalaemic, polarised AL storage environment combined with the oxygenated AL arrest/reperfusion strategy led to higher MVO_2 prior to switching to working mode, and this strategy appears to be more cardioprotective than all other Celsior treatment groups.

Another interesting finding, during the last minute of the 5 minute rewarming period, was that the MVO_2 of the AL cold storage group increased from 18.6 ± 1.0 with AL rewarm to 23.9 ± 2.5 μmol O_2/min/g dry wt when rewarmed with non-arrest KH alone (the vehicle of AL arrest solution) see Figure 5.6. This increase appears modest, as the KH rewarm group was a non-working beating heart. The higher MVO_2 was not due to higher coronary flow as the effluent flow was 12.8 ml/min at 5 mins rewarm compared with 13.6 ml/min for AL arrested hearts. It is possible that the extra 29% O_2 consumed by KH rewarm hearts was largely derived from greater myocardial mitochondrial metabolism and O_2 extraction, not from changes in coronary vascular resistance.

Normally, cardiac maintenance or basal metabolism represents about 20 to 30% of the total energy flux (Gibbs and Loiselle, 2001). While the maintenance metabolism for hearts arrested in cold AL with KH rewarm appeared to be 14% higher than those arrested in Celsior and rewarmed in KH, cardiac basal metabolism of arrested hearts is dependent on the ionic composition of the cardioplegic solution. Here the basal metabolism for Celsior stored hearts was higher than that reported by others for potassium arrested hearts. When converted using the following formula 1umol/min/gdrywt = 1.74 mWg^{-1} (Gibbs and Loiselle, 2001) the MVO_2 of the Celsior stored and warmed hearts converts to 20.4 mWg^{-1} which is slightly higher than 10 to 15 mWg^{-1} reported in other studies (Kira *et al.*, 1984; Bittl and Ingwall, 1986; Sternbergh *et al.*, 1989; Burkhoff *et al.*, 1990; Hanley *et al.*, 1994). This may be caused in part by the increased osmolality of Celsior solution (presence of lactobionate) when compared to the solutions used in these studies which were based on KCl (Hanley *et al.*, 1994). The basal cardiac metabolism following storage in AL solution, measured during the 5 minute rewarm while arrested in AL or KH, was still much higher (32.4 mWg^{-1} or 41.6 mWg^{-1}) during the 5 minute rewarm than the potassium arrested hearts. The increase in basal metabolism is much greater than that reported by others measured during polarised arrest with TTX (Kira *et al.*, 1984; Sternbergh *et al.*, 1989) and may be due to a restorative response as it resulted in significantly better functional recoveries especially in early reperfusion. As early as 1986, Allen and colleagues reported that the best myocardial recovery following global ischaemia in dogs occurred when the heart consumed oxygen in excess of basal demands (Allen *et al.*, 1986c). Further studies are required during this critical rewarm/reperfusion period following cold storage to elucidate the metabolic activities and their beneficial effects.

5.5.3 Possible reasons for the high maintenance metabolism during rewarming

In 1997 Lawton and co-workers reported a higher MVO_2 in isolated rabbit hearts during early reperfusion from 30 mins global arrest in hyperpolarising pinacidil compared with depolarising St Thomas Hospital solution (Plegisol) (Lawton *et al.*, 1997). No post-arrest functional differences were found between the two groups. Lawton and colleagues suggested that the higher MVO_2 following hyperpolarised arrest may be related to reparative processes or to a higher oxygen debt generated during ischaemia (Lawton *et al.*, 1997). In this study, the higher MVO_2 in AL cold hearts with AL rewarm does not appear to be related to repayment of oxygen debt since there was no excess lactate efflux or no metabolic acidosis (pH = 7.4) see Figures 5.7 and 5.8. Nor does the higher MVO_2 appear to arise from reparative O_2 consuming processes of viable myocytes, because polarising AL cardioplegia and rewarm showed superior functional recoveries compared to the Celsior groups this is shown in Figures 5.2, 5.3, 5.4 and 5.5 above. The higher MVO_2 during the AL arrest/reperfusion strategy is more likely linked to improved mitochondrial oxidative replenishment of ATP to support a new maintenance or steady-state metabolism. This new steady-state in AL cold and rewarm hearts could be a restorative response to the rewarming oxygenation challenge, not a reparative response to ischaemia-reperfusion injury as lactate was low and effluent pH was pH 7.4. The presence of exogenous adenosine in the AL solution, may have assisted in purine salvage and maintaining the adenylate stores within the myocardium and stabilizing ATP (Canyon and Dobson, 2006; Schulze *et al.*, 2007).

Further experiments are required to test these rewarm hypotheses, and to test the effect of different durations of reperfusion and doses of AL in both crystalloid and blood solutions during the early rewarming phase. It would also be of interest to measure MVO_2 during cold storage and transition to normothermic conditions and continue for the entire rewarm-reperfusion period. For logistical reasons, it is difficult to measure MVO_2 during cold storage. Dobson and Jones (2004) calculated MVO_2 in Langendorff arrested rat hearts during 2 min normothermic infusions of AL cardioplegia over a 4 hour arrest period (28 – 30 °C), and reported values of between 6 and 8 μmol O_2/min/g dry wt for coronary effluent perfusate flows of 10 to 14 ml/min. In the present study, the cold (4°C) stored AL arrested and rewarmed hearts had MVO_2 values 2.6 times this value for maintenance metabolism at 5 mins oxygenated reperfusion (Figure 5.6). This could represent increased metabolic activity due to the temperature transition from cold stored to warm, or perhaps it represents a better protected heart. The colder temperature adds another level of cardioprotection to the polarising AL stored hearts which recover better than those stored warm and intermittently flushed (69% vs 55% recovery of pre-arrest CO) as seen in the previous chapter.

5.5.4 Effect of rewarming with polarised AL arrest solution and possible prevention of stunning

One of the most striking results was the effect of rewarming on stunning in the Celsior group. In Celsior cold hearts there was a delay in the spontaneous return of aortic and coronary flows (Figures 5.2, 5.3), and a delay in return of sinus rhythm. After switching to working mode, the HR was highly variable and significantly lower during early and late reperfusion in this group (Figure 5.5). These suboptimal

outcomes are consistent with Celsior-related myocardial, vascular and electrical stunning. Stunning is defined as the loss of post-operative contractility during reperfusion without cell death (Braunwald and Kloner, 1982). The early functional deficits in our study were not found if Celsior rewarm was replaced with polarising AL rewarm (Table 5.1, Figures 5.2, 5.3, 5.4 and 5.5), which implicates depolarising hyperkalaemic reperfusion as a possible cause. Stunning in Celsior hearts was also associated with significant 9-fold higher lactate efflux and significantly lower effluent pH of 6.5 prior to switching to working mode (Figure 5.7 and 5.8). The higher lactate efflux and acidic pH at 5 min rewarm period were not due to changes in effluent coronary flows (11.0 ml/min versus 12.2 ml/min for Celsior cold with Celsior and Celsior cold with KH rewarm, respectively). The anaerobic production of ATP in the cold Celsior with Celsior rewarm was also significantly higher than any other group, and over 10-fold higher than cold Celsior rewarmed with AL arrest solution (Figure 5.9). This result in Celsior hearts is important because Neely and colleagues (1973) showed in isolated rat hearts that the decrease in contractile force of ischaemic hearts was also associated with increased tissue lactate. Similarly, Kobayshi and colleagues (2004) found that there was a significantly greater lactate release and impaired total calcium handling in early reperfusion in rat hearts arrested for 30 mins in a hyperpolarising nicorandil (100 μmol/L) when compared to hearts stored under similar conditions in 30 mM KCl.

In a human transplant study using Celsior cold-preservation, Wildhirt and colleagues also reported myocardial and possibly endothelial stunning (Wildhirt *et al.*, 2000). They reported a requirement for greater inotropic support within the first 5 postoperative days in patients receiving the Celsior preserved donor hearts and a

significant correlation between longer ischaemic times in the Celsior group and reduced coronary flow reserve implicating endothelial dysfunction. Furthermore, endothelin and inducible nitric oxide synthase gene expression were significantly higher in the Celsior group. Ventricular stunning is common in post-implanted denervated donor hearts and a key contributor to early graft failure during first 30 days post-transplantation (Kendall *et al.*, 1997; Obadia *et al.*, 1997; Bittner *et al.*, 1999). In summary, cold Celsior hearts reperfused for 5 min at 37°C prior to reanimation with warm AL cardioplegia, led to no stunning compared with rewarming with Celsior arrest solution alone.

Possible reasons for improved spontaneous return of function following oxygenated normokalaemic, polarising AL arrest/reperfusion strategy may include the following alterations to the reperfuion environment. Firstly, the return of myocardial and coronary vascular membrane voltages to their normal operating limits, may reduce Ca^{2+} loading and stunning (Snabaitis *et al.*, 1997a; Dobson, 2004; Sloots and Dobson, 2010); secondly, the provision of an environment favouring aerobic replenishment of ATP and a reduction in hydrogen ion accumulation seen during anaerobic respiration and finally, down regulation of markers of inflammation on myocytes and activated endothelial cells during rewarming-reperfusion (Sellke *et al.*, 1996) (Smolenski *et al.*, 2001).

5.6 Clinical Significance

During organ storage, rewarming and implantation, the donor heart enters and emerges from a hostile, cold depolarising environment where it is subjected to unnatural changes in cell membrane voltage, ionic and metabolic imbalances. In the

early 1990s, Wheeldon and co-workers from the Papworth Hospital reported that some form of reperfusion was used 55% of the time in 92 transplant centers worldwide, and most used intermittent flushes of cold blood or cold hyperkalaemic, oxygenated, depolarising crystalloid cardioplegia during implantation followed by normothermic blood reperfusion (Wheeldon *et al.*, 1992). In 1996 Pradas and colleagues showed in human transplantation that continuous warm reperfusion was technically feasible and appeared to provide improved myocardial protection (Pradas *et al.*, 1996). These reperfusion methods were largely translated from the extensive preclinical and clinical studies from Buckberg's group and Menache's group on different reperfusion strategies following cardioplegic arrest for coronary artery bypass graft or valvular surgery (Menasche *et al.*, 1984; Allen *et al.*, 1986a; Vinten-Johansen and Nakanishi, 1993). The more targeted concept of controlled reperfusion was developed following these studies (Osaki *et al.*, 2006; Beyersdorf, 2009).

This study has demonstrated another possible strategy for rewarming and reperfusing the donor heart, namely rewarming with normokalaemic, oxygenated, polarising AL arrest/reperfusion strategy. Cold or warm hyperkalaemic depolarising cardioplegia, combined with the effects of ischaemia, acidosis and temperature shifts, predisposes the heart to electrical and mechanical instability during reperfusion which can lead to post-operative stunning (Pradas *et al.*, 1996; Canyon and Dobson, 2004; Dobson, 2004; Canyon and Dobson, 2006). By contrast, restoring the heart's cell membrane potentials to their normal operational limits within the myocardium, with intact transmural and regional heterogeneities, may promote a more electrically and mechanically stable heart. The new AL cold-warm normokalaemic reperfusion strategy may also find utility in portable perfusion apparatuses employing controlled,

low-pressure infusions to preserve the human donor heart (Hassanein *et al.*, 1998);(Ozeki *et al.*, 2007). In summary, evolving technologies to minimise damage during rewarming and implantation of donor hearts and other organs; include: temperature control; haemodynamic control (unloaded, non-working mode); pressure control; and reperfusate control (Jahania *et al.*, 1999; Stoica *et al.*, 2001; Bethea *et al.*, 2003; Osaki *et al.*, 2006). We propose a fifth strategy, namely, membrane voltage control using normokalaemic, polarising strategies.

5.7 Conclusion

Early continuous reperfusion with a warm, oxygenated, normokalaemic polarising adenosine and lignocaine (AL) cardioplegia improved spontaneous functional recovery following 6 hours cold storage in the isolated rat heart. Rewarming cold Celsior hearts with AL solution appeared to reduce myocardial and microvascular stunning. The normokalaemic AL arrest and preservation solution may find utility during cold-to-warm 'wash' transitions and implantation of donor hearts.

CHAPTER 6: IMPROVING THE COMPOSITION OF THE AL PRESERVATION SOLUTION

6.1 Introduction

A major contributor to ischaemia-reperfusion injury has been inadequate preservation during cold storage and early reperfusion. Extended storage in hyperkalaemic preservation solutions which depolarise the myocyte membrane, leave the heart in a vulnerable state and can lead to calcium loading and subsequent microvascular and myocardial damage upon reperfusion (He and Yang, 1998); (Jovanovic *et al.*, 1998). This may play an important role in the activation of intracellular secondary messenger systems leading to enzyme activation, contractile dysfunction (Spinale, 1999) and an up-regulation of inflammatory markers upon reperfusion (Boros and Bromberg, 2006) resulting in acute and chronic rejection.

Strategies to attenuate calcium overload during ischaemia have included the addition of pharmacological interventions such as sarcolemmal calcium channel antagonists (Klein *et al.*, 1989), mitochondrial calcium uniporter antagonists (Carry *et al.*, 1989) and sodium hydrogen exchange inhibitors (Yasutake *et al.*, 1994). However, the results of clinical studies have been disappointing (Theroux *et al.*, 1998; Theroux *et al.*, 2000; Mentzer *et al.*, 2003). A strategy of increasing clinical interest is to modify calcium concentrations of existing cardioplegia and preservation solutions for both adult (Klein *et al.*, 1989; Chen *et al.*, 1996) and paediatric cardiac surgery (Allen *et al.*, 2001). Reducing extracellular calcium lowers the driving force for calcium entry into myocytes (Alto *et al.*, 1980; Ahn *et al.*, 1994). Lowering calcium also reduces the formation of precipitates from binding to bicarbonate and/or phosphate which

could damage microcirculation and lead to inadequate perfusion of the donor organ during storage (Odom, 1997).

Increasing magnesium is also known to reduce calcium loading (Lansman *et al.*, 1986). Magnesium has been referred to as a naturally-occurring calcium antagonist and is known to block membrane L-type calcium channels (Iseri and French, 1984; Tsukube *et al.*, 1996a; Mubagwa *et al.*, 2007). Magnesium has other cardioprotective properties including reduction of calcium loading (Steenbergen *et al.*, 1990), prevention of ATP depletion (Caputo *et al.*, 1998), preservation of ATPase activity (Brown *et al.*, 1991), inhibition of calcium outflow from the SR (Terada *et al.*, 1996), reduction of calcium loading of the mitochondrial matrix (Sunamori *et al.*, 1980; Sharikabad *et al.*, 2001) and inhibition of MPTP opening and cell death (Dhalla and Temsah, 2001; Racay, 2008). These cardioprotective effects may be additive as the combination of lower calcium and increased magnesium concentration in cardioplegic and preservation solutions has been shown to further improve protection of both adult and paediatric hearts (Geffin *et al.*, 1989; Allen *et al.*, 2001; Camara *et al.*, 2004).

Another strategy for reducing calcium loading and ischaemia reperfusion injury has been inhibition of mitochondrial permeability transition pore (MPTP) (Halestrap *et al.*, 2004) with the addition of drugs known to inhibit MPTP opening such as cyclosporine A (CYA) (Griffiths, 2000);(Suleiman *et al.*, 2001) and melatonin (Petrosillo *et al.*, 2009a) to preservation solutions. CYA is able to inhibit MPTP opening by preventing the binding of cyclophilin D to adenine nucleotide transporter (ANT), thereby preventing the pore forming conformation. Several

studies have demonstrated the effectiveness of CYA for reducing IRI in a number of animal models (Griffiths and Halestrap, 1993; Xie and Yu, 2007; Leshnower *et al.*, 2008) and in human clinical trials of myocardial infarction (Piot *et al.*, 2008). Griffiths and colleagues demonstrated in an isolated rat heart model that the protective affects were only seen at lower CYA concentrations (0.2 μM vs 1.0 μM) and particularly when applied just prior to or during early reperfusion. The addition of 0.2 μM CYA to the perfusate, immediately before arrest, restored ATP/ADP ratio and improved left ventricular developed pressure (LVDP) following 30 and 40 minutes of ischaemia, however, 1 μM cyclosporin A reversed the effects (Griffiths and Halestrap, 1993). Hausenloy and colleagues also demonstrated that CYA given at reperfusion not only restored LV function and ATP levels but also demonstrated the prevention of cell death (Hausenloy *et al.*, 2002).While, Xie and colleagues also reported similar results, with a reduction in infarct size, in an *invivo* model of IRI (Xie and Yu, 2007).

The aim of the studies from this chapter was to examine the effects of lowering calcium to 0.22 mM, increasing magnesium concentration to 2.6 mM, adjusting the adenosine and lignocaine concentrations, along with the addition of 0.2 μM CYA, on the functional recoveries of isolated rat hearts stored for 6 hours in normokalaemic, adenosine-lignocaine solution (4 °C).

6.2 Materials and Methods

6.2.1 Arrest solutions for normothermic induction and cold storage:

6.2.1.1 *Adenosine-Lignocaine (AL) solution*

The adenosine and lignocaine (AL) solution contained 200 μmol/L adenosine plus 500 μmol/L lignocaine in Krebs Henseleit buffer (pH 7.7 at 37°C) as described in Chapter 3 Materials and Methods and Appendix A: Table A4.

6.2.1.2 Adenosine lignocaine (200:500 μM) solution with low calcium and high magnesium

The adenosine (200 μM) and lignocaine (500 μM) with low calcium and high magnesium (AL(LowCa^{2+}:HighMg^{2+})**)** solution contained 200 μmol/L adenosine plus 500 μmol/L lignocaine in 10 mmol/L glucose containing modified Krebs Henseleit with low Ca^{2+}:High Mg^{2+} (Appendix A: Table A3) buffer (pH 7.7 at 37°C) as described in Chapter 3 Materials and Methods and Appendix A: Table A5.

6.2.1.3 Adenosine lignocaine (200:500 μM) solution with low calcium and high magnesium plus 0.2 uM cyclosporin A

The adenosine (200 μM) and lignocaine (500 μM) with low calcium and high magnesium (AL200:500μM**)** solution contained 200 μmol/L adenosine plus 500 μmol/L lignocaine in 10 mmol/L glucose containing modified Krebs Henseleit buffer with low Ca^{2+}:High Mg^{2+} (Appendix A: Table A3) and 0.2 μM CYA (pH 7.7 at 37°C) as described in Appendix A: Table A9.

6.2.1.4 Adenosine lignocaine (200:1000 µM) solution with low calcium and high magnesium

The adenosine (200 µM) and lignocaine (1000 µM) with low calcium and high magnesium (AL(200:1000 µM)**)** solution contained 200 µmol/L adenosine plus 1000 µmol/L lignocaine in 10 mmol/L glucose containing modified Krebs Henseleit with LowCa^{2+}:HighMg^{2+} (Appendix A: Table A3) buffer (pH 7.7 at 37°C) as described in Appendix A: Table A6.

6.2.1.5 Adenosine lignocaine (200:1500 µM) solution with low calcium and high magnesium

The adenosine (200 µM) and lignocaine (1500 µM) with low calcium and high magnesium (AL(200:1500 µM)**)** solution contained 200 µmol/L adenosine plus 1500 µmol/L lignocaine in 10 mmol/L glucose containing modified Krebs Henseleit with LowCa^{2+}:HighMg^{2+} (Appendix A: Table A3) buffer (pH 7.7 at 37°C) as described in Appendix A: Table A7.

6.2.1.6 Adenosine lignocaine (400:1000 µM) solution with low calcium and high magnesium

The adenosine (400 µM) and lignocaine (1000 µM) with low calcium and high magnesium (AL400:1000 µM**)** solution contained 400 µmol/L adenosine plus 1000 µmol/L lignocaine in 10 mmol/L glucose containing modified Krebs-Henseleit with Low Ca^{2+}:High Mg^{2+} (Appendix A: Table A3) buffer (pH 7.7 at 37°C) as described in Appendix A: Table A8.

6.2.2 Arrest solutions for 5 min warm reperfusion following cold storage

The reperfusion solutions were the same as described above except that they were actively bubbled with 95% O_2/5% CO_2 to achieve a pO_2 greater than 600 mmHg and

not recirculated. This was to facilitate slow warming and oxygenation during the early reperfusion of the hearts.

6.2.3 Experimental groups:

6.2.3.1 Study one: Low Ca^{2+}: High Mg^{2+}

Rats were randomly assigned to one of 6 groups:

1) **(AL-AL)** Hearts arrested and stored in AL(4°C) and rewarmed with oxygenated AL arrest/reperfusion strategy (n=8);
2) **(AL-AL(LowCa^{2+}:HighMg^{2+})** Hearts arrested and stored in AL (4°C) and rewarmed with oxygenated AL (LowCa^{2+}:HighMg^{2+}) arrest/reperfusion strategy (n=8);
3) **(AL(LowCa^{2+}:HighMg^{2+}-KH)** Hearts arrested and stored in AL (LowCa^{2+}:HighMg^{2+}) (4°C) and rewarmed with oxygenated KH (n=7);
4) **((AL(LowCa^{2+}:HighMg^{2+})-AL(LowCa^{2+}:HighMg^{2+}))** Hearts arrested and stored in AL (LowCa^{2+}:HighMg^{2+}) (4°C) and rewarmed with oxygenated AL (LowCa^{2+}:HighMg^{2+}) arrest/reperfusion strategy (n=8);
5) **(AL(LowCa^{2+}:HighMg^{2+})-AL)** Hearts arrested and stored in AL (LowCa^{2+}:HighMg^{2+}) (4°C) and rewarmed with oxygenated AL arrest/reperfusion strategy (n=8);
6) **(AL(LowCa^{2+}:HighMg^{2+})+CYA)** Hearts arrested and stored in AL (LowCa^{2+}:HighMg^{2+}) + CYA (4°C) and rewarmed with oxygenated AL (LowCa^{2+}:HighMg^{2+}) + CYA arrest/reperfusion strategy (n=8).

6.2.3.2 Study two: Optimising adenosine and lignocaine concentrations

Rats were randomly assigned to one of 4 groups:

1) AL(200:500 μM) cold (4°C) arrest, storage and reperfusion (n=6);
2) AL(200:1000 μM) cold (4°C) arrest, storage reperfusion (n=6);
3) AL(200:1500 μM) cold (4°C) arrest, storage reperfusion (n=6);
4) AL(400:1000 μM) cold (4°C) arrest, storage reperfusion strategy (n=6).

6.2.4 Study design

6.2.4.1 Study one: Low Ca^{2+}: High Mg^{2+}

Hearts were rapidly removed from anaesthetised rats and placed in ice-cold heparinised modified KH buffer. Details of heart preparation, attachment and perfusion are described in Chapter 3 Materials and Methods. Briefly, hearts were attached to a Langendorff apparatus and perfused at a pressure head of 90 cm H_2O (68 mmHg). The hearts were then equilibrated in working mode for 15 minutes prior to taking baseline measurements (see Figure 6.1). Hearts were then switched to Langendorff mode and arrested with a 5 minute normothermic (37ºC) induction dose of the cardioplegic solution (AL, AL (LowCa^{2+}:HighMg^{2+}), or AL(LowCa^{2+}:HighMg^{2+})+CYA). Hearts were removed after the induction dose and placed in 50 ml centrifuge tubes contining the same solution and placed in a refrigerated water (4°C) bath for 6 hours. Hearts were then reattached in Langendorff mode with oxygenated KH, AL Cardioplegia or AL(LowCa^{2+}:HighMg^{2+}), or AL(LowCa^{2+}:HighMg^{2+})+ CYA as the perfusate once cannulated (5 minutes) hearts were then switched to working mode and the

functional measurements made at 5, 10, 15 30, 45 and 60 minutes of reperfusion. See Figure 6.1 below.

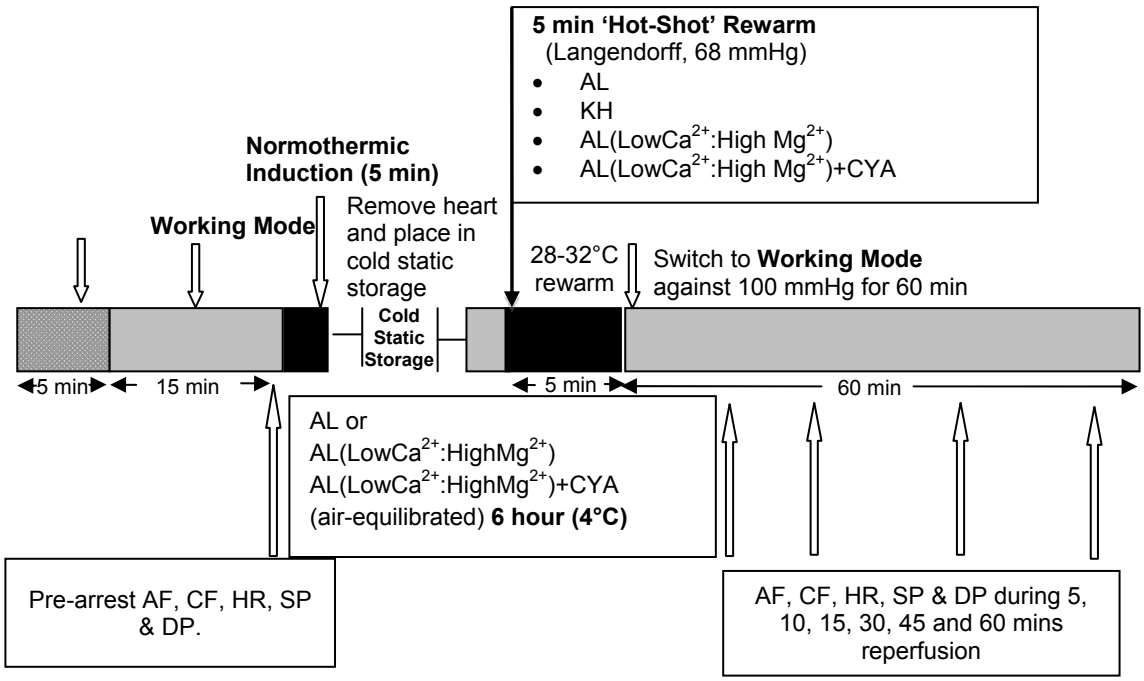

Figure 6.1: Experimental protocol for Chapter 6: Study 1
Isolated rat hearts (n=47) were placed in cold static storage for 6 hours in one of 3 cardioplegic solutions and rewarmed with either the same solution or KH. The experimental groups are as follows: 1) AL cardioplegia with AL rewarm (AL-AL); 2) AL cardioplegia with AL(LowCa^{2+}:HighMg^{2+})(AL-AL(LowCa^{2+}:HighMg^{2+}); rewarm 3) AL(LowCa^{2+}:HighMg^{2+}) cardioplegia with KH rewarm (AL(LowCa^{2+}:HighMg^{2+})-KH); 4) AL(LowCa^{2+}:HighMg^{2+}) cardioplegia with AL(LowCa^{2+}:HighMg^{2+}) rewarm (AL(LowCa^{2+}:HighMg^{2+})- AL(LowCa^{2+}:HighMg^{2+})) 5) AL(LowCa^{2+}:HighMg^{2+}) cardioplegia with AL rewarm (AL(LowCa^{2+}:HighMg^{2+})-AL); 6) AL(LowCa^{2+}:HighMg^{2+})+ CYA cardioplegia with AL(LowCa^{2+}:HighMg^{2+})+CYA rewarm (AL(LowCa^{2+}:HighMg^{2+})+CYA).

6.2.4.2 Study two: Optimising adenosine and lignocaine concentrations

Hearts were rapidly removed from anaesthetised rats and placed in ice-cold heparinised modified KH buffer. Details of heart preparation, attachment and perfusion are described in Chapter 3 Materials and Methods. Briefly, hearts were

attached to a Langendorff apparatus and perfused at a pressure head of 90 cm H_2O (68 mmHg). The hearts were then equilibrated in working mode for 15 minutes prior to taking initial or baseline measurements. See Figure 6.2 below. Hearts were then switched to Langendorff mode and arrested with a 5 minute normothermic (37ºC) induction dose of the AL(LowCa^{2+}:HighMg^{2+}) cardioplegic containing either: 1) 200 μM adenosine and 500 μM lignocaine (AL (200:500 μM)), 2) 200 μM adenosine and 1000 μM lignocaine (AL(200:1000 μM)), 3) 200 μM adenosine and 1500 μM lignocaine (AL(200:1500 μM)) or 4) 400 μM adenosine and 1000 μM lignocaine (AL(400:1000 μM)). Hearts were removed after the induction dose and placed in a 50 ml centrifuge tube containing the same solution and placed in a refrigerated water (4°C) bath for 6 hours. Hearts were then reattached in Langendorff mode while perfused with the same solution which was reoxygenated, once cannulated (5 minutes); hearts were then switched to working mode and the functional measurements made at 10, 15 30, 45 and 60 minutes of reperfusion. See Figure 6.2 below.

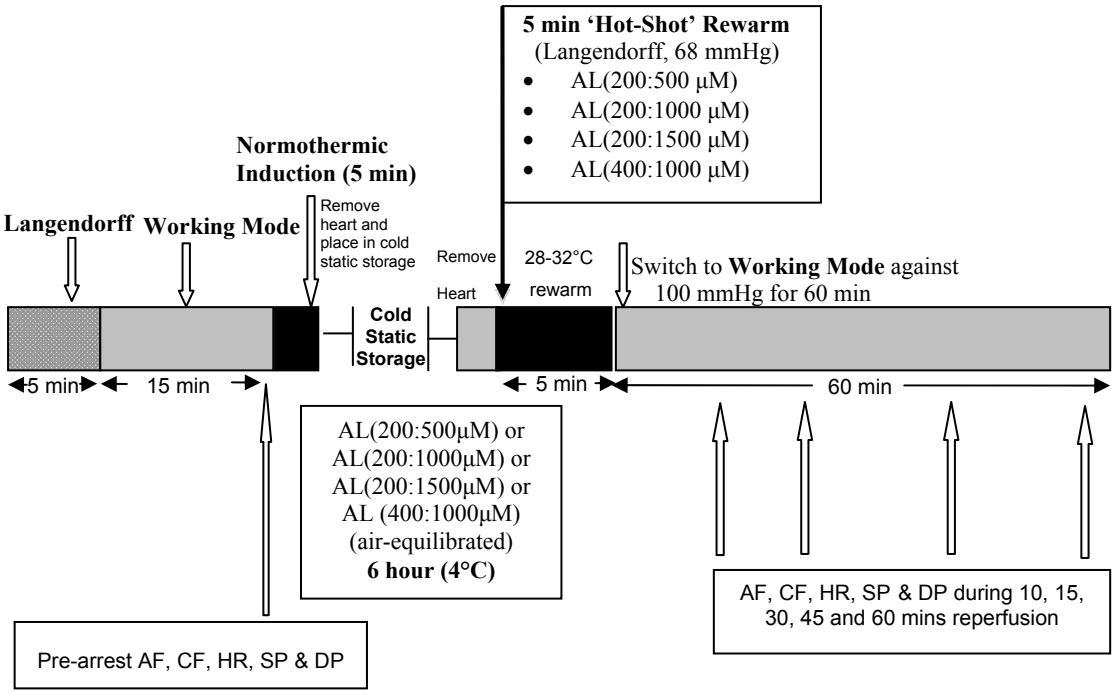

Figure 6.2: Experimental protocol for Chapter 6: Study 2
Isolated rat hearts (n=26) were placed in cold static storage for 6 hours in 1) AL(200:500 μM) cardioplegia with AL(200:500 μM) rewarm (n=6), 2) AL(200:1000 μM) cardioplegia with AL(200:1000 μM) rewarm (n=6), 3) AL(200:1500 μM) cardioplegia with AL(200:1500 μM) rewarm (n=6), 4) AL(400:1000 μM) cardioplegia with AL(400:1000 μM) rewarm (n=6).

6.2.5 Duration of unstable heart rhythm during reperfusion

The number and duration of episodes of unsatble heart rhythm during reperfusion were counted and timed as an estimate of the stability of the heart following cold storage. Lignocaine at higher doses has been reported to be proarrhythmic (Waller, 1981a). Episodes were counted as the number of disruptions to regular rhythm during the 60 minutes of reperfusion from the pressure trace of aortic pressures. No ECG was recorded so it was not possible to determine whether the electrical disturbances were atrial or ventricular.

6.3 Results

6.4 Study One: Low Ca^{2+} :High Mg^{2+}

6.4.1 Time to arrest

There was no significant difference in time taken to arrest with AL preservation solutions with varying combinations of low and normal calcium and high and normal magnesium (Figure 6.3).

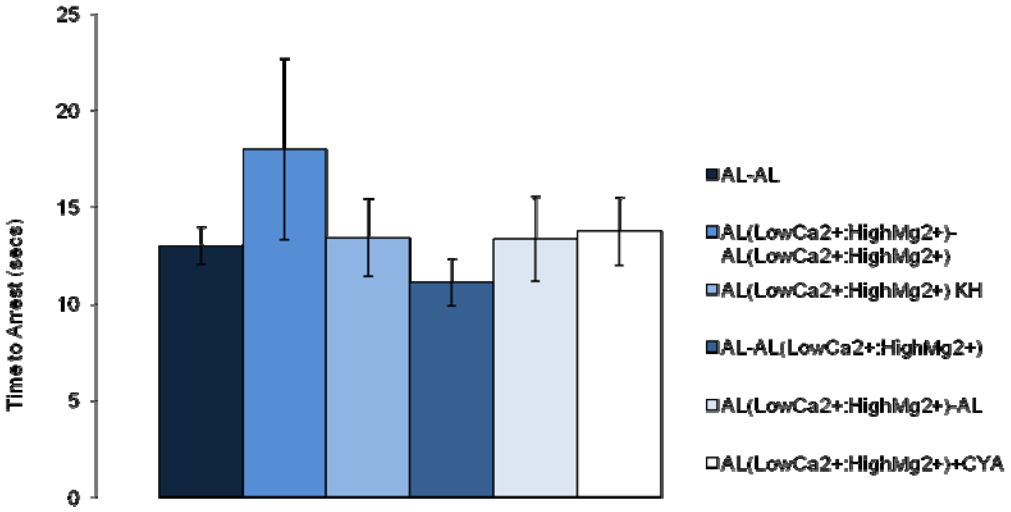

Figure 6.3: Time taken to arrest for hearts perfused with AL preservation solutions with different combinations of calcium and magnesium
AL-AL (n=8); AL(LowCa^{2+}:HighMg^{2+})-AL(LowCa^{2+}:HighMg^{2+})(n=8); AL(LowCa^{2+}:HighMg^{2+})-KH (n=7); AL-AL(LowCa^{2+}:HighMg^{2+}) (n=8); AL(LowCa^{2+}:HighMg^{2+})-AL (n=8); AL(LowCa^{2+}:HighMg^{2+})+CYA (n=8).
There was no significant difference between the time taken to arrest the heart for the different solutions ($p<0.05$). Data presented as mean ± SEM.

6.4.2 Time to first beat

Time to first beat was significantly longer in hearts rewarmed with low calcium preservation solutions (Figure 6.4).

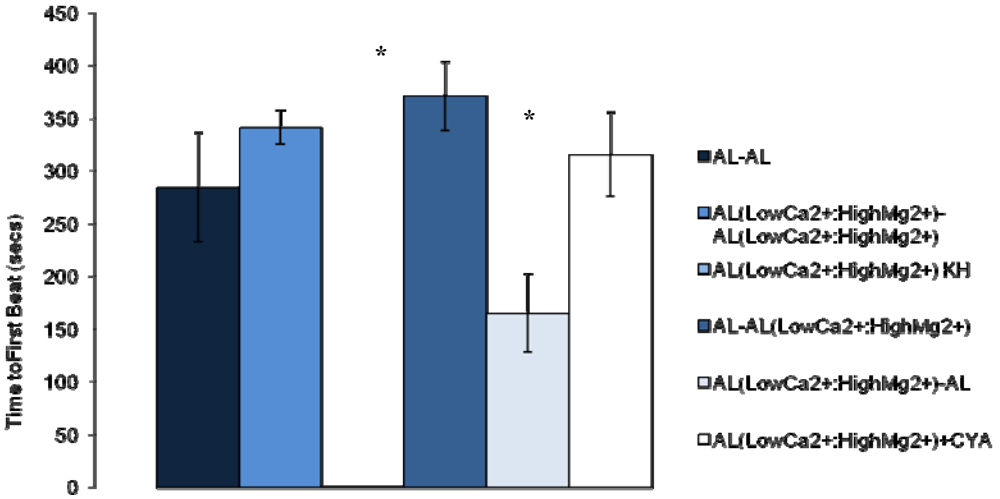

Figure 6.4: Time taken to first beat for hearts stored and rewarmed in AL preservation solutions with different combinations of calcium and magnesium
Data presented as mean ± SEM.
AL-AL (n=8); AL(LowCa^{2+}:HighMg^{2+})-AL(LowCa^{2+}:HighMg^{2+})(n=8);
AL(LowCa^{2+}:HighMg^{2+})-KH (n=7); AL-AL(LowCa^{2+}:HighMg^{2+})(n=8);
AL(LowCa^{2+}:HighMg^{2+})-AL (n=8); AL(LowCa^{2+}:HighMg^{2+})+CYA (n=8).
* denotes significant difference of AL(LowCa^{2+}:HighMg^{2+}) rewarmed hearts from those rewarmed in normal physiological levels of calcium and magnesium ($p<0.05$).

6.4.3 Time to aortic flow

The group rewarmed with KH returned aortic flow significantly faster than groups which remained arrested throughout the rewarm period. Although hearts stored in AL and rewarmed in AL(LowCa^{2+}:HighMg^{2+}) took 790 ± 55 seconds to reach aortic flow while this was significantly longer than the time required for hearts stored in AL and rewarmed in AL to recover AF, it was not significantly different from all other treatment groups (Figure 6.5).

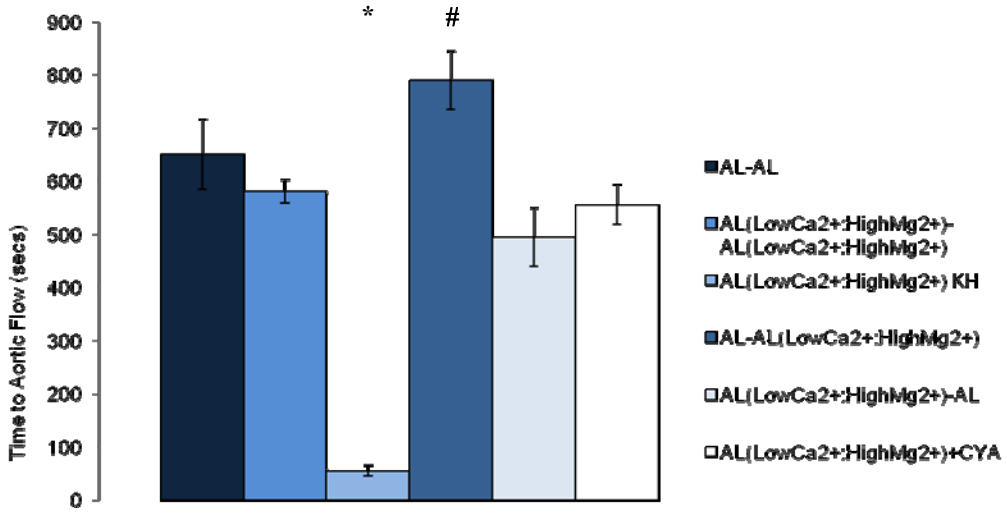

Figure 6.5: Time taken to return aortic flow for hearts stored and rewarmed in AL preservation solutions with different combinations of calcium and magnesium
Data presented as mean ± SEM (n=47). AL-AL (n=8);
AL(LowCa^{2+}:HighMg^{2+})-AL(LowCa^{2+}:HighMg^{2+})(n=8); AL(LowCa^{2+}:HighMg^{2+})-KH(n=7);
AL- AL(LowCa^{2+}:HighMg^{2+})(n=8); AL(LowCa^{2+}:HighMg^{2+})-AL (n=8);
AL(LowCa^{2+}:HighMg^{2+})+CYA (n=8).
* denotes significant difference of KH rewarmed hearts from those rewarmed in AL cardioplegic solution ($p<0.05$).
# denotes significant difference of hearts stored in AL and rewarmed with AL(LowCa^{2+}:HighMg^{2+}) from those stored and rewarmed in AL cardioplegic solution ($p<0.05$).

6.4.4 Functional recoveries

Functional properties prior to and following cold storage are shown in Table 6.1 and Figs 6.6, 6.7, 6.8 and 6.9. During pre-arrest, there were no significant differences between the five groups in AF, CF, CO, HR, SP, DP or RPP (Table 6.1).

Table 6.1: Functional parameters (AF, CF & CO) of isolated working rat hearts following 6 hours cold storage and rewarm with AL preservation solution containing different combinations of calcium and magnesium

Arrest and reperfusion strategies adenosine-lignocaine (AL) containing physiological calcium (1.12mM) and magnesium (0.5mM) or AL(Low Ca^{2+}:High Mg^{2+}) containing low calcium (0.22mM) and high magnesium (2.6mM). Data presented as mean ± SEM.

Time during Pre-Arrest and Reperfusion	Treatment	n	Aortic Flow (ml/min)	Coronary Flow (ml/min)	Cardiac Output (ml/min)
15 mins Pre-Arrest	AL 6 hr arrest				
	+AL rewarm	8	60 ± 3	22 ± 2	82 ± 4
	+AL(Low Ca^{2+}:High Mg^{2+}) rewarm	8	66 ± 3	22 ± 1	88 ± 3
	AL(Low Ca^{2+}:High Mg^{2+}) 6 hr arrest				
	+ Krebs Henseleit	7	68 ± 2	22 ± 2	90 ± 3
	+ AL (Low Ca^{2+}:High Mg^{2+}) rewarm	8	65 ± 2	22 ± 2	87 ± 3
	+ AL rewarm	8	65 ± 3	20 ± 1	86 ± 3
	AL(Low Ca^{2+}:High Mg^{2+})+CYA	8	61 ± 4	18 ± 1	79 ± 4
5 mins Recovery	AL 6 hr arrest				
	+AL rewarm	8	4 ± 4	2 ± 2	6 ± 6
	+AL(Low Ca^{2+}:High Mg^{2+}) rewarm	8	0 ± 0	0 ± 0	0 ± 0
	AL(Low Ca^{2+}:High Mg^{2+}) 6 hr arrest				
	+ Krebs Henseleit	7	16 ± 2	12 ± 1	28 ± 3
	+ AL (Low Ca^{2+}:High Mg^{2+}) rewarm	8	0 ± 0	0 ± 0	0 ± 0
	+ AL rewarm	8	6 ± 6	3 ± 3	9 ± 9
	AL(Low Ca^{2+}:High Mg^{2+})+CYA	8	0 ± 0	0 ± 0	0 ± 0
10 mins Recovery	AL 6 hr arrest				
	+AL rewarm	8	19 ± 5	13 ± 3	32 ± 7
	+AL(Low Ca:High Mg) rewarm	8	9 ± 6	4 ± 3	13 ± 9 $^{€}$
	AL(Low Ca:High Mg) 6 hr arrest				
	+ Krebs Henseleit	7	30 ± 3	14 ± 1	44 ± 4
	+ AL (Low Ca:High Mg) rewarm	8	20 ± 3	19 ± 1	39 ± 2
	+ AL rewarm	8	33 ± 3 §	18 ± 1 $^{\#‡}$	51 ± 3 ‡
	AL(Low Ca:High Mg)+CYA	8	36 ± 7 ^{ī}	18 ± 2 $^{¥!}$	54 ± 9
15 mins Recovery	AL 6 hr arrest				
	+AL rewarm	8	25 ± 3	16 ± 1	40 ± 3
	+AL(Low Ca^{2+}:High Mg^{2+}) rewarm	8	27 ± 4	18 ± 2	45 ± 3
	AL(Low Ca^{2+}:High Mg^{2+}) 6 hr arrest				
	+ Krebs Henseleit	7	30 ± 2	13 ± 1	43 ± 3
	+ AL (Low Ca^{2+}:High Mg^{2+}) rewarm	8	21 ± 3	15 ± 2	36 ± 5
	+ AL rewarm	8	31 ± 4	15 ± 2	46 ± 5
	AL(Low Ca^{2+}:High Mg^{2+})+CYA	8	31 ± 4	14 ± 1	45 ± 5
30 mins Recovery	AL 6 hr arrest				
	+AL rewarm	8	37 ± 3	16 ± 1	52 ± 4
	+AL(Low Ca^{2+}:High Mg^{2+}) rewarm	8	35 ± 5	15 ± 1	49 ± 5
	AL(Low Ca^{2+}:High Mg^{2+}) 6 hr arrest				
	+ Krebs Henseleit	7	37 ± 4	14 ± 1	51 ± 5
	+ AL (Low Ca^{2+}:High Mg^{2+}) rewarm	8	35 ± 3	18 ± 2	53 ± 4
	+ AL rewarm	8	45 ± 3	17 ± 2	62 ± 4 ‡
	AL(Low Ca^{2+}:High Mg^{2+})+CYA	8	42 ± 4	17 ± 1	59 ± 6
45 mins Recovery	AL 6 hr arrest				
	+AL rewarm	8	39 ± 4	16 ± 1	55 ± 5
	+AL(Low Ca^{2+}:High Mg^{2+}) rewarm	8	38 ± 5	16 ± 1	54 ± 5
	AL(Low Ca^{2+}:High Mg^{2+}) 6 hr arrest				
	+ Krebs Henseleit	7	32 ± 3	14 ± 1	45 ± 5
	+ AL (Low Ca^{2+}:High Mg^{2+}) rewarm	8	36 ± 3	19 ± 2	55 ± 3
	+ AL rewarm	8	44 ± 3 $^{§\#}$	17 ± 1 $^{\#}$	61 ± 4 $^{§\#‡}$
	AL(Low Ca^{2+}:High Mg^{2+})+CYA	8	45 ± 4 $^{¥!}$	18 ± 2 $^{!}$	63 ± 5 $^{¥!ī}$

60 mins Recovery	AL 6 hr arrest				
	+AL rewarm	8	39 ± 3	18 ± 2	57 ± 4
	+AL(Low Ca^{2+}:High Mg^{2+}) rewarm	8	39 ± 5	17 ± 1	56 ± 6
	AL(Low Ca^{2+}:High Mg^{2+}) 6 hr arrest				
	+ Krebs Henseleit	7	33 ± 4	15 ± 1	48 ± 4
	+ AL (Low Ca^{2+}:High Mg^{2+}) rewarm	8	35 ± 3	20 ± 2	55 ± 4
	+ AL rewarm	8	45 ± 2[§#]	19 ± 2	64 ± 3
	AL(Low Ca^{2+}:High Mg^{2+})+CYA	8	44 ± 4	19 ± 2	63 ± 5[!]

[€] AL with AL (Low Ca^{2+}:High Mg^{2+}) and all other groups $p<0.05$
[†] AL (Low Ca^{2+}:High Mg^{2+}) with AL(Low Ca^{2+}:High Mg^{2+}) rewarm and AL (Low Ca^{2+}:High Mg^{2+}) with KH (rewarm) $p<0.05$;
[#] AL (Low Ca^{2+}:High Mg^{2+}) with AL rewarm and AL (Low Ca^{2+}:High Mg^{2+}) with KH (rewarm) $p<0.05$;
[§] AL (Low Ca^{2+}:High Mg^{2+}) with AL rewarm vs AL (Low Ca^{2+}:High Mg^{2+}) with AL (Low Ca^{2+}:High Mg^{2+}) rewarm $p<0.05$;
[‡] AL (Low Ca^{2+}:High Mg^{2+}) with AL rewarm vs AL with AL rewarm $p<0.05$;
[¥] AL(Low Ca^{2+}:High Mg^{2+})+CYA with AL AL $p<0.05$;
[ĩ] AL(Low Ca^{2+}:High Mg^{2+})+CYA with AL (Low Ca^{2+}:High Mg^{2+}) with AL (Low Ca^{2+}:High Mg^{2+}) $p<0.05$;
[!] AL(Low Ca^{2+}:High Mg^{2+})+CYA with AL (Low Ca^{2+}:High Mg^{2+}) with KH (rewarm) $p<0.05$.

Table 6.2: Functional parameters (HR, SP, DP & RPP) of isolated working rat hearts following 6 hours cold storage and rewarm with AL preservation solution containing different combinations of calcium and magnesium

Arrest and reperfusion strategies adenosine-lignocaine (AL) containing physiological calcium (1.12mM) and magnesium (0.5mM) or AL(Low Ca^{2+}:High Mg^{2+}) containing low calcium (0.22mM) and high magnesium (2.6mM). Data presented as mean ± SEM.

Time during Pre-Arrest and Reperfusion	Treatment	n	Heart Rate (bpm)	Systolic Pressure	Diastolic Pressure (mmHg)	Rate Pressure Product beats.mmHg/min
15 mins Pre-Arrest	AL 6 hr arrest					
	+AL rewarm	8	282 ± 13	129 ± 2	60 ± 0	36245±1470
	+AL(Low Ca^{2+}:High Mg^{2+}) rewarm	8	271 ± 4	128 ± 1	60 ± 0	34678 ± 754
	AL(Low Ca^{2+}:High Mg^{2+}) 6 hr arrest					
	+ Krebs Henseleit	7	296 ± 11	130 ± 0	60 ± 0	38464 ±1366
	+ AL (Low Ca^{2+}:HighMg^{2+}) rewarm	8	268 ± 8	129 ± 1	60 ± 0	34528 ± 898
	+ AL rewarm	8	282 ± 7	129 ± 1	59 ± 1	36510 ± 845
	AL(Low Ca:High Mg)+CYA	8	269 ± 9	125 ± 2	59 ± 1	33621 ±1431
5 mins Recovery	AL 6 hr arrest					
	+AL rewarm	8	17 ± 9	13 ± 8	4 ± 4	706 ± 584
	+AL(Low Ca^{2+}:High Mg^{2+}) rewarm	8	3 ± 3$^{€}$	2 ± 2	1 ± 1$^{€}$	30 ± 30$^{€}$
	AL(Low Ca^{2+}:High Mg^{2+}) 6 hr arrest					
	+ Krebs Henseleit	7	231 ± 23	110 ± 1	68 ± 1	25426 ± 2513
	+ AL (Low Ca^{2+}:HighMg^{2+}) rewarm	8	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	+ AL rewarm	8	61 ± 33	35 ± 14	20 ± 6	5356 ± 4625
	AL(Low Ca:High Mg)+CYA	8	6 ± 5	6 ± 4	4 ± 3	94 ± 75
10 mins Recovery	AL 6 hr arrest					
	+AL rewarm	8	195 ± 41	91 ± 19	46 ± 10	23105 ±5132
	+AL(Low Ca:High Mg) rewarm	8	76 ± 39$^{€}$	46 ± 19	26 ± 7$^{€}$	8373 ± 5261$^{€}$
	AL(Low Ca:High Mg) 6 hr arrest					
	+ Krebs Henseleit	7	259 ± 14	114 ± 2	66 ± 2	29369 ±1426
	+ AL (Low Ca:High Mg) rewarm	8	213 ± 11	125 ± 2	62 ± 1	26483 ±1106
	+ AL rewarm	8	262 ± 11	120 ± 3	62 ± 1	31431 ±1375
	AL(Low Ca:High Mg)+CYA	8	235 ± 28	116 ±	55 ± 5	29423 ±4213
15 mins Recovery	AL 6 hr arrest					
	+AL rewarm	8	252 ± 14	113 ± 3	66 ± 2	28548 ± 1735
	+AL(Low Ca^{2+}:High Mg^{2+}) rewarm	8	254 ± 13	119 ± 4	63 ± 1	30156 ± 1334
	AL(Low Ca^{2+}:High Mg^{2+}) 6 hr arrest					
	+ Krebs Henseleit	7	256 ± 16	114 ± 2	66 ± 2	29032 ± 1589
	+ AL (Low Ca^{2+}:HighMg^{2+}) rewarm	8	249 ± 18	109 ± 3	68 ± 1	27300 ± 2229
	+ AL rewarm	8	279 ± 13	111 ± 3	64 ± 2	31155 ± 1853
	AL(Low Ca:High Mg)+CYA	8	276 ± 16	111 ± 2	66 ± 1	30491 ± 1770
30 mins Recovery	AL 6 hr arrest					
	+AL rewarm	8	281 ± 13	116 ± 2	63 ± 2	32545 ±1773
	+AL(Low Ca^{2+}:High Mg^{2+}) rewarm	8	266 ± 10	113 ± 2	64 ± 2	29895 ±1012
	AL(Low Ca^{2+}:High Mg^{2+}) 6 hr arrest					
	+ Krebs Henseleit	7	269 ± 12	116 ± 3	64 ± 2	31303 ±1696
	+ AL (Low Ca^{2+}:High Mg^{2+}) rewarm	8	276 ± 12	119 ± 2	64 ± 2	32875 ±1264
	+ AL rewarm	8	293 ± 12$^{\#}$	118 ± 2	61 ± 1	34441 ±1663
	AL(Low Ca:High Mg)+CYA	8	286 ± 13$^{!}$	115 ± 3	63 ± 2	32969 ±1895$^{!}$
45 mins Recovery	AL 6 hr arrest					
	+AL rewarm	8	284 ± 14	114 ± 3	63 ± 2	32623 ± 1899
	+AL(Low Ca^{2+}:High Mg^{2+}) rewarm	8	280 ± 11	114 ± 3	63 ± 1	32016 ± 1496
	AL(Low Ca^{2+}:High Mg^{2+}) 6 hr arrest					
	+ Krebs Henseleit	7	260 ± 12	116 ± 3	65 ± 1	30107 ± 1570
	+ AL (Low Ca^{2+}:HighMg^{2+}) rewarm	8	279 ± 10	118 ± 1	63 ± 1	32736 ± 1052
	+ AL rewarm	8	291 ± 12$^{\#}$	117 ± 2	60 ± 1	34121 ± 1800
	AL(Low Ca:High Mg)+CYA	8	307 ± 9$^{!}$	116 ± 3	63 ± 2	35683 ± 1353$^{!}$

60 mins	AL 6 hr arrest					
Recovery	+AL rewarm	8	297 ± 13[∫]	114 ± 3	64 ± 2	33986 ±1709
	+AL(Low Ca^{2+}:High Mg^{2+}) rewarm	8	286 ± 12[Ψ]	114 ± 3	64 ± 2	32722 ±1621
	AL(Low Ca^{2+}:High Mg^{2+}) 6 hr arrest					
	+ Krebs Henseleit	7	263 ± 10	118 ± 1	64 ± 2	30881 ± 1177
	+ AL (Low Ca^{2+}:High Mg^{2+}) rewarm	8	283 ± 11[†]	117 ± 1	63 ± 1	33328 ± 1131
	+ AL rewarm	8	299 ± 11[#]	116 ± 2	60 ± 1	35161 ± 1697
	AL(Low Ca:High Mg)+CYA	8	313 ± 9[!]	116 ± 3	63 ± 2	36335 ± 304[¥!ĩ]

[€] AL with AL (Low Ca^{2+}:High Mg^{2+}) and all other groups $p<0.05$
[Ψ] AL with AL (Low Ca^{2+}:High Mg^{2+}) and AL (Low Ca^{2+}:High Mg^{2+}) with KH rewarm $p<0.05$
[†]AL (Low Ca^{2+}:High Mg^{2+}) with AL(Low Ca^{2+}:High Mg^{2+}) rewarm and AL (Low Ca^{2+}:High Mg^{2+}) with KH (rewarm) $p<0.05$;
[#] AL (Low Ca^{2+}:High Mg^{2+}) with AL rewarm and AL (Low Ca^{2+}:High Mg^{2+}) with KH (rewarm) $p<0.05$;
[∫] AL with AL rewarm vs AL(Low Ca^{2+}:High Mg^{2+}) with KH rewarm $p<0.05$;
[¥] AL(Low Ca^{2+}:High Mg^{2+})+CYA with AL AL $p<0.05$;
[ĩ] AL(Low Ca^{2+}:High Mg^{2+})+CYA with AL (Low Ca^{2+}:High Mg^{2+}) with AL (Low Ca^{2+}:High Mg^{2+}) $p<0.05$;
[!] AL(Low Ca^{2+}:High Mg^{2+})+CYA with AL (Low Ca^{2+}:High Mg^{2+}) with KH (rewarm) $p<0.05$.

6.4.5 Recovery of aortic flow

Storing hearts in AL: Hearts preserved in AL cold and rewarmed with AL at 5 minutes reperfusion had generated 7% of pre-arrest AF while those rewarmed with AL(Low Ca^{2+}:High Mg^{2+}) remained arrested. By 10 mins, however, the AF for the AL rewarm group, had increased 2-fold to 32% compared to 14% with AL(Low Ca^{2+}:High Mg^{2+}) rewarm (Table 6.1, Figure 6.6); however, this increase was not significant ($p>0.05$). After 60 mins of reperfusion, AF increased to 65% in hearts stored and rewarmed with AL and 59% for those stored in AL and rewarmed in AL(Low Ca^{2+}:High Mg^{2+}) which was not significantly different ($p>0.05$).

Storing hearts in AL(Low Ca^{2+}:High Mg^{2+}): The AL(Low Ca^{2+}:High Mg^{2+}) cold storage group rewarmed with KH and AL(Low Ca^{2+}:High Mg^{2+}) recovered 44% and 31% of pre-arrest AF, respectively, by 10 mins and 49 and 54% respectively by 60 mins; again this was not significantly different ($p>0.05$) (Table 6.1, Figure 6.6). By contrast hearts stored in the cold AL(Low Ca^{2+}:High Mg^{2+}) and rewarmed with AL solution returned 69% of their pre-arrest AF by 60 mins and this was significantly better ($p<0.05$) than rewarming in either AL(Low Ca^{2+}:High Mg^{2+}) or KH alone.

Storing hearts in AL(Low Ca^{2+}:High Mg^{2+})+CYA: Hearts stored and rewarmed in the same solution augmented with 0.2 μM cyclosporine A (CYA) returned 72% of pre-arrest values, which was significantly better ($p<0.05$) than rewarming with AL(Low Ca^{2+}:High Mg^{2+}) or KH alone but was not significantly different from the AL(Low Ca^{2+}:High Mg^{2+}) AL rewarm group. (Table 6.1, Figure 6.6).

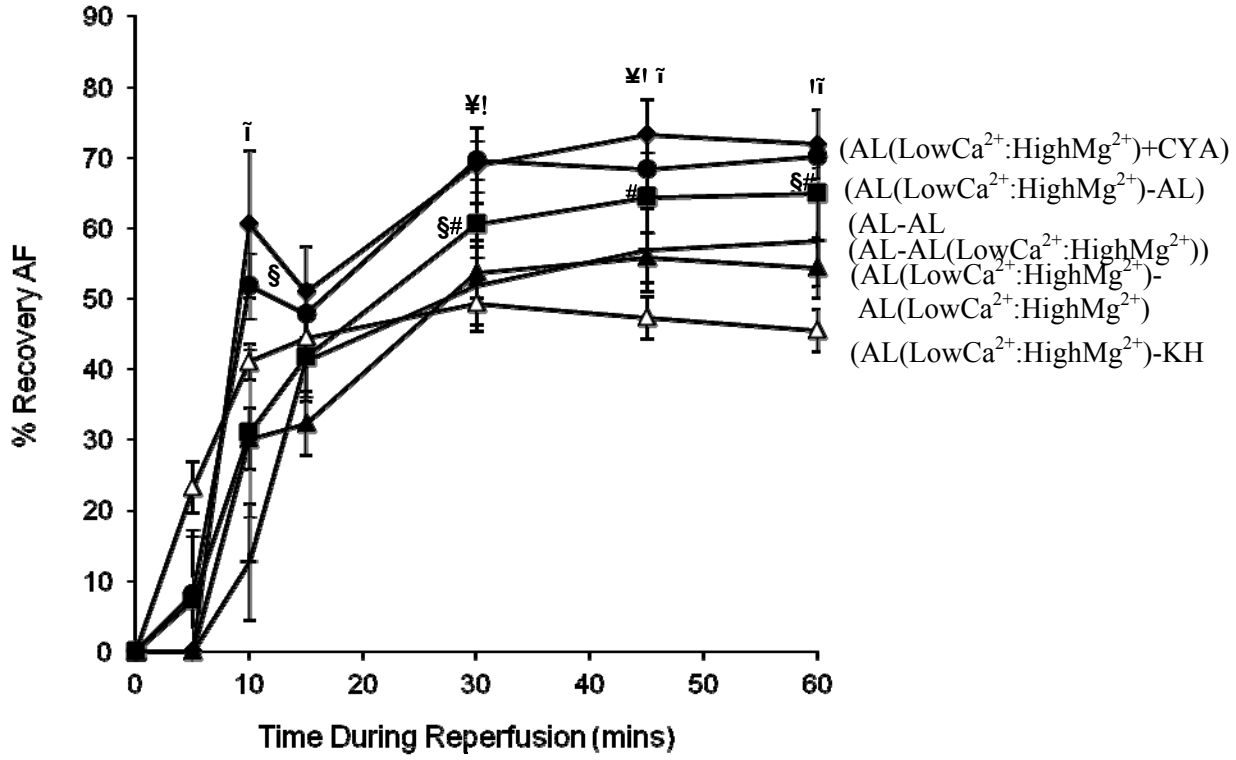

Figure 6.6: Recovery of aortic flow (%AF) following 6 hours of cold storage in AL preservation solutions with different combinations of calcium and magnesium
Data presented as mean ± SEM.
♦ AL(Low Ca^{2+}:High Mg^{2+})+CYA (n=8);● AL(Low Ca^{2+}:High Mg^{2+})-AL(n=8);■ AL-AL;+ AL-AL(Low Ca^{2+}:High Mg^{2+})(n=8);▲ AL(Low Ca^{2+}:High Mg^{2+})-AL(Low Ca^{2+}:High Mg^{2+})(n=8);△ AL(Low Ca^{2+}:High Mg^{2+})-KH (n=7)
AL (Low Ca^{2+}:High Mg^{2+}) with AL rewarm and AL (Low Ca^{2+}:High Mg^{2+}) with KH (rewarm);
§ AL (Low Ca^{2+}:High Mg^{2+}) with AL rewarm vs AL (Low Ca^{2+}:High Mg^{2+}) with AL (Low Ca^{2+}:High Mg^{2+}) rewarm; ¥ AL(Low Ca^{2+}:High Mg^{2+})+CYA with AL AL; ĩ AL(Low Ca^{2+}:High Mg^{2+})+CYA with AL (Low Ca^{2+}:High Mg^{2+}) with AL (Low Ca^{2+}:High Mg^{2+}); ! AL(Low Ca^{2+}:High Mg^{2+})+CYA with AL (Low Ca^{2+}:High Mg^{2+}) with KH (rewarm). Statistical level of significance was $p<0.05$.

6.4.6 Recovery of coronary flow

Storing hearts in AL: There was a rapid spontaneous return of CF in the cold AL group rewarmed with AL cardioplegia with 9% and 59% of pre-arrest values recovered at 5 and 10 mins (Table 6.1 and Figure 6.7). This was significantly different from the AL group rewarmed with AL(LowCa^{2+}:HighMg^{2+}), which did not return any coronary flow at 5 mins ($p<0.05$). There was no significant difference

($p>0.05$) between these two treatment groups after the 10 minute reperfusion time point.

Storing hearts in AL(Low Ca^{2+}:High Mg^{2+}): Hearts stored in AL(LowCa^{2+}:HighMg^{2+}) and rewarmed with KH and AL(LowCa^{2+}:HighMg^{2+}) had returned 64% and 86% respectively of pre-arrest CF by 10 mins and this improved further to 68% and 91% by 60 mins, however; this was not significantly different ($p>0.05$). By contrast, hearts stored in AL(LowCa^{2+}:HighMg^{2+}) and rewarmed in AL performed significantly better ($p<0.05$) returning 90 and 95% of pre-arrest CF at 10 and 60 minutes reperfusion.

Storing hearts in AL(Low Ca^{2+}:High Mg^{2+})+CYA: By 10 minutes the AL(LowCa^{2+}:HighMg^{2+})+CYA group had recovered 100% of CF and this further improved to 106% by 60 mins. This was significantly different the those rewarmed in KH alone but not significantly different from those rewarmed in AL and AL(LowCa^{2+}:HighMg^{2+})(Table 6. 1, Figure 6.7).

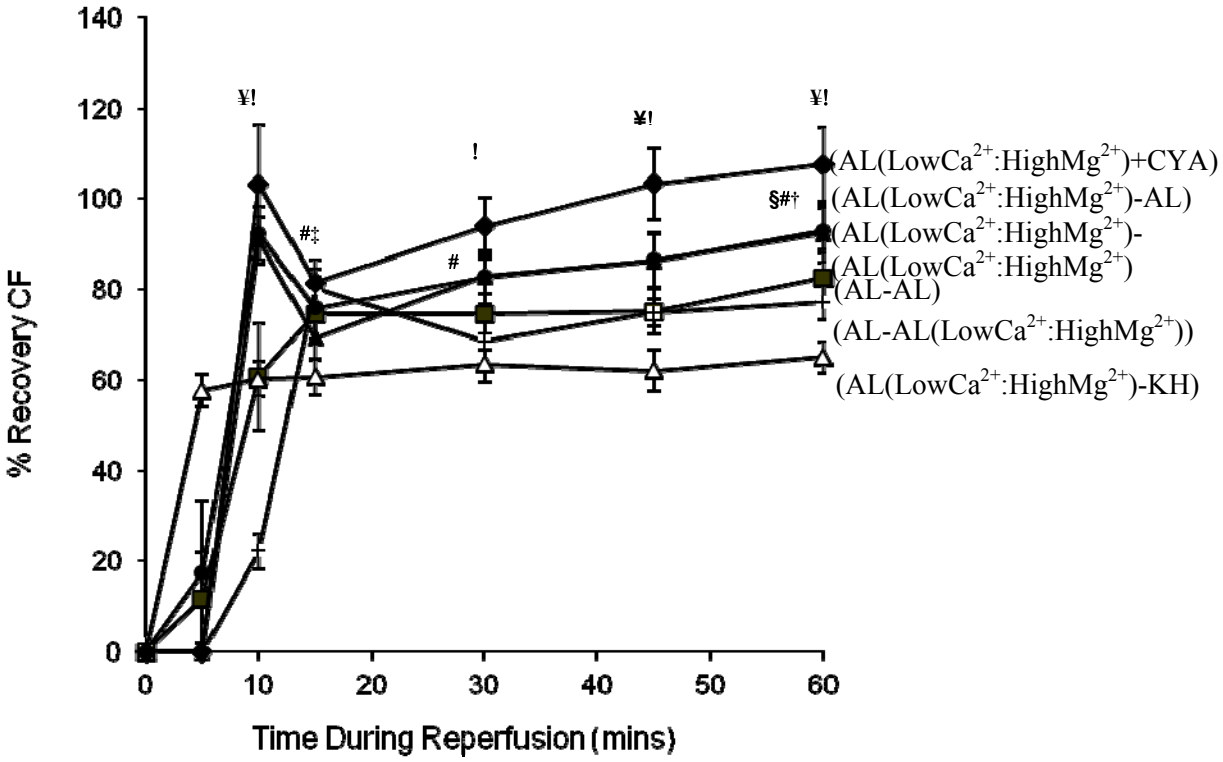

Figure 6.7: Recovery of coronary flow (%CF) following 6 hours of cold storage in AL preservation solutions with different combinations of calcium and magnesium
Data presented as mean ± SEM.
♦ AL(Low Ca^{2+}:High Mg^{2+})+CYA (n=8);● AL(Low Ca^{2+}:High Mg^{2+})-AL (n=8);■ AL-AL (n=8);AL-AL(Low Ca^{2+}:High Mg^{2+}) (n=8);▲ AL(Low Ca^{2+}:High Mg^{2+})-AL(Low Ca^{2+}:High Mg^{2+}) (n=8);△ AL(Low Ca^{2+}:High Mg^{2+})-KH (n=7).
† AL (Low Ca^{2+}:High Mg^{2+}) with AL(Low Ca^{2+}:High Mg^{2+}) rewarm and AL (Low Ca^{2+}:High Mg^{2+}) with KH (rewarm) ;# AL (Low Ca^{2+}:High Mg^{2+}) with AL rewarm and AL (Low Ca^{2+}:High Mg^{2+}) with KH (rewarm); § AL (Low Ca^{2+}:High Mg^{2+}) with AL rewarm vs AL (Low Ca^{2+}:High Mg^{2+}) with AL (Low Ca^{2+}:High Mg^{2+}) rewarm ;‡ AL (Low Ca^{2+}:High Mg^{2+}) with AL rewarm vs AL with AL rewarm ; ¥ AL(Low Ca^{2+}:High Mg^{2+})+CYA with AL AL;**!** AL(Low Ca^{2+}:High Mg^{2+})+CYA with AL (Low Ca^{2+}:High Mg^{2+}) with KH (rewarm). Statistical level of significance was $p<0.05$.

6.4.7 Recovery of cardiac output

Storing hearts in AL: The cold AL group rewarmed with AL cardioplegia returned 7% of pre-arrest CO at 5 min, while the AL group rewarmed with AL(Low Ca^{2+}:High Mg^{2+}) remained arrested. By 10 mins the AL rewarmed with AL group had significantly improved ($p<0.05$) to 39% when compared to the AL(Low

Ca^{2+}:High Mg^{2+}) rewarmed group which recovered 15%. All groups rewarmed with AL(Low Ca^{2+}:High Mg^{2+}) did not return CO at the 5 minute measurement and again this was significantly different ($p<0.05$) from hearts rewarmed with solutions containing normal physiological calcium. After 60 minutes of reperfusion hearts stored in AL and rewarmed with AL and AL(Low Ca^{2+}:High Mg^{2+}) had returned 70% and 64% of their pre-arrest CO which were not significantly different ($p>0.05$).

Storing hearts in AL(Low Ca^{2+}:High Mg^{2+}): Hearts stored in AL(LowCa^{2+}:HighMg^{2+}) and then rewarmed with KH and AL recovered 36% and 11% of pre-arrest CO respectively at 5 mins, while the group rewarmed with AL(Low Ca^{2+}:High Mg^{2+}) remained arrested. Although the recovery of the KH rewarmed group was significantly better at 5 mins ($p<0.05$), by 10 mins the recovered CO was not significantly different from the other rewarm groups ($p>0.05$). After 60 mins of reperfusion hearts stored in AL(Low Ca^{2+}:High Mg^{2+}) and rewarmed in KH and AL(Low Ca^{2+}:High Mg^{2+}) had returned 53% and 63% of pre-arrest CO (not significant ($p>0.05$)). In contrast hearts arrested and stored in AL(Low Ca^{2+}:High Mg^{2+}) and rewarmed with AL, with normal physiological calcium, returned a significantly better 74% of pre-arrest cardiac output following 60 minutes reperfusion ($p<0.05$). (Table 6.1, Figure 6.9). In fact, hearts stored in AL(Low Ca^{2+}:High Mg^{2+}) and rewarmed with AL recovered significantly higher CO at 10, 30 and 60 mins than those arrested and stored in AL with physiological concentrations of calcium and magnesium ($p<0.05$) (Table 6.1; Figure 6.9).

Storing hearts in AL(Low Ca^{2+}:High Mg^{2+})+CYA: Those hearts stored and rewarmed in AL(Low Ca^{2+}:High Mg^{2+})+CYA returned 80% of pre-arrest cardiac

output, which was significantly better than those hearts stored in AL(Low Ca^{2+}:High Mg^{2+}) and rewarmed in either KH or AL(Low Ca^{2+}:High Mg^{2+}) but not significantly different from those rewarmed in AL ($p>0.05$).

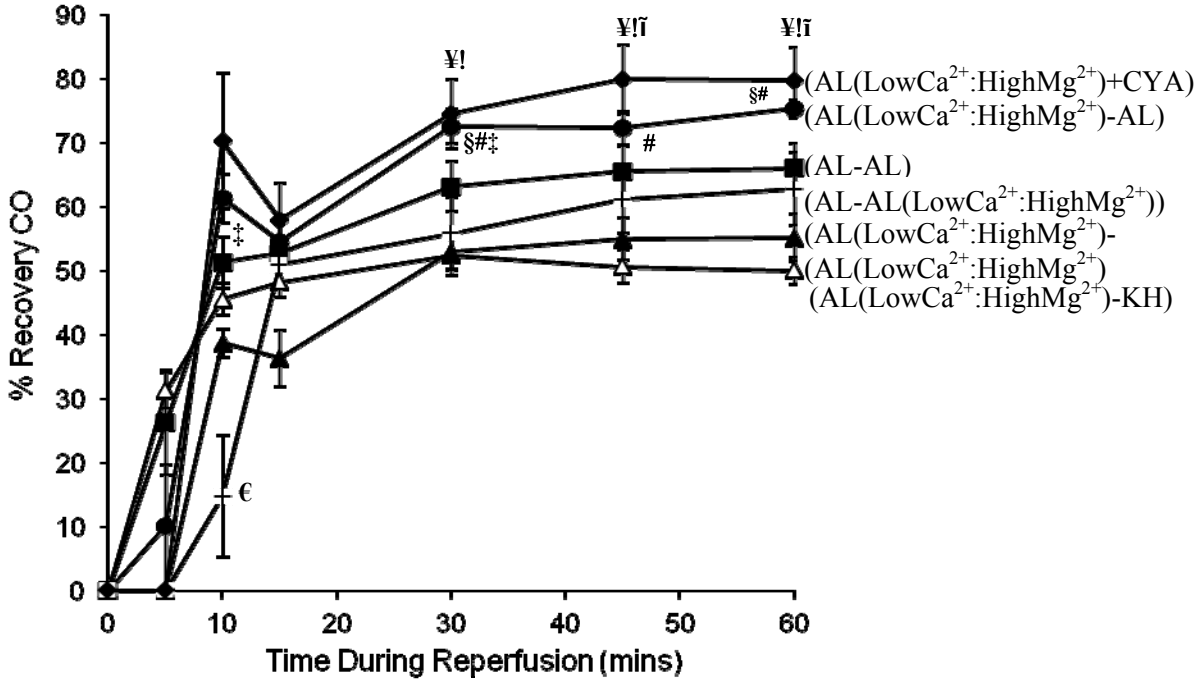

Figure 6.8: Recovery of pre-arrest cardiac output (%CO) following 6 hours cold storage with AL preservation solutions with different combinations of calcium and magnesium
♦ AL(Low Ca^{2+}:High Mg^{2+})+CYA (n=8);● AL(Low Ca^{2+}:High Mg^{2+})-AL (n=8);■ AL-AL (n=8);AL-AL(Low Ca^{2+}:High Mg^{2+}) (n=8);▲ AL(Low Ca^{2+}:High Mg^{2+})-AL(Low Ca^{2+}:High Mg^{2+}) (n=8);△ AL(Low Ca^{2+}:High Mg^{2+})-KH (n=7). Data presented as mean ± SEM.
€ AL with AL (Low Ca^{2+}:High Mg^{2+}) and all other groups
AL (Low Ca^{2+}:High Mg^{2+}) with AL rewarm and AL (Low Ca^{2+}:High Mg^{2+}) with KH (rewarm) $p<0.05$;
§ AL (Low Ca^{2+}:High Mg^{2+}) with AL rewarm vs AL (Low Ca^{2+}:High Mg^{2+}) with AL (Low Ca^{2+}:High Mg^{2+}) rewarm $p<0.05$;
‡ AL (Low Ca^{2+}:High Mg^{2+}) with AL rewarm vs AL with AL rewarm $p<0.05$;
¥ AL(Low Ca^{2+}:High Mg^{2+})+CYA with AL AL $p<0.05$;
ĩ AL(Low Ca^{2+}:High Mg^{2+})+CYA with AL (Low Ca^{2+}:High Mg^{2+}) with AL (Low Ca^{2+}:High Mg^{2+}) $p<0.05$;
! AL(Low Ca^{2+}:High Mg^{2+})+CYA with AL (Low Ca^{2+}:High Mg^{2+}) with KH (rewarm) $p<0.05$

6.4.8 Recovery of heart rate

Storing hearts in AL: At 5 min reperfusion after switching from Langendorff to working mode, the AL cold group with AL rewarm spontaneously returned a heart rate of 17 and 195 beats min^{-1} at 5 and 10 mins of reperfusion (Table 6.2, Figure 6.8). The AL arrest storage and AL(Low Ca^{2+}:High Mg^{2+}) rewarm group was still arrested at 5 mins and returned only 76 beats min^{-1} at 10 mins of reperfusion. The higher heart rate for the AL rewarm group at 5 and 10 mins was significant ($p<0.05$) and was possibly due to the presence of physiologic levels of calcium in the AL solution. After 30 minutes of reperfusion there was no significant difference between the AL or AL(Low Ca^{2+}:High Mg^{2+}) rewarm strategies (281 vs 266 beats min^{-1} at 30 mins and 297 and 286 beats min^{-1} at 60 minutes) ($p>0.05$).

Storing hearts in AL(Low Ca^{2+}:High Mg^{2+}): Both groups stored and rewarmed with the lower calcium and higher magnesium concentrations were slow to recover heart rate: with the AL(LowCa^{2+}:HighMg^{2+}) rewarm and the AL(Low Ca^{2+}:High Mg^{2+})+CYA arrest storage and rewarm groups remaining arrested at 5 mins. On the other hand hearts stored in AL(LowCa^{2+}:HighMg^{2+}) and rewarmed in solutions containing higher calcium concentrations, KH and AL, returned significantly higher heart rates of 227 and 61 beats min^{-1}. By 10 mins reperfusion all groups had spontaneously recovered heart rate; with hearts rewarmed with KH, AL(Low Ca^{2+}:High Mg^{2+}) and AL returning 88%, 79%, and 93% respectively of their pre-arrest heart rates and these were not significantly different. However, by 30 mins while all rewarm groups increased their heart rates by around 17% both the AL(LowCa^{2+}:HighMg^{2+}) and AL rewarm groups had significanty higher heart rates than those rewarmed in KH alone ($p<0.05$) (Table 6.2). By 60 mins all hearts stored

in AL(LowCa^{2+}:HighMg^{2+}) and rewarmed in AL regardless of the calcium and magnesium content had recovered significantly higher heart rates than those rewarmed in KH alone ($p<0.05$) (Table 6.2, Figure 6.8).

Storing hearts in AL(Low Ca^{2+}:High Mg^{2+})+CYA: The AL(Low Ca^{2+}:High Mg^{2+})+CYA group returned following 60 minutes of reperfusion a heart rate of 313 beats min^{-1} which was 116% of baseline and this was also significantly higher than the KH rewarm group ($p<0.05$) (Figure 6.8).

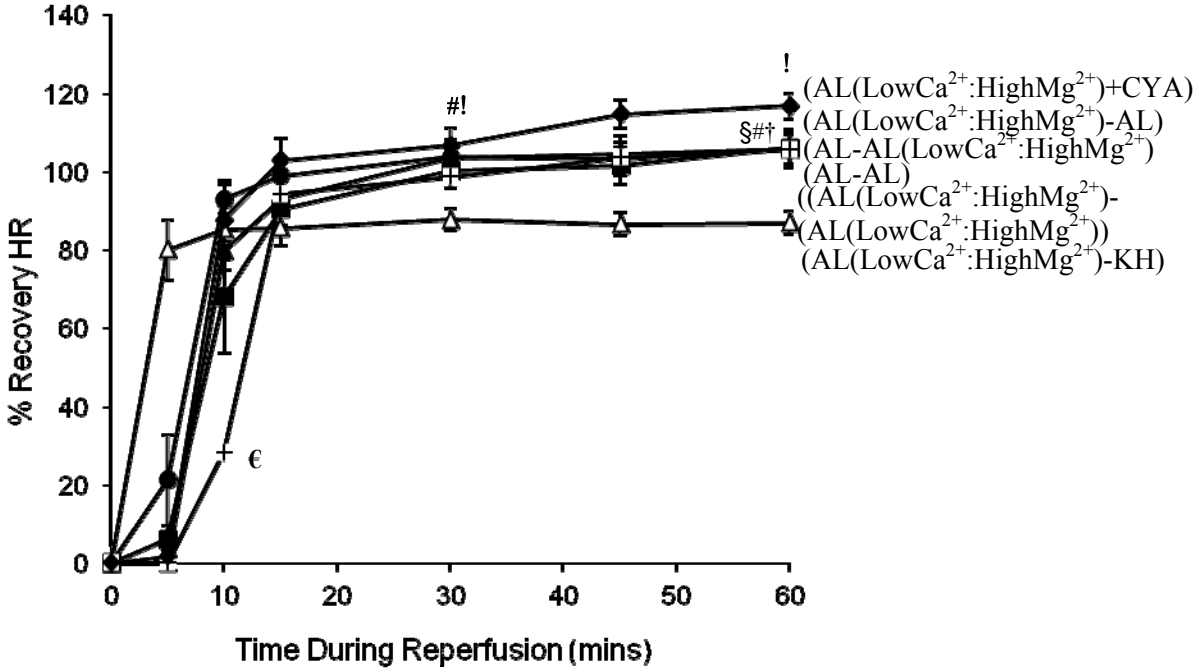

Figure 6.9: Recovery of pre-arrest heart rate (%HR) following 6 hours cold storage with different combinations of calcium and magnesium

Data presented as mean ± SEM.

♦ AL(Low Ca^{2+}:High Mg^{2+})+CYA (n=8);● AL(Low Ca^{2+}:High Mg^{2+})-AL (n=8);■ AL-AL (n=8); AL-AL(Low Ca^{2+}:High Mg^{2+}) (n=8);▲ AL(Low Ca^{2+}:High Mg^{2+})-AL(Low Ca^{2+}:High Mg^{2+}) (n=8); △ AL(Low Ca^{2+}:High Mg^{2+})-KH (n=7).

€ AL with AL (Low Ca^{2+}:High Mg^{2+}) and all other groups

† AL (Low Ca^{2+}:High Mg^{2+}) with AL(Low Ca^{2+}:High Mg^{2+}) rewarm and AL (Low Ca^{2+}:High Mg^{2+}) with KH (rewarm) $p<0.05$;

AL (Low Ca^{2+}:High Mg^{2+}) with AL rewarm and AL (Low Ca^{2+}:High Mg^{2+}) with KH (rewarm) $p<0.05$;

§ AL (Low Ca^{2+}:High Mg^{2+}) with AL rewarm vs AL (Low Ca^{2+}:High Mg^{2+}) with AL (Low Ca^{2+}:High Mg^{2+}) rewarm $p<0.05$;

! AL(Low Ca^{2+}:High Mg^{2+})+CYA with AL (Low Ca^{2+}:High Mg^{2+}) with KH (rewarm) $p<0.05$.

6.4.9 Recovery of systolic and diastolic pressures and rate pressure product

Storing hearts in AL: Although there were no significant differences in developed diastolic pressures between the groups, the AL(Low Ca^{2+}:High Mg^{2+}) rewarm groups again failed to generate high systolic pressures at 5 min when compared with groups rewarmed with physiological levels of calcium, and this was also reflected in lower rate-pressure products at the different reperfusion times (Table 6.2). At 10

mins, the group of hearts stored in AL and rewarmed with AL generated 2 fold higher pressures and rate pressure products than the group stored under similar conditions but rewarmed with AL(Low Ca^{2+}:High Mg^{2+}). This group had generated a systolic/diastolic pressure of 46/26 mmHg and RPP of 8373 ± 5261 by 10 minutes and this was significantly different from all other groups ($p<0.05$). However, after 30 minutes reperfusion the systolic/diastolic pressures were not significantly different between the groups ($p>0.05$) (Table 6.2).

Storing hearts in AL(Low Ca^{2+}:High Mg^{2+}) or AL(Low Ca^{2+}:High Mg^{2+})+CYA: There were no significant differences between the recovered pressures for hearts arrested and stored in AL(Low Ca^{2+}:High Mg^{2+}) and rewarmed with KH, AL(Low Ca^{2+}:High Mg^{2+}) and AL, as well as the group arrested, stored and rewarmed in AL(Low Ca^{2+}:High Mg^{2+})+CYA ($p>0.05$).

6.4.10 Myocardial oxygen consumption, coronary vascular resistance and pH during the last minute of 5 minute rewarm

Storing hearts in AL: At the end of rewarming phase, the MVO_2 for cold AL preservation groups in Langendorff mode with AL or AL(Low Ca^{2+}:High Mg^{2+}) rewarm were 18.3 ± 1.0 and 22.7 ± 4.2 μmol O_2/min/g dry wt respectively (Figure 6.10).

Storing hearts in AL(Low Ca^{2+}:High Mg^{2+}): The MVO_2 for the cold AL(Low Ca^{2+}:High Mg^{2+}) group rewarmed with KH, AL(Low Ca^{2+}:High Mg^{2+}) or AL arrest were 31.6 ± 3.0, 17.6 ± 1.6 and 25.3 ± 3.8 μmol O_2/min/g dry wt respectively (Figure 6.10). The higher MVO_2 for the KH rewarm group was possibly due to the fact that the heart was not arrested but beating throughout the rewarm period (not

significant (p>0.05)); similar results were reported in the study described in Chapter 5.

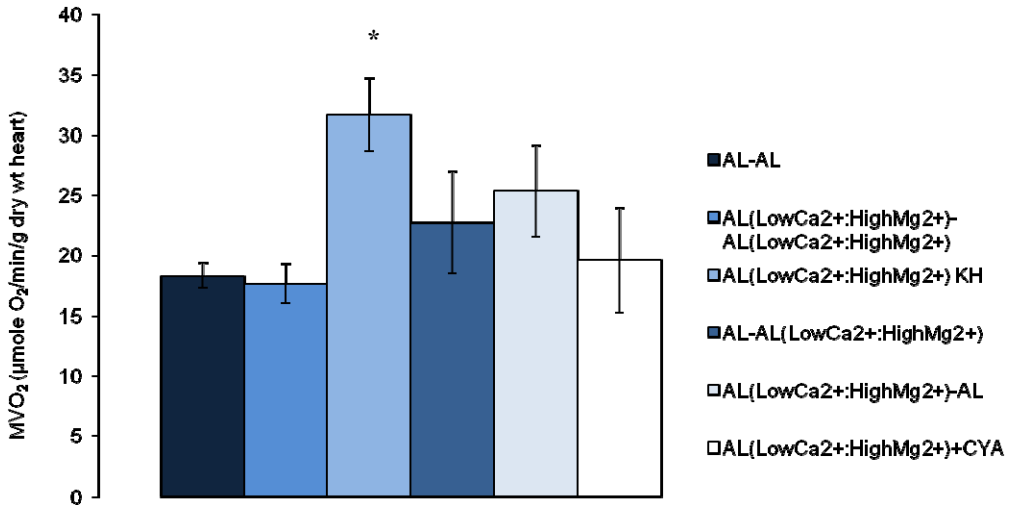

Figure 6.10: Myocardial oxygen consumption (MVO_2) during the 5 minute rewarm for AL preservation solutions with different combinations of calcium and magnesium
AL-AL (n=8); AL(LowCa^{2+}:HighMg^{2+})- AL(LowCa^{2+}:HighMg^{2+}) ; AL(LowCa^{2+}:HighMg^{2+}) – KH (n=7); AL- AL(LowCa^{2+}:HighMg^{2+}) (n=8); AL(LowCa^{2+}:HighMg^{2+})-AL (n=8); AL(LowCa^{2+}:HighMg^{2+})+CYA (n=8). Data presented as mean ± SEM.
* denotes significant difference of KH rewarmed hearts from those rewarmed in AL cardioplegic solution (p<0.05).

Storing hearts in AL: The coronary effluent flows in the final minute for hearts arrested and stored in AL and rewarmed in AL and AL(Low Ca^{2+}:High Mg^{2+}) were 12.9 ± 0.6 and 15.2 ± 1.0 ml respectively.

Storing hearts in AL(Low Ca^{2+}:High Mg^{2+}): For those arrested and stored in AL(LowCa^{2+}:HighMg^{2+}) and rewarmed in KH, AL(Low Ca^{2+}:HighMg^{2+}) and AL were 17.1 ± 1.2, 13.9 ± 0.7 and 14.9 ± 0.7 ml respectively. The coronary vascular resistance for each of the groups is shown in Figure 6.11; these ranged from 0.33 to 0.40 but were not significantly different (p>0.05).

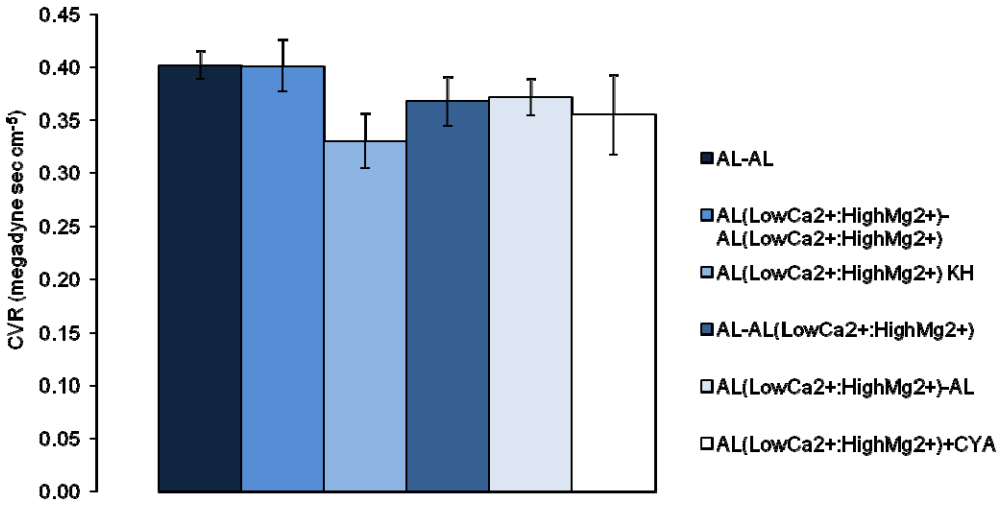

Figure 6.11: Coronary vascular resistance during 5 minute rewarming for AL preservation solutions with different combinations of calcium and magnesium
AL-AL (n=8); AL(LowCa^{2+}:HighMg^{2+})- AL(LowCa^{2+}:HighMg^{2+}) ; AL(LowCa^{2+}:HighMg^{2+}) –KH (n=7); AL- AL(LowCa^{2+}:HighMg^{2+}) (n=8); AL(LowCa^{2+}:HighMg^{2+})-AL (n=8); AL(LowCa^{2+}:HighMg^{2+})+CYA (n=8). Data presented as mean ± SEM. There was no significant difference between the groups for CVR during the 5 minutes rewarming ($p<0.05$).

The pH of the coronary effluent collected in the last minute of the 5 minute rewarming period ranged between 7.41 and 7.49 for all the groups (Figure 6.12) and were not significantly different ($p>0.05$).

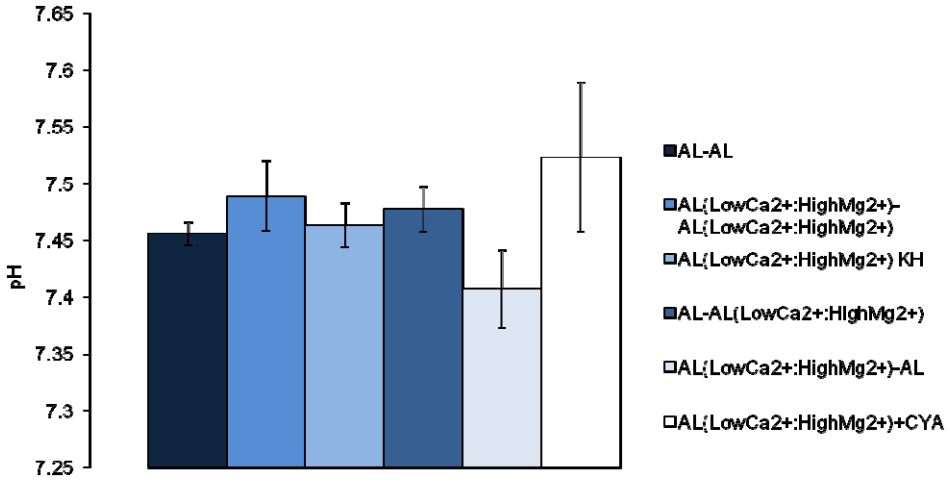

Figure 6.12: Effluent pH during 5 minute rewarm for AL preservation solutions with different combinations of calcium and magnesium
AL-AL (n=8); AL(LowCa^{2+}:HighMg^{2+})- AL(LowCa^{2+}:HighMg^{2+}) ; AL(LowCa^{2+}:HighMg^{2+}) – KH (n=7); AL- AL(LowCa^{2+}:HighMg^{2+}) (n=8); AL(LowCa^{2+}:HighMg^{2+})-AL (n=8); AL(LowCa^{2+}:HighMg^{2+})+CYA (n=8). Data presented as mean ± SEM.
There was no significant difference between the groups for pH during the 5 minutes rewarming ($p<0.05$).

6.5 Study Two: Optimising Adenosine and Lignocaine Concentrations for 6 Hour Preservation

6.5.1 Functional recoveries

Functional measurements of aortic flow, coronary flow, heart rate, cardiac output, systolic pressure, diastolic pressure and rate pressure product were not significantly different in the pre-arrest measurements (Table 6.3).

Table 6.3: Functional parameters (AF, CF and CO) of isolated working rat hearts following 6 hours cold storage and rewarm with AL preservation solution containing various combinations of adenosine and lignocaine

Time during Pre-arrest and Reperfusion	Cold Arrest + Rewarm Treatment	n	Aortic Flow (ml/min)	Coronary Flow (ml/min)	Cardiac Output (ml/min)
15 mins Pre-Arrest	AL(200:500 µM)	8	65 ± 2	21 ± 2	87 ± 3
	AL(200:1000 µM)	6	62 ± 1	22 ± 1	84 ± 1
	AL(200:1500 µM)	6	59 ± 1	21 ± 1	80 ± 2
	AL(400:1000 µM)	6	64 ± 2	24 ± 1	88 ± 2
10 mins Recovery	AL(200:500 µM)	8	20 ± 3	19 ± 1	37 ± 2
	AL(200:1000 µM)	6	15 ± 5	11 ± 3	32 ± 5
	AL(200:1500 µM)	6	16 ± 9	10 ± 4	26 ± 12
	AL(400:1000 µM)	6	8 ± 7	16 ± 4	24 ± 10
15 mins Recovery	AL(200:500 µM)	8	21 ± 3	15 ± 2	36 ± 5
	AL(200:1000 µM)	6	19 ± 4	13 ± 2	32 ± 6
	AL(200:1500 µM)	6	35 ± 2	19 ± 2	54 ± 3
	AL(400:1000 µM)	6	31 ± 8	17 ± 3	37 ± 12
30 mins Recovery	AL(200:500 µM)	8	35 ± 3	18 ± 3	53 ± 4
	AL(200:1000 µM)	6	33 ± 1	15 ± 1	48 ± 2
	AL(200:1500 µM)	6	50 ± 2*#	18 ± 1	68 ± 2#
	AL(400:1000 µM)	6	42 ± 5	21 ± 2†	63 ± 6‡
45 mins Recovery	AL(200:500 µM)	8	36 ± 3	19 ± 2	55 ± 3
	AL(200:1000 µM)	6	35 ± 2	16 ± 2	51 ± 4
	AL(200:1500 µM)	6	50 ± 2*#	19 ± 1	69 ± 2*#
	AL(400:1000 µM)	6	46 ± 3†‡	22 ± 2	67 ± 4†‡
60 mins Recovery	AL(200:500 µM)	8	35 ± 3	20 ± 2	55 ± 4
	AL(200:1000 µM)	6	32 ± 2	16 ± 2	48 ± 3
	AL(200:1500 µM)	6	50 ± 2*#	20 ± 1	70 ± 2*#
	AL(400:1000 µM)	6	44 ± 2†‡	22 ± 1	66 ± 3†‡

Data presented as mean ± SEM.
* Denotes AL(200:1500 µM) significantly different AL(200:500 µM)
# Denotes AL(200:1500 µM) significantly different AL(200:1000 µM)
† Denotes AL(400:1000 µM) significantly different AL(200:500 µM)
‡ Denotes AL(200:1000 µM) significantly different AL(200:1000 µM)

6.5.2 Recovery of aortic flow

Hearts preserved in AL(200:500 µM) recovered 31% of their pre-arrest AF at 10 minutes of reperfusion and this improved to 54% by 60 minutes (Table 6.3, Figure 6.13). If the lignocaine concentration is doubled or tripled each generated 24% and 27% of pre-arrest AF at 10 mins respectively and this was not significantly different from the standard lignoocaine concentration ($p>0.05$). However, by 60 mins the

AL(200:1500 μM) group had improved to 85% of its pre-arrest AF and this was significantly better than the AL(400:1000 μM) which had recovered 52% ($p<0.05$) (Table 6.3 and Figure 6.13). Doubling both the adenosine and lignocaine concentrations resulted in worse (13% of pre-arrest AF) recovery at 10 minutes but this improved to 69% by 60 minutes and was significantly higher than for AL(200:500 μM) and AL(200:1000 μM) but not significantly different from AL(200:1500 μM) ($p>0.05$).

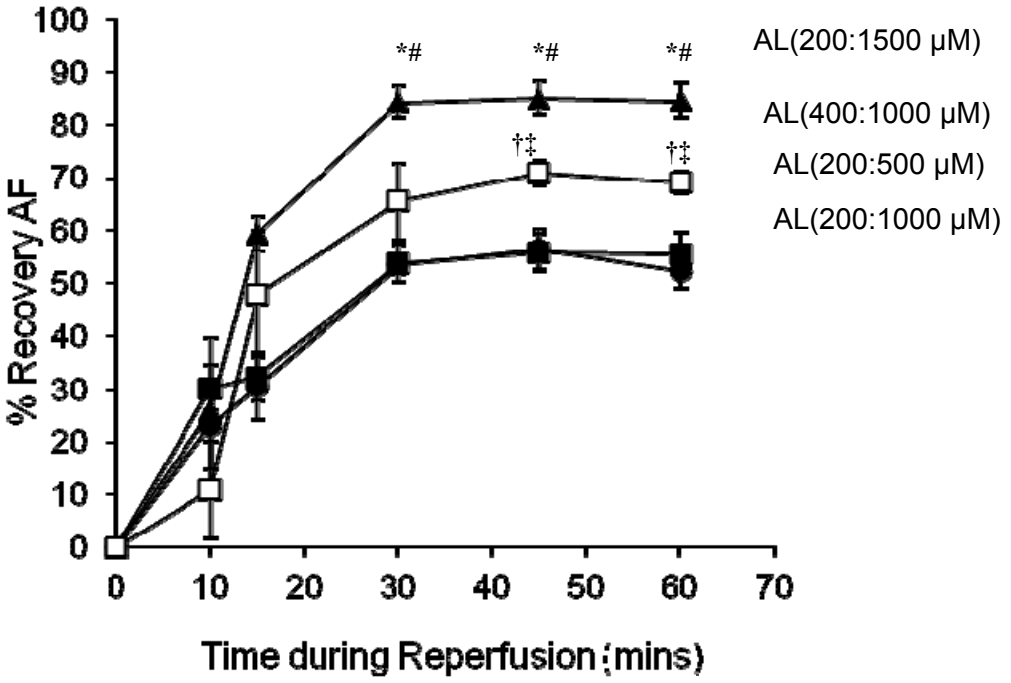

Figure 6.13: Recovery of aortic flow (%AF) following 6 hours cold storage in AL preservation solution containing various combinations of adenosine and lignocaine
■ AL(200:500 μM) (n=6); ● AL(200:1000 μM) (n=6);▲ AL(200:1500 μM) (n=6); □AL(400:1000 μM) (n=6). Data presented as mean ± SEM.
***** Denotes AL(200:1500 μM) significantly different AL(200:500 μM)
# Denotes AL(200:1500 μM) significantly different AL(200:1000 μM)
† Denotes AL(400:1000 μM) significantly different AL(200:500 μM)
‡ Denotes AL(200:1000 μM) significantly different AL(200:1000 μM)

6.5.3 Recovery of coronary flow

There was a rapid spontaneous return of CF in the AL(200:500 μM) group with 90% and 71% of pre-arrest values recovered at 10 and 15 minute time points (Table 6.3 and Figure 6.14). This was not significantly different from the AL(200:1000 μM) group or the AL(200:1500 μM) groups which returned 50% and 48% coronary flow at 10 mins and 59% and 90% at 15 minutes. By 60 mins there was no significant difference between the AL(200:500 μM) and the other groups. Although the AL(400:1000 μM) group was significantly better with a return of coronary flow of 88% at 30 minutes, by 60 mins this improvement was no longer significant (Table 6.3, Figure 6.14).

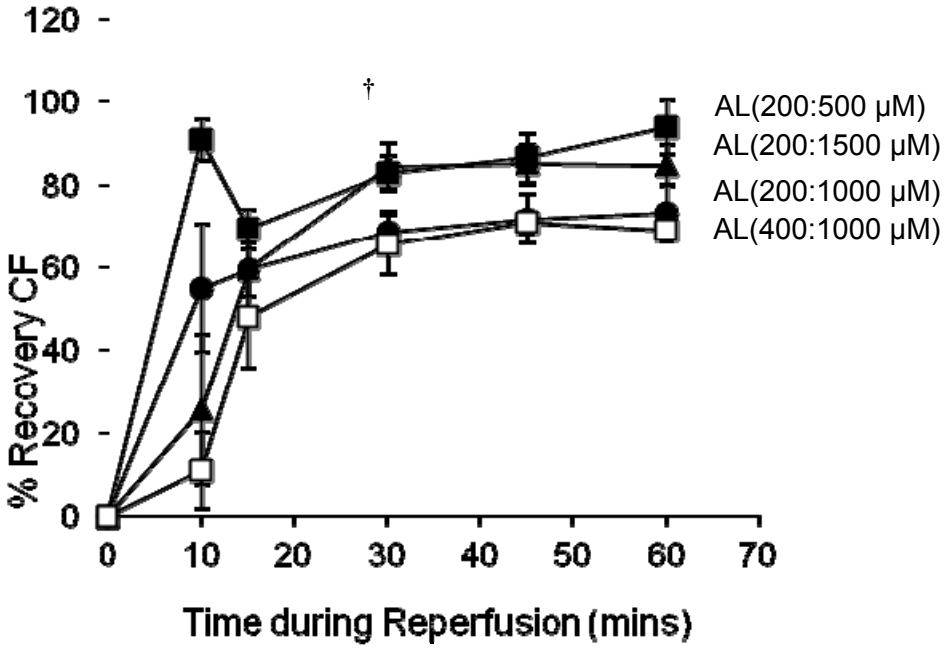

Figure 6.14: Recovery of coronary flow (%CF) following 6 hours cold storage in AL preservation solution containing various combinations of adenosine and lignocaine
■ AL(200:500 µM) (n=6); ● AL(200:1000 µM) (n=6);▲ AL(200:1500 µM) (n=6);
□AL(400:1000 µM) (n=6). Data presented as mean ± SEM.
***** Denotes AL(200:1500 µM) significantly different AL(200:500 µM)
# Denotes AL(200:1500 µM) significantly different AL(200:1000 µM)
† Denotes AL(400:1000 µM) significantly different AL(200:500 µM)
‡ Denotes AL(200:1000 µM) significantly different AL(200:1000 µM)

6.5.4 Recovery of cardiac output

Hearts arrested and stored in AL(200:500 µM) group returned 42 and 41% of pre-arrest CO respectively at 10 mins, and by 15 mins and this improved to 63% by 60 minutes of reperfusion. Doubling the lignocaine concentration in the AL(200:1000 µM) did not significantly improve the cardiac output at any time point with 38%, 38% and 57% at 10, 15 and 60 mins respectively (See Figure 6.16). By contrast, tripling the lignocaine concentration, as in the AL(200:1500 µM) group resulted in 33%, 68% and 88% recovery of CO at 10, 15 and 60 mins of reperfusion and this improvement was significant by 60 minutes ($p<0.05$). The AL(400:1000 µM) group also showed significantly better recoveries with 27%, 42%

and 75% at 10, 15 and 60 minutes of reperfusion (Table 6.3; Figure 6.16). Although the recovery by 60 minutes was more than 10% less than the AL(200:1500 μM) group, it was not significantly different (p>0.05).

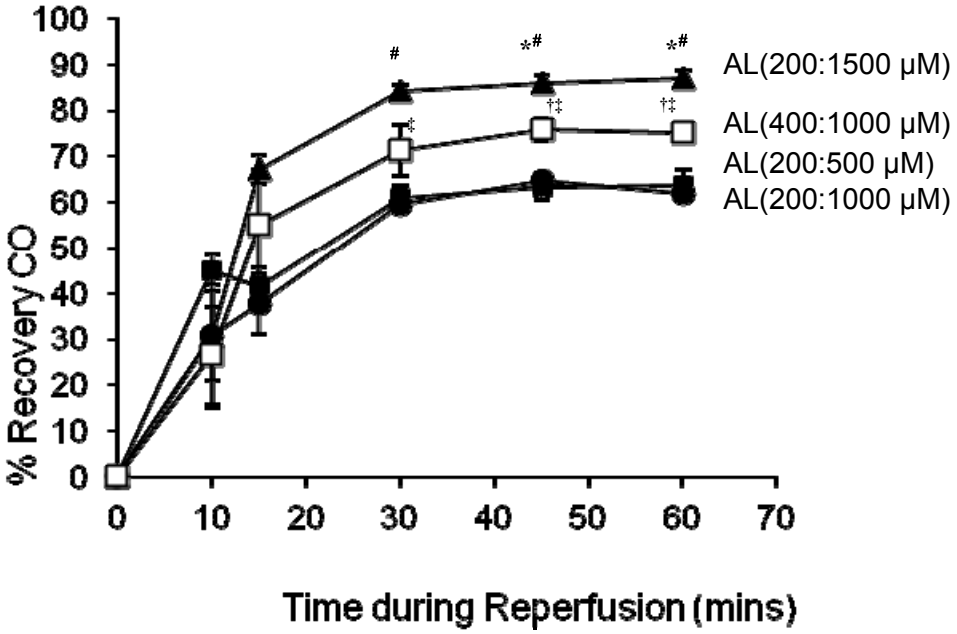

Figure 6.15: Recovery of cardiac output (%CO) following 6 hours cold storage with various combinations of adenosine and lignocaine
■ AL(200:500 μM) (n=6); ●AL(200:1000 μM) (n=6);▲ AL(200:1500 μM) (n=6);
□ AL(400:1000 μM) (n=6). Data presented as mean ± SEM.
* Denotes AL(200:1500 μM) significantly different AL(200:500 μM)
# Denotes AL(200:1500 μM) significantly different AL(200:1000 μM)
† Denotes AL(400:1000 μM) significantly different AL(200:500 μM)
‡ Denotes AL(200:1000 μM) significantly different AL(200:1000 μM)

6.5.5 Recovery of heart rate

Ten minutes after switching from Langendorff rewarm to working mode, the AL(200:500 μM) group spontaneously returned a heart rate that was 93% of pre-arrest heart rate and by 60 minutes reperfusion, had fully recovered (106%) (Table 6.4, Figure 6.15). In contrast the AL(200:1000 μM), AL(200:1500 μM) and

AL(400:1000 μM) groups recovered 59% and significantly lower 33% and 31% of pre-arrest heart rate by 10 mins respectively, however; by 60 mins these groups had fully recovered ($p<0.05$) (Table 6.4, Figure 6.15).

Table 6.4: Functional parameters (HR, SP, DP and RPP) of isolated working rat hearts following 6 hours cold storage and rewarm with AL preservation solution containing various combinations of adenosine and lignocaine

Time during Pre-arrest and Reperfusion	Cold Arrest + Rewarm Treatment	n	Heart Rate (bpm)	Systolic Pressure (mmHg)	Diastolic Pressure (mmHg)	Rate Pressure Product (beats.mmHg/min)
15 min Pre-Arrest	AL(200:500 μM)	8	268 ± 8	129 ± 1	60 ± 0	34528 ± 899
	AL(200:1000 μM)	6	282 ± 7	128 ± 1	60 ± 0	36210 ± 941
	AL(200:1500 μM)	6	282 ± 9	128 ± 1	60 ± 0	35919 ± 943
	AL(400:1000 μM)	6	283 ± 3	129 ± 1	60 ± 0	36592 ± 349
10 min Recovery	AL(200:500 μM)	8	213 ±11	125 ± 2	62 ± 1	26483 ± 1106
	AL(200:1000 μM)	6	165 ± 38	82 ± 16	48 ± 8	17327 ± 4843
	AL(200:1500 μM)	6	93 ± 42*	61 ± 24	28 ± 8	12240 ± 6488
	AL(400:1000 μM)	6	88 ± 43†	40 ± 18†	22 ± 9†	8508 ± 5146†
15 min Recovery	AL(200:500 μM)	8	249 ± 18	109 ± 2	68 ± 1	27300 ± 2230
	AL(200:1000 μM)	6	223 ± 37	94 ± 11	57 ± 7	23493 ± 4161
	AL(200:1500 μM)	6	254 ± 13	120 ± 5	62 ± 1	30088 ± 763
	AL(400:1000 μM)	6	206 ± 47	90 ± 19	48 ± 8†	23948 ± 6516
30 min Recovery	AL(200:500 μM)	8	276 ± 12	119 ± 2	64 ± 2	32875 ± 1264
	AL(200:1000 μM)	6	284 ± 12	109 ± 2	67 ± 2	31029 ± 1436
	AL(200:1500 μM)	6	275 ± 11	123 ± 2#	61 ± 1	33620 ± 1172
	AL(400:1000 μM)	6	299 ± 11	123 ± 4‡	60 ± 2	36723 ±1936
45 mins Recovery	AL(200:500 μM)	8	279 ± 10	118 ± 1	63 ± 1	32736 ± 1052
	AL(200:1000 μM)	6	284 ± 11	127 ± 3	59 ± 1	36194 ± 1591
	AL(200:1500 μM)	6	285 ± 10	108 ± 3	66 ± 2	30684 ± 1118
	AL(400:1000 μM)	6	294 ± 11	123 ± 4	58 ± 1	36272 ± 1839
60 min Recovery	AL(200:500 μM)	8	283 ± 11	117 ± 1	63 ± 1	33328 ± 1132
	AL(200:1000 μM)	6	293 ± 10	107 ± 3	67 ± 2	31244 ±1348
	AL(200:1500 μM)	6	291 ± 10	124 ± 3#	58 ± 1#	36109 ± 1601
	AL(400:1000 μM)	6	294 ± 10	123 ± 4‡	59 ± 2‡	36120 ± 1360

Data presented as mean ± SEM.
* Denotes AL(200:1500 μM) significantly different AL(200:500 μM)
# Denotes AL(200:1500 μM) significantly different AL(200:1000 μM)
† Denotes AL(400:1000 μM) significantly different AL(200:500 μM)
‡ Denotes AL(200:1000 μM) significantly different AL(200:1000 μM)

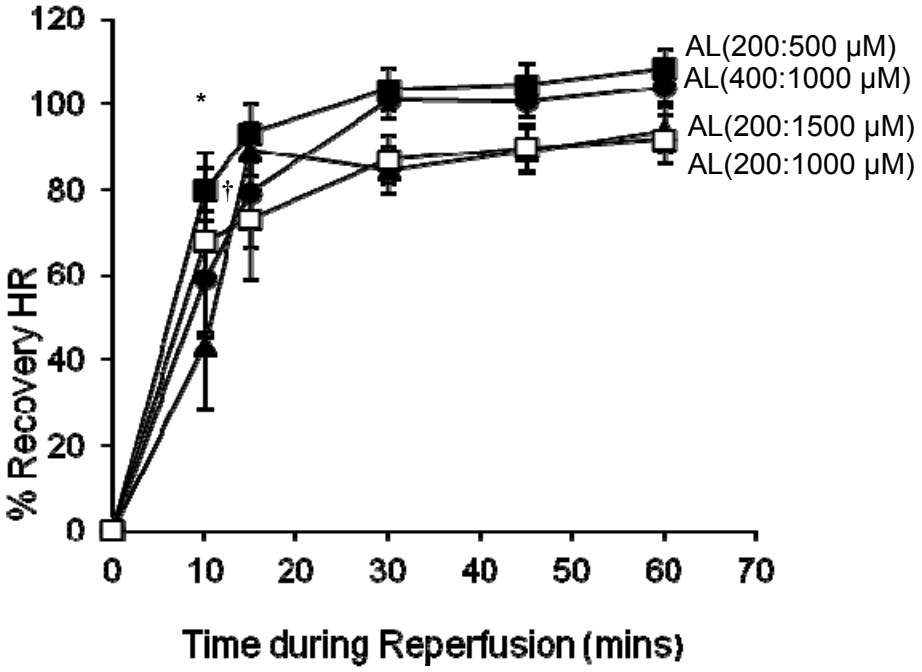

Figure 6.16: Recovery of heart rate (%HR) following 6 hours cold storage with various combinations of adenosine and lignocaine
■ AL(200:500 µM) (n=6); □ AL(200:1000 µM) (n=6);▲ AL(200:1500 µM) (n=6);● AL(400:1000 µM) (n=6). Data presented as mean ± SEM.
***** Denotes AL(200:1500 µM) significantly different AL(200:500 µM)
# Denotes AL(200:1500 µM) significantly different AL(200:1000 µM)
† Denotes AL(400:1000 µM) significantly different AL(200:500 µM)
‡ Denotes AL(200:1000 µM) significantly different AL(200:1000 µM)

6.5.6 Recovery of systolic and diastolic pressures and rate pressure product

The AL(200:500 µM) group recovered 97% of systolic pressure and 103% of diastolic pressure in the first 10 mins of reperfusion. All other groups were slow to recover SP and DP at the same time point (Table 6.4). While the AL(200:1000 µM) group had a 40% lower SP and a 20% lower DP, the AL(200:1500 µM) and the AL(400:1000 µM) group had 50% and more than 60% lower SP and DP respectively at 10 mins reperfusion. The AL(200:500 µM) group was able to maintain SP and DP until 60 mins of reperfusion with 91% and 105% of pre-arrest SP and DP respectively. The developed pressures for the AL(200:1000 µM) group increased to 107 mmHg and 67 mmHg which were 84 and 112% respectively. In contrast the

AL(200:1500 μM) recovered 97% of both SP and DP to 124 mmHg and 58 mmHg by the end of 60 mins reperfusion and this was significantly better ($p<0.05$) then the AL(200:500 μM) and AL(200:1000 μM) groups. After a washout period (10-15 mins) the AL(400:1000 μM) group recovered pressures to achieve 95 and 98% of pre-arrest SP and DP at 60 mins of reperfusion and this was significantly better ($p<0.05$) than both the AL(200:500 μM) and AL(200:1000 μM) groups. Although, the AL(400:1000 μM) group had a significantly lower RPP at 10 mins than the AL(200:500 μM) group, the recovery of rate pressure product (RPP) for the four groups was not significantly different after 60 mins of reperfusion ($p>0.05$) (Table 6.4).

6.5.7 Coronary vascular resistance during 5 minute rewarm

Coronary vascular resistance during the 5 minute rewarm for the 4 groups is shown in Figure 6.16. The CVR for the AL(200:500 μM) and AL(200:1000 μM) groups were 0.40 megadyne/sec/cm^{-5} and 0.41 megadyne/sec/cm^{-5} respectively and this was in contrast to the other two groups AL(200:1500 μM) and AL(400:1000 μM) which were lower, 0.32 megadyne/sec/cm^{-5} (not significant $p=0.064$) and 0.31 megadyne/sec/cm^{-5} (significant $p<0.05$) respectively (See Figure 6.17).

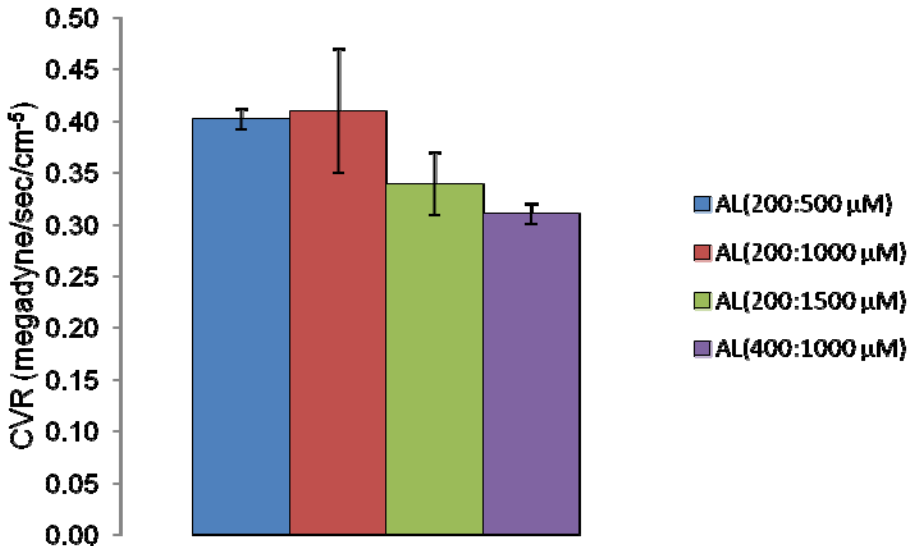

Figure 6.17 Coronary vascular resistance (CVR) in the last minute of the 5 minute rewarm with various combinations of adenosine and lignocaine
Data presented as mean ± SEM.* Denotes AL(400:1000 µM) significantly different than

6.5.8 Episodes of unstable heart rhythm during reperfusion

During the 60 minutes of reperfusion the duration of unstable heart rhythmn was measured over the 60 minute reperfusion period and averaged over the group, these are presented in Figure 6.18. The AL(200:1500 µM) group showed a significantly increased duration of arrhythmias (5/6 hearts, experiencing up to 2652 seconds duration) when compared to the other three groups: AL(200:500 µM) (4/8 hearts, all experiencing less than 70 seconds), AL(200:1000 µM) (3/6 hearts, all experiencing less than 21 seconds) and AL(400:1000 µM) (2/6 hearts, all experiencing less than 50 seconds duration) which although much greater in number was not significantly different ($p>0.05$).

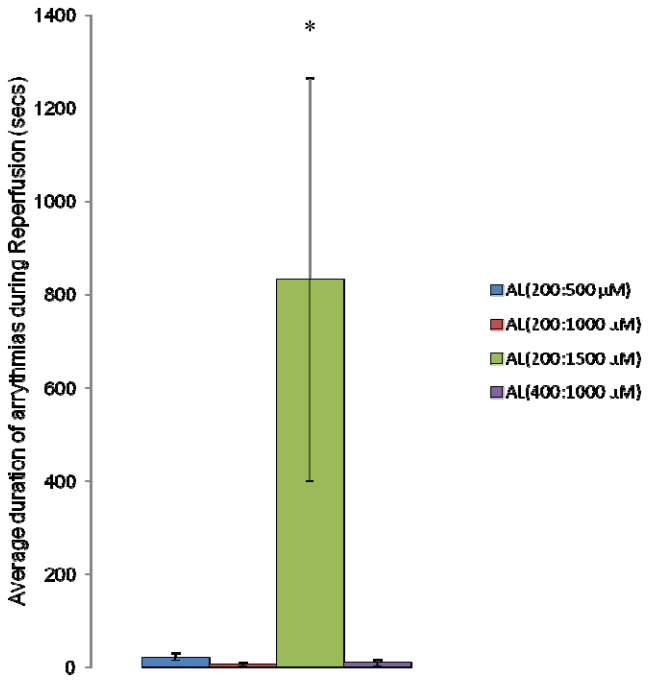

Figure 6.18: Average duration of arrhythmias during 60 minutes of reperfusion following storage and rewarm with various combinations of adenosine and lignocaine
The average duration in seconds of arrhythmias during the 60 minutes reperfusion period following 6 hours cold static storage in AL solution with various combinations of adenosine and lignocaine concentrations. Data presented as mean ± SEM.

6.6 Discussion

6.6.1 Study one: Low calcium: High magnesium

Early functional recovery of hearts arrested and stored for 6 hours in AL(Low Ca^{2+}:HighMg^{2+}) and rewarmed with AL solution was significantly better than those arrested, stored and rewarmed in AL solution. Furthermore, rewarming hearts stored in AL(Low Ca^{2+}:HighMg^{2+}) with AL significantly improved recovery of AF, CF and CO when compared to hearts stored in the same solution but rewarmed in AL(Low Ca^{2+}:HighMg^{2+}) or KH alone ($p<0.05$). The addition of 0.2 µM CYA to the AL preservation solution during arrest, storage and reperfusion did not further improve recovery of AF, CF or CO in the isolated rat heart.

6.6.1.1 Effect of lowering Ca^{2+} and increasing Mg^{2+} during arrest and storage on functional recoveries

6.6.1.1.1 Time to arrest, first beat and aortic flow

Reducing the calcium concentration and increasing the magnesium concentration of AL solution during arrest made no significant difference to the time taken to arrest the heart; this is interesting as other groups have shown that lowering the calcium of hyperkalaemic solutions has been associated with faster arrest times (Bers and Despa, 2006; Chambers and Fallouh, 2010). Adenosine and lignocaine in combination have been shown in other studies to bring about a rapid arrest of the isolated rat heart (Dobson, 2004; Rudd and Dobson, 2009) and this study has shown that this is independent of the calcium concentration of the arrest solution.

Time to first beat was significantly longer for those hearts rewarmed in AL solution with lower calcium and higher magnesium concentrations. This is not surprising as extracellular calcium concentration is an important determinant of contractility; the delay in recommencement of contractility could possibly be due to calcium uptake by the myocytes (Makazan *et al.*, 2009).

6.6.1.1.2 Recovery of aortic flow, cardiac output and heart rate

Lowering the calcium concentration (0.22 mM) and increasing the magnesium concentration (2.6 mM) of the AL arrest and storage solution significantly improved early recovery of CF and CO at 10 and 30 minutes of reperfusion but had minimal effect on the recovery at 60 minutes reperfusion of AF (69 vs 65%) and CO (74 vs 70%) when compared with hearts stored under similar conditions in AL cardioplegic solution with physiological levels of calcium and magnesium. The strategy of

lowering calcium and increasing the magnesium concentration of hyperkalaemic solutions has been used to ameliorate calcium loading during storage and reperfusion of both adult (Fremes *et al.*, 1995; Tsukube *et al.*, 1996a) and neonatal hearts (Kronon *et al.*, 1997); (Obadia *et al.*, 1997; Stowe *et al.*, 2000).

However, despite many studies demonstrating benefits, no consensus on the optimum concentrations of calcium and magnesium in preservation solutions has been reached. This is possibly because optimal calcium and magnesium concentrations depend on the ionic concentration and temperature of the cardioplegic solution and the length of the ischaemic episode (Boggs *et al.*, 1987; Geffin *et al.*, 1989; Robinson and Harwood, 1991; Ahn *et al.*, 1994; Fukuhiro *et al.*, 2000; An *et al.*, 2003; Malhotra *et al.*, 2003; Camara *et al.*, 2004). Robinson and Harwood (1991) found that lowering the calcium concentration of St Thomas No. 2 (Plegisol) from 1.2 mmol/L to 0.6 mmol/L resulted in a dramatic improvement of cardiac function. They demonstrated in isolated rat hearts an increase from 22% to 86% recovery of pre-arrest aortic flow following 5 hours of hypothermic (20°C) intermittent arrest. This was confirmed by Baker and colleagues (1991), who found lower calcium concentration was particularly important for neonatal hearts during prolonged (6 hr) hypothermic (14°C) global ischaemia in immature rabbit hearts.

Clinically however, it is difficult to achieve extremely low calcium levels in blood without the use of chelating agents; therefore, magnesium is often added to help limit calcium loading in low calcium and normocalcaemic solutions (Kronon *et al.*, 1997). Magnesium has been described as a natural calcium antagonist because it limits calcium entry into the cell via competition for the sarcolemmal calcium channels

(Headrick *et al.*, 1998) and the reduction of sarcoplasmic reticulum release (Lansman *et al.*, 1986; Tsukube *et al.*, 1996a). Replenishing magnesium in ischaemic hearts may also diminish ischaemic ATP depletion upon reperfusion (Brown *et al.*, 1991).

The value of lowering the calcium level and increasing the magnesium levels of a normokalaemic AL preservation solution is not known. Theoretically, arresting the myocyte at or near its resting membrane potential, should protect the heart because there should be fewer transmembrane ionic shifts and therefore calcium loading should be limited (Watanabe *et al.*, 2002). However, Sunamori and colleagues (Sunamori *et al.*, 2001), found that despite inducing a polarised arrest with a potassium-free lignocaine-containing cardioplegia during a 6 hr hypothermic arrest, while the calcium loading was significantly reduced when compared to UW it was not entirely ameliorated. These studies and the study described here, indicate that it may be efficacious to lower the calcium level in a normokalaemic, polarising preservation solution for extended storage of hearts in a normokalaemic AL solution.

6.6.1.1.3 Recovery of Coronary Flow

In the current study, storing hearts in AL solution with a lower calcium and increased magnesium concentration improved post-storage CF by 13 %; however, this was not significant ($p>0.05$). Previous studies have shown that calcium homeostasis is crucial for protection of both the endothelial (Suttorp *et al.*, 1989) and smooth muscle (Ingemansson *et al.*, 1996) components of the coronary vasculature. A reduction of calcium and an increase in magnesium concentrations of hyperkalaemic solutions can limit the increase in pathological calcium loading and

vasoconstriction seen with high potassium concentrations (Kimblad *et al.*, 1991; Kronon *et al.*, 1999; Sharikabad *et al.*, 2001). Despite the fact that the improvement in CF was not significant here, lowering the calcium and increasing the magnesium of the AL solution may provide extra protection to the coronary vasculature. Further studies are required to elucidate the extent of preservation of vascular endothelium, measuring endothelial dependent and non-dependent responses following storage, may give more information regarding the improvement in coronary flows (Kevelaitis *et al.*, 1999).

6.6.1.1.4 Recovery of CVR, MVO_2 and pH during 5 minute rewarm

There were no significant differences in CVR and pH of isolated rat hearts during the 5 minute rewarm with AL solution following 6 hours cold storage in AL(Low Ca^{2+}:High Mg^{2+}). This finding is similar to that from the studies in Chapter 5. The MVO_2 of the KH rewarm group was significantly higher than the AL rewarm group, possibly due to the fact that the KH rewarm group was a non-working beating heart. Studies in Chapter 5 have also shown that AL arrest during the 5 minute rewarm resulted in 18.6 μmol/O_2/min/d dry wt, which was significantly higher than the Celsior (hyperkalaemic) rewarm group 11.7 μmol/O_2/min/d dry wt. However, in this study no significant difference was seen in the MVO_2 of groups rewarmed with AL with or without lower calcium and higher magnesium concentrations (22.7 vs 18.3 μmol/O_2/min/d dry wt). A study by Burkhoff and colleagues (1990) showed a decrease in MVO_2 when the calcium concentration of the hyperkalaemic preservation solution was lowered (Burkhoff *et al.*, 1990). Reperfusion of ischaemic myocytes with a warmed oxygenated solution results in an abrupt return of metabolism, ATP generation and a restoration of ionic pump functions and can lead

to further calcium loading (Shirai *et al.*, 1993; Griffiths *et al.*, 1998). However, maintaining a non-depolarised arrest during rewarming and reanimation of hearts stored in cold AL solution appears to be cardioprotective irrespective of the calcium and magnesium concentration of the AL solution. Given the faster and significantly better recoveries of hearts stored in AL(Low Ca^{2+}:HighMg^{2+}) and rewarmed in AL, this strategy was chosen for the studies in the next chapter.

6.6.1.2 Reperfusion with the AL is protective regardless of the calcium and magnesium concentration

Reperfusion of the donor heart following prolonged cold storage represents an opportunity to protect the donor heart from reperfusion injury. Therefore, the composition of reperfusion solutions has become an important target for prevention of ischaemic reperfusion injury (Carrier *et al.*, 1996; Cope *et al.*, 1996; Fiocchi *et al.*, 2000). This study has shown that reperfusing hearts stored in AL(Low Ca^{2+}:High Mg^{2+}) preservation solution and rewarming with an oxygenated, normokalaemic AL strategy is superior to reperfusing with the same solution with lower calcium and higher magnesium concentration or with KH a solution with the same physiological calcium and magnesium concentrations but without the AL.

Several studies have shown that a reduction of calcium in the initial reperfusate solution can protect the heart from reperfusion injury (Shine and Douglas, 1983; Allen *et al.*, 1986a; Kuroda *et al.*, 1986; Chambers *et al.*, 1992). However, these studies have been largely conducted following regional ischaemia or involve the use of hyperkalaemic cardioplegic solutions. The optimal concentration of calcium in a reperfusate solution appears to be dependent on the temperature and concentration of other ions in the reperfusate. Kuroda and colleagues (1986) performed a dose

response to determine the optimal calcium concentration in the presence of potassium and magnesium and found that the optimal calcium concentration for a solution with normal potassium and magnesium concentrations was 0.5 mM and if the magnesium concentration was increased, higher concentrations of calcium up to physiological levels could be tolerated. This finding was confirmed by Follette and co-workers (Follette *et al.*, 1981).

In these studies, a lower calcium concentration did not improve functional recovery and this has been described by others (Chambers *et al.*, 1992). This may be due in part to the increased magnesium concentration. Kuroda and colleagues (1986) also found that the presence of magnesium negated the importance of lower calcium concentration. This has also been demonstrated in normokalaemic solutions by Menache and colleagues (Menasche *et al.*, 1984). Further increasing the magnesium concentration of the AL preservation solution could be of value and future studies are required to investigate the efficacy of supra-physiologic levels of magnesium in the AL solution. Others have reported superior recoveries following the addition of up to 16 mmol/L magnesium to both low and high potassium solutions (Geffin *et al.*, 1989; Yamaguchi *et al.*, 2007) particularly in paediatric hearts (Kronon *et al.*, 1997).

6.6.1.3 The addition of Cyclosporin A (CYA) to AL for arrest and reperfusion does not further improve functional recoveries

The addition of CYA, a potent immunosuppressant, to the AL(Low Ca^{2+}:High Mg^{2+}) preservation solution for the arrest, storage and reperfusion of isolated rat hearts significantly improved the recovery of AF (72 vs 54%) and resulted in a 15% greater recovery of CF, although this was not significant. Recovery of AF, CF and CO was also significantly better than hearts stored in AL(Low Ca^{2+}:High Mg^{2+}) and

rewarming in KH alone. Several studies involving experimental models of IRI have reported that low levels of CYA inhibited calcium-dependent permeability of the mitochondrial inner membrane and therefore MPTP opening and cell death (Altschuld *et al.*, 1992; Griffiths and Halestrap, 1993; Griffiths, 2000; Hausenloy *et al.*, 2002); (Leshnower *et al.*, 2008). Cyclosporin A and it's reported inhibition of MPTP opening may attenuate lethal myocardial injury as it has also been shown in human trials to infarct size in patients suffering myocardial infarction (Piot *et al.*, 2008).

However, recoveries of hearts arrested, stored and reperfused with AL(Low-Ca^{2+}:High Mg^{2+}) + 0.2 μM CYA were not significantly different from those arrested and stored in AL(Low Ca^{2+}:High Mg^{2+}) and rewarmed in AL with physiological levels of calcium and magnesium. This is an interesting finding, since other studies have demonstrated remarkable cardioprotection with the addition of CYA to hyperkalaemic solutions (Duan *et al.*, 2011;(Pritzwald-Stegmann, 2011). The reduced effect of CYA on functional recoveries of rat hearts in this study may in part be due to the non-depolarising paradigm for arresting the myocyte; opening of the MPTP may already be inhibited by AL or other components of the AL preservation solution. Adensoine has been reported to bring about its cardioprotective actions through opening of K_{ATP} channels, a proposed trigger of IPC, this may prevent MPTP opening. Others have demonstrated that A_1 receptor agonists can mediate the second window of protection afforded by IPC, through MPTP inhibition (Hausenloy *et al.*, 2004). High magnesium concentrations through calcium channel blockade and antioxidant effect may also inhibit the opening of MPTP (Halestrap *et al.*, 2004). Further studies are required to elucidate the

mechanism by which AL preservation solution provides improved recoveries in isolated rat hearts and this could shed some light on the failure of CYA to significantly improve functional recoveries in this study.

CYA may not be the drug of choice for MPTP opening inhibition and cardioprotection. CYA has recently been reported to have unwanted side-effects through its interactions with cyclophillins other than cyclophophillin D (Halestrap and Pasdois, 2009). It has also been demonstrated that there are limits to CYA's ability to maintain MPTP opening inhibition during increasing oxidative stress (Halestrap, 1991; Griffiths and Halestrap, 1995; Basso *et al.*, 2005). Due to these implications and the evidence that long-term treatment with higher doses of CYA is been associated with renal damage and other detrimental effects (Curtis *et al.*, 1986; Burke *et al.*, 1994;Rahamimoff *et al.*, 2007) the addition of CYA to AL preservation solution was not pursued.

6.6.2 Study two: Optimising adenosine and lignocaine concentrations

To further improve the composition of the AL preservation solution for use over extended cold (4°C) ischaemic times, the aim of this study was to determine the optimal combination of adenosine and lignocaine concentrations. Previous studies have reported temperature dependence for lignocaine's activity as a local anaesthetic (Powell, 1987) and cardioprotective agent (Asano *et al.*, 2003). Similarly adenosine has also been reported to show less efficacy at lower temperatures (Katayama *et al.*, 1997b).

In this study, a combination of AL (200:1500 μM) was found to significantly improve functional recoveries of hearts stored for 6 hours (4°C) when compared to AL(200:500 μM); see Table 6.3, Figure 6.16. This group, however, was associated with a greater number of episodes and duration of post-ischaemic arrhythmias (Figure 6.18), which could indicate lignocaine toxicity. While doubling the lignocaine concentration to 1000 μM in combination with 200 μM adenosine did not improve functional recovery, doubling both the adenosine and lignocaine concentrations AL(400:1000 μM) showed a significant improvement in functional recoveries of CO (63 vs 75%) (see Figure 6.16) for hearts stored for 6 hours in cold storage. Functional recoveries of this group were not significantly different from the AL(200:1500 μM) group, but also showed a low CVR during the 5 minute rewarm and a lower number and duration of reperfusion arrhythmias.

Concerns have been raised about the use of lignocaine as a primary arresting agent in cardioplegic and preservation solutions due to its potential for arrhythmic and neurological toxicity and it has not seen clinical uptake, despite its use at lower doses as an anti-arrhythmic and local anaesthetic (Waller, 1981a; Anderson *et al.*, 1990). Yamaguchi et al (Yamaguchi *et al.*, 2007) in a canine model of cardiac bypass surgery used a similar dose (1.4mM) of lignocaine in combination with 5.5 mM magnesium and found the plasma concentration of lignocaine reached 0.08 mM which is much greater than the therapeutic index of lignocaine in man: 0.03-0.04 mM (Foldes *et al.*, 1960; Fallouh and Chambers, 2007). This concentration is close to the AL(200:1500 μM) concentrations and may be the reason for increased electrical instability of the heart during reperfusion. Others have reported an increased number of re-enterant arrhythmias with the use of lignocaine particularly

in injured myocardium, although both studies used a rapid injection of lignocaine at 2mg/Kg in canine hearts (El-Sherif *et al.*, 1977; Yin *et al.*, 1997).

Although both the AL(200:1500 μM) and AL(400:1000 μM) groups were associated with reduced CVR during the 5 minute rewarm, the CVR for these were not significantly different from the other groups, mainly due to the larger SEM (see Figure 6.17). The lower CVR during the rewarm period, however, did not translate into a significant improvement in recovery of coronary flow during reperfusion.

6.7 Conclusion

Lowering the calcium (0.22 mM) and increasing the magnesium concentration (2.6 mM) in the arrest and storage solutions and rewarming in AL solution with physiological levels of calcium and magnesium gave optimum recoveries for both AF (69%), CF (95%) and CO (74%) and this was not further improved by the addition of MPTP inhibitor CYA which did not provide any additional benefit AF (72%), CF (106%) and CO (80%). Increasing the adenosine and lignocaine concentration further improved recovery of AF (69%), CF (92%) and CO (75%) without the increased electrical instability seen with higher doses of lignocaine. This study has determined an improved formula for the AL preservation solution for further extending safe cold preservation times beyond 6 hours.

CHAPTER 7: EIGHT HOURS OF COLD STORAGE USING MODIFIED AL SOLUTION: TOWARDS THERAPEUTIC SUSPENDED ANIMATION

"For successful short- and long-term outcome after HTx [heart transplantation] a technically perfect procedure must involve careful myocardial protection strategy that avoids damage from ischaemia and reperfusion (i.e., ischaemic storage period, implantation phase and reperfusion period) and brain death."

Beyersdorf 2004.

7.1 Introduction

The studies from the current chapter combine results from previous chapters to investigate the performance of the new AL preservation paradigm over an eight hour cold storage period. The effect of the addition of insulin and melatonin was also investigated for use during extended cold storage.

The benefits of inhibition of MPTP opening, through the addition of CYA to the AL solution during arrest storage and reanimation of the isolated rat heart were demonstrated in the studies of Chapter 6. However, the safety of the use of CYA, particularly in compromised patients, has been questioned. Other studies have shown the use of other MPTP inhibitors to be cardioprotective. Gao et al. demonstrated superior recoveries in a working rat heart model, the addition of 0.1mmol/L of melatonin to St Thomas (Plegisol) resulting in greater than 80% recovery of cardiac output after 12 hours of cold static storage (Gao *et al.*, 2003). Petriosillo et al. also

demonstrated that melatonin, in physiological concentrations, strongly protected integral proteins involved in mitochondrial oxidative metabolism from ROS damage (Petrosillo *et al.*, 2004). In further studies the same group were able to demonstrate that melatonin also prevented mitochondrial NAD^+ release, cytochrome c release and cardiolipin oxidation in an isolated rat heart model of ischemia reperfusion injury (Petrosillo *et al.*, 2009a). Melatonin was therefore chosen for its ability to protect the heart against ischaemia-reperfusion injury (Petrosillo *et al.*, 2009a). This protection has been attributed to its free- radical scavenging of both oxygen and nitrogen free radicals and anti-oxidant properties (Lochner *et al.*, 2006);(Okatani *et al.*, 2001). Melatonin has been shown to reduce infarct size (Lagneux *et al.*, 2000; Sahna *et al.*, 2005), improve mitochondrial function (Petrosillo *et al.*, 2009a), improve post ischaemic performance in a working rat heart model (Dobsak *et al.*, 2003) and reduce the incidence of post ischaemic arrhythmias (Sahna *et al.*, 2005). In humans, melatonin at physiological concentrations has been shown to correlate with protection from acute cardiac events such as MI and CABG (Sokullu *et al.*, 2009). Insulin was chosen for its ability to protect against ischaemia reperfusion injury via the phosphatidyl inositol 3´kinase-Akt dependent pathway (Gao *et al.*, 2002; Fischer-Rasokat and Doenst, 2003; Ji *et al.*, 2010). Mechanisms proposed for insulins effects include enhanced glucose uptake during hypothermia (Orme and Kelly, 1977), post ischaemic substrate utilisation (Taegtmeyer *et al.*, 1998), enhanced eNOS activity, and down regulated iNOS gene expression (Ji *et al.*, 2010), vasodilation (Baron, 1994) and direct insulin effects on the heart (Doenst *et al.*, 1999; Jonassen *et al.*, 2001). The cardioprotective benefits of insulin addition has been demonstrated with Glucose-Insulin-Potassium cardioplegic solution which has

been well studied showing benefits in the peri-operative period (Kjellman *et al.*, 2000; Elvenes *et al.*, 2002; Quinn *et al.*, 2005).

The aim of the current study is to investigate the effect of melatonin and insulin with the modified AL solution on functional recovery of isolated rat hearts over 8 hours of cold static storage and compare this with the recoveries of hearts stored in Celsior and HTK (Custodial).

7.2 Materials and Methods

7.2.1 Experimental groups

Rats were randomly assigned to one of 4 groups (n = 8 each group):

1) AL(LowCa^{2+}:HighMg^{2+});

2) 2xAL (LowCa^{2+}:HighMg^{2+}) + insulin +melatonin (ALMI);

3) Celsior;

4) HTK (Custodial) solution.

7.2.2 Study design

Hearts were rapidly removed from anaesthetised rats and placed in ice-cold heparinised modified KH buffer. Details of heart preparation, attachment and perfusion are described in Chapter 3 Materials and Methods. Briefly, hearts were attached to a Langendorff apparatus and perfused at a pressure head of 90 cm H_2O (68 mmHg). The hearts were then equilibrated in working mode for 15 minutes prior to taking baseline measurements (see Figure 7.1). Hearts were then switched to Langendorff mode and arrested with a 5 minute normothermic (37ºC) induction dose of the cardioplegic solution (AL (LowCa^{2+}:HighMg^{2+}), ALMI, HTK (Custodial), or

Celsior). Hearts were removed after the induction dose and placed in a centrifuge tube containing 50 ml of the same solution and placed in a refrigerated water bath for 8 hours. Hearts were then reattached in Langendorff mode while arrested in the same solution (5 minute rewarm). Once hearts were canulated, they were then switched to working mode for functional measurements at 5, 10, 15 30, 45 and 60 minutes of reperfusion. See Figure 7.1 below.

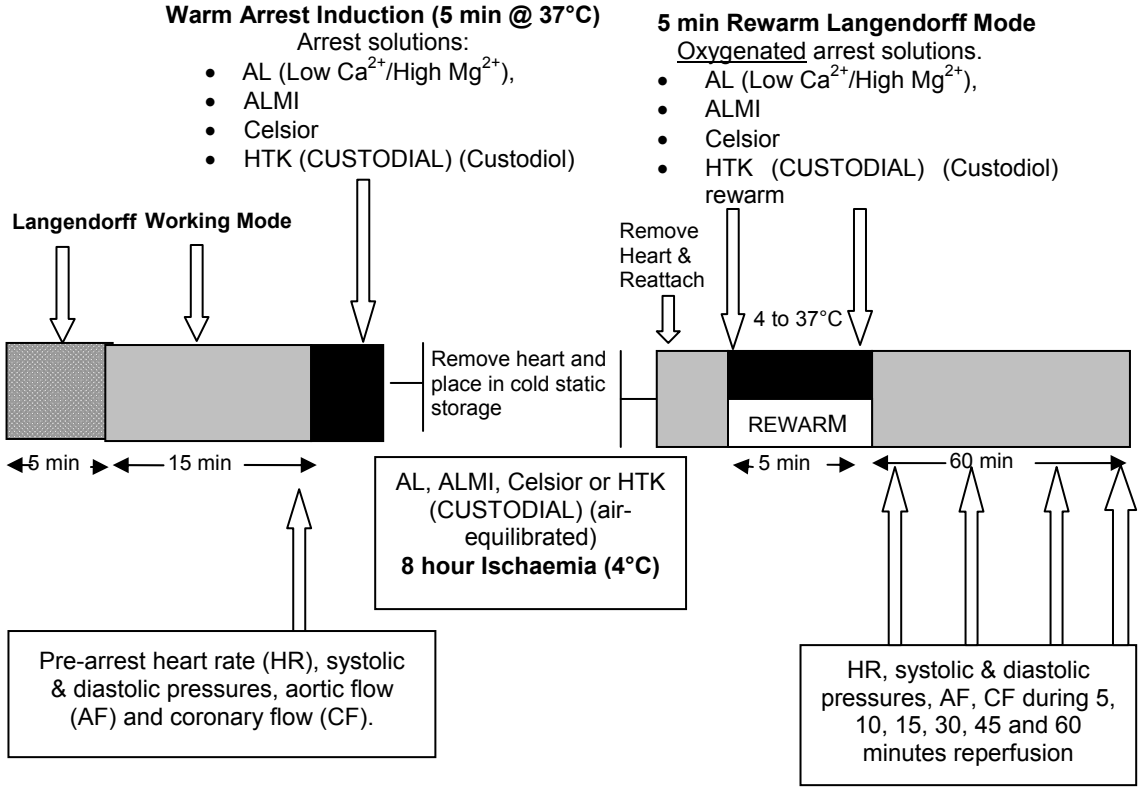

Figure 7.1: Experimental protocol for studies in Chapter 7.
Isolated rat hearts were placed in cold static storage for 8 hours in the following preservation solutions: 1.) AL (Low Ca^{2+}/M:High Mg^{2+}) 2) AL was doubled in concentration with melatonin (100uM) and insulin (0.01IU/ml)(ALMI) 3) HTK (CUSTODIAL) (Histidine-tryptophan-ketoglutarate, Custodial) and 4) Celsior. Each group was rewarmed in Langendorff mode for 5 min with their respective oxygenated arrest solution (see Appendix A and Materials and Methods for details).

7.3 Results

Heart functional properties before and following cold storage in the four different preservation solutions are shown in Tables 7.1 and 7.2 and Figures 7.2 and 7.3. During pre-arrest, there were no significant differences between the groups in AF, CF, CO, HR, DP, SP or RPP (Table 7.1).

Table 7.1: Functional parameters of isolated working rat hearts during different rewarm reperfusion strategies following 8 hour cold storage in AL preservation or Celsior and HTK (Custodial) solutions.

Time during Pre-Arrest and Reperfusion	Cold Arrest + Rewarm Treatment	n	Aortic Flow (ml/min)	Coronary Flow (ml/min)	Cardiac Output (ml/min)
15 mins Pre-Arrest	AL ($LowCa^{2+}$:$HighMg^{2+}$)	8	60 ± 2	20 ± 1	80 ± 2
	ALMI	8	62 ± 1	22 ± 1	85 ± 2
	HTK	8	54 ± 2	19 ± 1	73 ± 2
	Celsior	8	63 ± 4	21 ± 2	85 ± 5
5 mins Recovery	AL ($LowCa^{2+}$:$HighMg^{2+}$)	8	0 ± 0	1 ± 1	1 ± 1
	ALMI	8	3 ± 3	2 ± 2	5 ± 5
	HTK	8	0 ± 0	1 ± 1	1 ± 1
	Celsior	8	0 ± 0	3 ± 2	3 ± 2
10 mins Recovery	AL ($LowCa^{2+}$:$HighMg^{2+}$)	8	11 ± 6	11 ± 3	22 ± 9†
	ALMI	8	14 ± 7	8 ± 4	22 ± 11
	HTK	8	0 ± 0	2 ± 1	2 ± 1
	Celsior	8	1 ± 1	8 ± 2	9 ± 3
15 mins Recovery	AL ($LowCa^{2+}$:$HighMg^{2+}$)	8	22 ± 5†	15 ± 1†	37 ± 6
	ALMI	8	31 ± 6*	20 ± 2§#	51 ± 6
	HTK	8	0 ± 0	1 ± 1	1 ± 1
	Celsior	8	2 ± 1	10 ± 2‡	12 ± 3
30 mins Recovery	AL ($LowCa^{2+}$:$HighMg^{2+}$)	8	28 ± 5†	15 ± 2†	43 ± 1†
	ALMI	8	44 ± 2*	18 ± 1§#	62 ± 3*
	HTK	8	0 ± 0	2 ± 1	2 ± 1
	Celsior	8	8 ± 4	10 ± 2‡	18 ± 6‡
45 mins Recovery	AL ($LowCa^{2+}$:$HighMg^{2+}$)	8	30 ± 5†	15 ± 1†	45 ± 6†
	ALMI	8	46 ± 2*	18 ± 1§#	65 ± 2*
	HTK	8	1 ± 1	1 ± 1	2 ± 2
	Celsior Cardioplegia	8	11 ± 5	10 ± 2‡	21 ± 7‡
60 min Recovery	AL ($LowCa^{2+}$:$HighMg^{2+}$)	8	30 ± 5†	14 ± 2†	44 ± 6†
	ALMI	8	47 ± 3*	19 ± 1§#	66 ± 3*
	HTK	8	1 ± 1	2 ± 1	3 ± 2
	Celsior	8	11 ± 5	10 ± 2‡	21 ± 7‡

No significant differences between pre-arrest groups. * ALMI significantly different from all other groups $p<0.05$; † AL ($LowCa^{2+}$:$High\ Mg^{2+}$) significantly different from HTK or Celsior or both$p<0.05$; § ALMI significantly different from Celsior only $p<0.05$; # ALMI significantly different from HTK only $p<0.05$; ‡ Celsior significantly different from HTK $p<0.05$

Table 7.2: Functional parameters of isolated working rat hearts during different rewarm reperfusion strategies following 8 hour cold storage in AL preservation or Celsior and HTK (Custodial) solutions.

Time during Pre-Arrest and Reperfusion	Cold Arrest + Rewarm Treatment	n	Heart Rate (bpm)	Systolic Pressure (mmHg)	Diastolic Pressure (mmHg)	Rate Pressure Product (beats.mmHg/min)
15 mins Pre-Arrest	AL (LowCa^{2+}:High Mg^{2+})	8	280 ± 7	124 ± 2	60 ± 0	34561 ± 584
	ALMI	8	303 ± 7	128 ± 1	60 ± 0	38836 ± 957
	HTK	8	275 ± 8	125 ± 1	60 ± 0	34404 ± 943
	Celsior	8	286 ± 12	126 ± 2	60 ± 0	36041 ± 1268
5 mins Recovery	AL (LowCa^{2+}:High Mg^{2+})	8	4 ± 4	3 ± 3	1 ± 1	80 ± 80
	ALMI	8	25 ± 25	16 ± 16	8 ± 8	3250 ± 3250
	HTK	8	22 ± 7	14 ± 5	9 ± 3	446 ± 201
	Celsior	8	48 ± 13	31 ± 9	19 ± 3	1998 ± 935
10 mins Recovery	AL (LowCa^{2+}:High Mg^{2+})	8	139 ± 44	71 ± 17	40 ± 10	14673 ± 5320
	ALMI	8	118 ± 50	51 ± 20	25 ± 10	13078 ± 6316
	HTK	8	39 ± 21	15 ± 9	10 ± 5	1795 ± 1377
	Celsior	8	176 ± 39	66 ± 10	48 ± 8	13944 ± 3680
15 mins Recovery	AL (LowCa^{2+}:High Mg^{2+})	8	254 ± 21	108 ± 3	63 ± 2	27461 ± 2426
	ALMI	8	265 ± 16#	118 ± 5§#	63 ± 1	30898 ± 1705§#
	HTK	8	32 ± 21	9 ± 7	6 ± 5	1163 ± 966
	Celsior	8	184 ± 34‡	72 ± 8	52 ± 7	14580 ± 3759‡
30 mins Recovery	AL (LowCa^{2+}:High Mg^{2+})	8	293 ± 22	110 ± 3	63 ± 2	32155 ± 2515
	ALMI	8	294 ± 16#	121 ± 3§#	60 ± 0#	35294 ± 1496§#
	HTK	8	41 ± 27	1 3 ± 11	10 ± 9	2411 ± 2221
	Celsior	8	213 ± 32‡	88 ± 8‡	59 ± 5‡	19998 ± 3834‡
45 mins Recovery	AL (LowCa^{2+}:High Mg^{2+})	8	282 ± 20	111 ± 3	63 ± 2	31176 ± 2289
	ALMI	8	299 ± 14#	118 ± 3#	60 ± 0#	35097 ± 1068§#
	HTK	8	52 ± 28	13 ± 12	10 ± 9	2642 ± 2451
	Celsior	8	231 ± 34‡	91 ± 7‡	61 ± 4‡	22211 ± 4100‡
60 min Recovery	AL (LowCa^{2+}:High Mg^{2+})	8	283 ± 19	111 ± 3	63 ± 2	31518 ± 2346
	ALMI	8	314 ± 12#	117 ± 3#	60 ± 0#	36800 ± 1021§#
	HTK	8	44 ± 30	14 ± 12	10 ± 9	3396 ± 2781
	Celsior	8	247 ± 28‡	89 ± 6‡	62 ± 4‡	21473 ±3642‡

No significant differences between pre-arrest groups
***** ALMI significantly different from all other groups $p<0.05$
† AL (LowCa^{2+}:High Mg^{2+}) significantly different from HTK or Celsior or both $p<0.05$
§ ALMI significantly different from Celsior only $p<0.05$
# ALMI significantly different from HTK only $p<0.05$
‡ Celsior significantly different from HTK $p<0.05$

7.3.1 Recovery of aortic flow, coronary flow and cardiac output

Baseline values of aortic flow ranged between 54 and 63 ml min^{-1} and there were no significant differences between the treatment groups. Five minutes after switching to working mode, the ALMI heart group was the only group that generated aortic flow

(1% or 3 ml/min) (Table 7.1, Figure 7.2). By 10 mins, AL (LowCa^{2+}:HighMg^{2+}) and ALMI hearts had spontaneously generated 18 and 22% of their respective baseline AF whereas HTK (Custodial) hearts had zero flow (0%) and Celsior hearts had 1 ml/min (2% of pre-arrest). At 30 mins, AL and ALMI hearts generated 47 and 71% of their pre-arrest AF and this increased to 50% and 76% at 60 min reperfusion. By contrast, hearts preserved in HTK (Custodial) still had not recovered and Celsior hearts generated 13 and 17% of pre-arrest AF after 30 and 60 mins of reperfusion (Table 7.1, Figure 7.2).

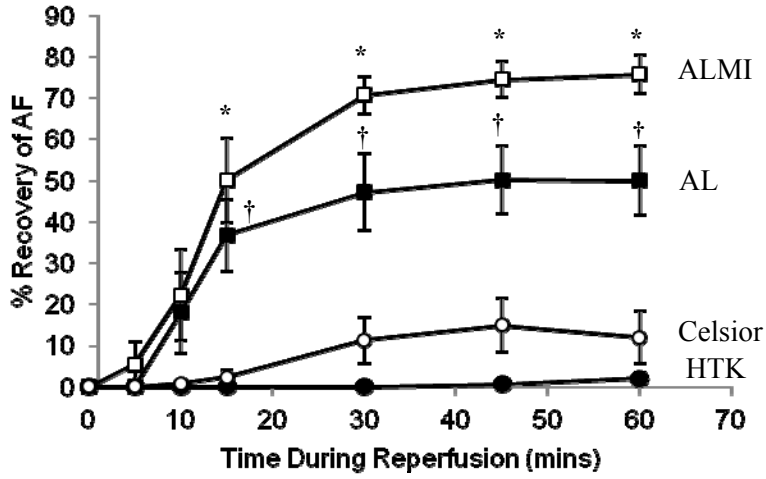

Figure 7.2: Recovery of aortic flow (%AF) following 8 hours of arrest, storage and rewarm with the different preservation solutions
Percentage recovery of pre-arrest aortic flow (AF) during 60 min reperfusion in working mode immediately following 5 min of rewarming oxygenated reperfusion in Langendorff mode with the following solutions: 1) AL (Low Ca^{2+}/High Mg^{2+}) (■) (n=8), 2) ALMI (□) (n=8), 3) HTK (●)(n=8) and 4) Celsior (○) (n=8). Statistical significance ($p<0.05$) were as follows:
* ALMI significantly different from all groups
† AL significantly different from Celsior and HTK

The spontaneous return of CF following 5 minutes of reperfusion for AL (LowCa^{2+}:HighMg^{2+}), ALMI hearts, HTK (Custodial) and Celsior were 1%, 9%, 5% and 14% respectively of baseline values, and at 10 minutes CF had increased to 55%, 36%, 11% and 38% respectively (Table 7.1, Figure 7.3). Following 30 minutes of reperfusion the ALMI hearts had increased their CF 2.3 fold (82% of pre-arrest) while the Celsior and AL (LowCa^{2+}:HighMg^{2+}) hearts had increased by 10 and 20% respectively. By the end of the reperfusion period, the return of CF for AL (LowCa^{2+}:HighMg^{2+}) and ALMI hearts were both significantly better (70% and 86% respectively) than the Celsior hearts which recovered 48% of their baseline values. HTK (Custodial) hearts failed to recvover baseline CF, which remained at 11% of pre-arrest value (2 ml min^{-1}) for the duration of the reperfusion period (Table 7.1, Figure 7.3).

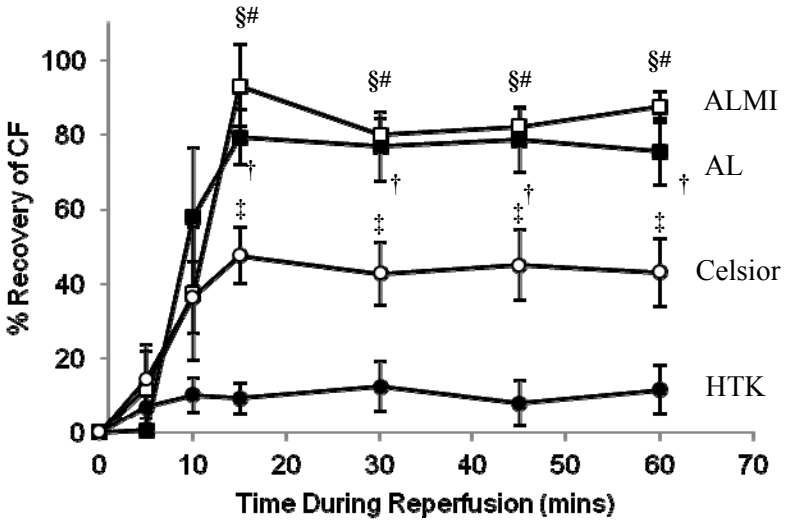

Figure 7.3: Recovery of coronary flow (%CF) following 8 hours of arrest, storage and rewarm with the different preservation solutions.
Recovery of pre-arrest coronary flow (CF) during 60 min reperfusion in working mode immediately following 5 min of oxygenated reperfusion in Langendorff mode.
1) AL (LowCa^{2+}/HighMg^{2+}) (■), 2) ALMI (□), 3) HTK (●) and 4) Celsior (○).
Statistical significance ($p<0.05$) were as follows:
† AL (LowCa^{2+}:HighMg^{2+}) significantly different from Celsior and HTK
§ ALMI significantly different from Celsior
# ALMI significantly different from HTK
‡ Celsior significantly different from HTK

The recovery of cardiac output (CO) at 5 mins for all groups in working mode ranged between 1 to 6% and at 10 mins between, 3 and 28%; however, there was no significant difference between any of the groups at either time point (Table 7.1, Figure 7.4). By 30 mins reperfusion, the AL(LowCa^{2+}:HighMg^{2+}) and ALMI hearts had doubled and tripled their outputs respectively, achieving significantly better CO recovery than either Celsior or HTK (Custodial) hearts ($p<0.05$). The Celsior hearts also doubled their output and this was significantly better than the HTK (Custodial) hearts which failed to recover CO above 3% of the pre-arrest value (Table 7.1, Figure 7.4). At 60 mins the ALMI hearts increased to 78% of pre-arrest CO values and this was significantly better than the AL hearts (55% pre-arrest output), and

Celsior hearts (25% pre-arrest output) ($p<0.05$). HTK (Custodial) failed to recover CO over the 60 mins reperfusion period (Table 7.1, Figure 7.4).

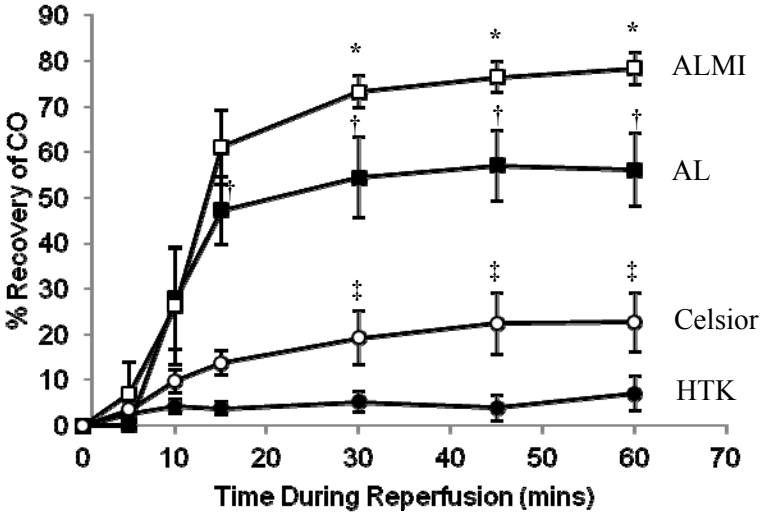

Figure 7.4: Recovery of cardiac output (%CO) following 8 hours of arrest, storage and rewarm with the different preservation solutions
Recovery of pre-arrest cardiac output (CO) during 60 min reperfusion in working mode immediately following 5 min of oxygenated reperfusion in Langendorff mode.
1) AL($LowCa^{2+}$/$HighMg^{2+}$) (■), 2) ALMI (□), 3) HTK (●) and 4) Celsior (○).
Statistical significance ($p<0.05$) were as follows:
* ALMI significantly different from all groups
† AL significantly different from Celsior and HTK
‡ Celsior significantly different from HTK

7.3.2 Recovery of systolic and diastolic developed pressures

The systolic and diastolic pressures are shown in Table 7.2 and Figure 7.5a and b respectively. Baseline values ranged between 124/60 and 128/60 and there were no significant differences between the groups. At 5 mins, the AL ($LowCa^{2+}$:$HighMg^{2+}$) and ALMI hearts generated 2% and 13% of baseline systolic pressures respectively, which rapidly increased to 57% and 40% at 10 min (Table 7.2, Figure 7.5a and b). At 30 and 60 mins reperfusion, the AL($LowCa^{2+}$:$HighMg^{2+}$) and ALMI had

recovered 89 to 94 % of baseline systolic pressures and had fully recovered diastolic pressures. Celsior hearts showed a faster recovery of baseline pressures at 5 mins (25% SP and 32% DP), and at 10 mins had recovered 52% systolic and 80% diastolic pressures. Although by 30 and 60 mins, Celsior hearts increased systolic pressure to 70% of baseline, this pressure did not generate a significantly better AF recovery than the AL(LowCa^{2+}:HighMg^{2+}) and ALMI hearts, despite the full recovery of diastolic pressure (98 to 100%). HTK (Custodial) hearts, again, failed to recover, with no increase in systolic pressure above 14 mmHg (11% baseline) and diastolic was not above 10 mmHg (17% baseline) over the 60 mins reperfusion period.

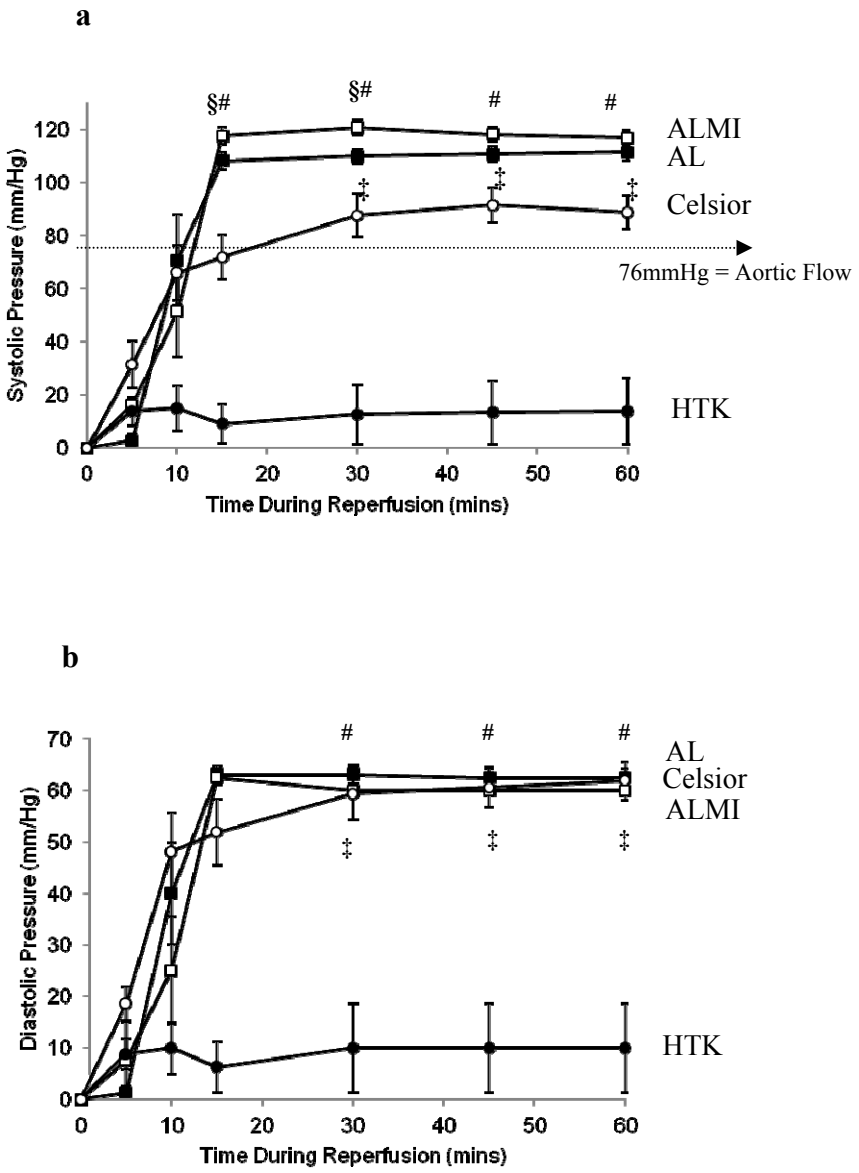

Figure 7.5: Recovery of systolic and diastolic pressures following 8 hours of arrest, storage and rewarm with the different preservation solutions
Recovery of pre-arrest systolic (SP) and diastolic pressures (DP) immediately following 5 min of oxygenated reperfusion in Langendorff mode.
a: Recovery of Systolic pressure (mm/Hg)
b: Recovery of Diastolic pressure (mm/Hg) during 60 min reperfusion in working mode

1) AL($LowCa^{2+}$/$HighMg^{2+}$) (■), 2) ALMI (□), 3) HTK (●) and 4) Celsior (○).
Statistical significance ($p<0.05$) were as follows:
§ ALMI significantly different from Celsior
# ALMI significantly different from HTK
‡ Celsior significantly different from HTK

7.3.3 Recovery of heart rate and rate-pressure product

Spontaneous return of heart rate is shown in Table 7.2 and Figure 7.6. There was no significant dofference in baseline rates for HR in all groups, these ranged between 275 and 303 beats min^{-1}. At 5 minutes of reperfusion, the AL(LowCa^{2+}:HighMg^{2+}), ALMI and HTK (Custodial) hearts had recovered heart rates between 1 and 8% of baseline; however, the Celsior hearts recovered double that rate (17%). At 10 mins the AL(LowCa^{2+}:HighMg^{2+}) and ALMI hearts increased their rates to 50 and 39% baseline respectively, and Celsior hearts were beating at 62% baseline. The HTK (Custodial) hearts increased their heart rate from 14 to 16% of baseline at 10 mins reperfusion, and this did not change over the 60 min reperfusion. By 30 mins of reperfusion, AL(LowCa^{2+}:HighMg^{2+}) and ALMI hearts returned 105 and 97% of their baseline heart rates respectively, while Celsior hearts had 75% of baseline rate. At 60 mins, full recovery of heart rate was found in the AL (Low Ca^{2+}:HighMg^{2+}) and ALMI hearts, and 86% recovery in the Celsior hearts; all three groups recoveries were significantly better than the HTK hearts.

Baseline rate-pressure product (RPP) ranged between 34,404 and 38,836 beats.mmHg min^{-1} and was not significantly different for the four preservation groups (Table 7.2) ($p<0.05$). By 5 mins of reperfusion, return of RPP was low in all groups. By 10 mins of reperfusion, the AL(LowCa^{2+}:HighMg^{2+}) and ALMI hearts generated 42% and 34% of baseline RPP, and the Celsior hearts had 39% (Table 7.2). By contrast the HTK (Custodial) hearts failed to recover and generated 5 to 10% baseline RPP over the entire 60 mins reperfusion period. By 30 mins of reperfusion, the AL (Low Ca^{2+}:HighMg^{2+}) and ALMI hearts had recovered 91-93% their pre-arrest RPP and were not significantly different. The ALMI hearts

had recovered 95% by 60 mins reperfusion and this was significantly better than the Celsior or HTK (Custodial) hearts. The Celsior hearts returned 55% and 59% of their RPP at 30 mins and 60 mins respectively and this was significantly better than the HTK (Custodial) hearts ($p<0.05$).

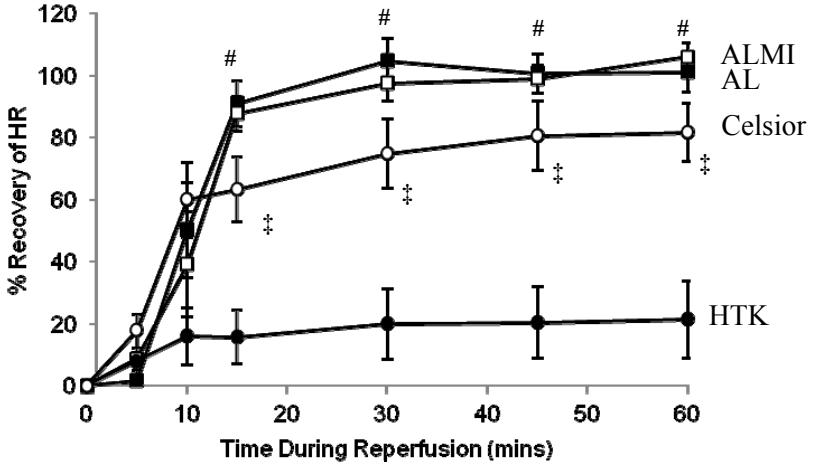

Figure 7.6: Recovery of heart rate (%HR) following 8 hours of arrest, storage and rewarm with the different preservation solutions.
Recovery of pre-arrest heart rate (HR) during 60 mins reperfusion in working mode immediately following 5 min of oxygenated reperfusion in Langendorff mode.
1) AL low Ca^{2+}/highMg^{2+} (■), 2) ALMI (□), 3) HTK (●) and 4) Celsior (○).
Statistical significance ($p<0.05$) were as follows:
§ ALMI significantly different from Celsior
# ALMI significantly different from HTK
‡ Celsior significantly different from HTK

7.3.4 Maintenance myocardial oxygen consumption in the last minute of the 5 min rewarming phase prior to switching to working mode

At the end of rewarming phase, the MVO_2 for AL(Low Ca^{2+}:HighMg^{2+}), ALMI, HTK (Custodial) and Celsior hearts in Langendorff mode perfused with their oxygenated arrest solutions were 23.0 ± 5, 20 ± 4, 15 ± 1 and 10 ± 2 μmol O_2/min/g dry wt respectively (see Figure 7.7 below). The MVO_2 of the

AL (Low Ca^{2+}:HighMg^{2+}) and ALMI groups were significantly higher than the Celsior group ($p<0.05$). The coronary effluent flows in the final minute for AL (Low Ca^{2+}:HighMg^{2+}), ALMI groups were 13.7 ± 0.9 and 14.2 ± 2.3 ml respectively, and for HTK (Custodial) and Celsior groups were 11.1 ± 0.9 and 10.8 ± 1.4 ml respectively and there were no significant differences between the groups. Therefore, the higher MVO_2's are not due to higher effluent flows.

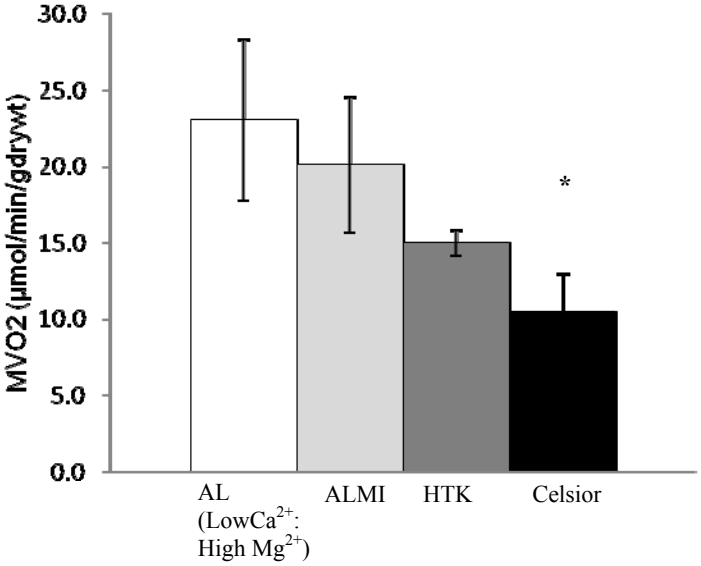

Figure 7.7: MVO_2 during 5 minute rewarming following 8 hours of arrest, storage and rewarm with the different preservation solutions
Myocardial oxygen consumption for the different treatment groups during the 5 minute rewarming period in Langendorff mode. Statistical significance ($p<0.05$).
*Celsior was significantly different than AL (AL (Low Ca^{2+}:HighMg^{2+}) and ALMI.

7.3.5 Lactate and troponin output in the last minute of 5 minute rewarm

The lactate outputs for AL (Low Ca^{2+}:HighMg^{2+}), ALMI, HTK (Custodial) and Celsior hearts perfused with their oxygenated arrest solutions were 1.8 ± 0.8, 1.5 ± 0.7, 2.6 ± 0.7 and 3.2 ± 1.4 µmol lactate/min/g dry wt (Figure 7.8). No troponin T

was detected in the coronary effluent of AL (Low Ca^{2+}:HighMg^{2+}) or ALMI hearts after rewarming. In contrast the troponin T levels for the HTK (Custodial) and Celsior hearts were 0.08 and 0.24 μg/ml of effluent (see Figure 7.9 below).

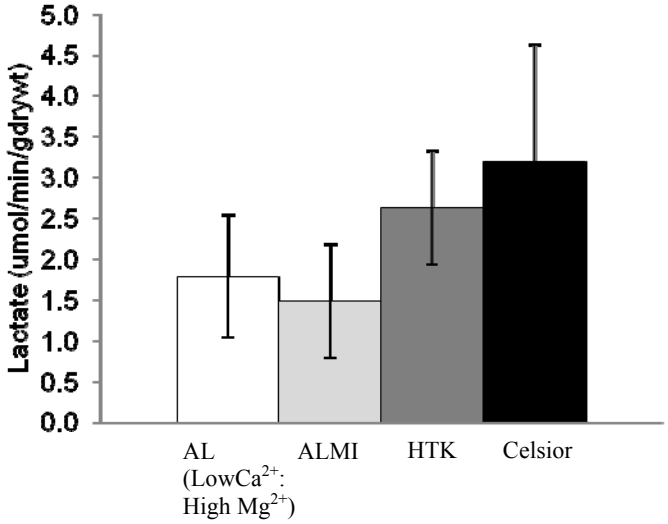

Figure 7.8: Lactate release during 5 minute rewarming following 8 hours of arrest, storage and rewarm with the different preservation solutions.
Lactate output (μmol/min/g drywt heart) measured in the last minute of the 5 minute rewarm period. Differences were not significant ($p<0.05$)

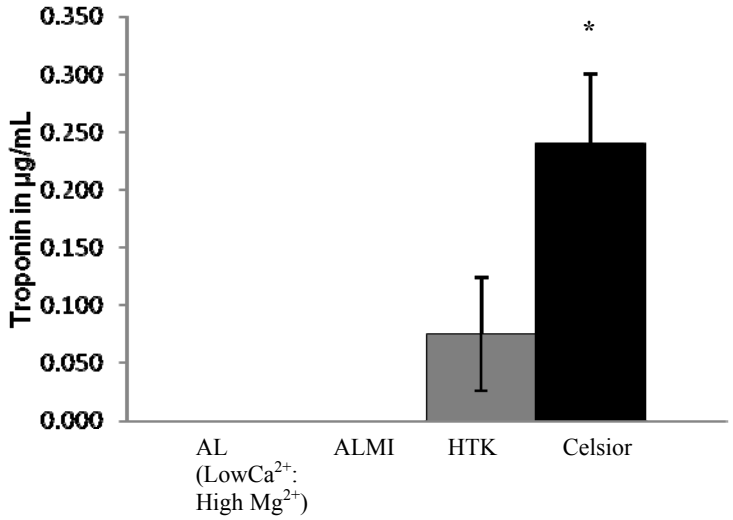

Figure 7.9: Troponin T release during 5 minute rewarming following 8 hours of arrest, storage and rewarm with the different preservation solutions.release during 5 minute rewarming.
Troponin T measured in the last minute of the 5 minute rewarm period. Statistical significance ($p<0.05$). *Celsior was significantly different than AL and ALMI.

7.4 Discussion

This chapter has shown that hearts arrested and stored in the AL preservation solution with low Ca^{2+}/high Mg^{2+} (0.22 mM/2.6 mM) recovered 55% cardiac output, 101% heart rate, 90 to 105% developed pressures and no detectable troponin T (<0.03 µg/ml) following 8 hours of cold storage. If the adenosine and lignocaine concentrations were doubled in the AL($LowCa^{2+}$:$HighMg^{2+}$) preservation solution and with the addition of melatonin (100 µM) and insulin (0.01 IU/ml) (ALMI), the return of CO increased to 78% significantly better than all other groups. By contrast, HTK hearts failed to recover function returning 4% CO, 16% HR and 11 to 17% developed pressures. These hearts showed significant myocyte death with a troponin T level of 0.13 µg/ml measured in the effluent from the 5 minute rewarm. Despite an 86% return of heart rate, Celsior hearts, failed to return adequate left

ventricular pump function demonstrated by the poor recoveries of AF which were significantly less than either AL group. After 8 hours of cold storage these hearts had 0.24 μg/ml troponin T release into the effluent collected during the 5 mins rewarm and returned 25% of baseline CO after 60 mins of reperfusion.

7.4.1 Higher maintenance metabolism during the 5 min rewarming phase

As shown in Chapter 5 a higher maintenance metabolism was seen during the 5 minute rewarm period following 8 hours of AL preservation. The myocardial oxygen consumption (MVO_2) in oxygenated, normokalaemic, polarised arrested hearts (AL(Low Ca^{2+}:HighMg^{2+}), ALMI) prior to switching to working mode were 1.5 times higher than oxygenated HTK (Custodial) arrested hearts (Figure 7.7), and 2 times higher than Celsior stored hearts. The higher MVO_2 values were associated with similar magnitudes of reduction in effluent lactate in all groups (Figure 7.8). As discussed earlier, the higher MVO_2 and lower effluent lactate in both AL (Low Ca^{2+}:High Mg^{2+}) preservation groups is indicative of increased reliance on mitochondrial oxidative phosphorylation to replenish the ATP during the rewarm reperfusion period in AL hearts compared with hyperkalaemic HTK (Custodial) and Celsior groups. These differences in MVO_2 among the groups were not due to differences in coronary effluent outflows, which ranged from 11 to 14 ml/min, and therefore must be of metabolic origin. The higher troponin T levels in the hyperkalaemic HTK (Custodial) and Celsior groups were also indicative of myocardial cell damage in these hearts sustained during the ischaemic storage and 5 min rewarming/reperfusion phase. No troponin T level was detected in the effluent of the AL (Low Ca^{2+}:High Mg^{2+}) or ALMI hearts (see Figure 7.9 below).

7.4.2 Functional recoveries in AL, Celsior and HTK (Custodial) solutions after 8 hours of cold storage

These studies from Chapter 7 have shown that hearts stored in AL(LowCa^{2+}:HighMg^{2+}) with and without melatonin and insulin (ALMI) for eight hours spontaneously returned of 78% and 55% of pre-arrest cardiac output with full recovery of heart rate and developed pressures. This is in direct contrast to hearts stored in Celsior and HTK (Custodial) which recovered less than 25% of their pre-arrest values. This result is consistent with data from the previous chapters. Lowering the calcium concentration (0.22 mM) and increasing the magnesium concentration (2.6 mM) of the AL preservation solution during arrest and storage (6hours), resulted in the recovery of 64% CO. This was associated with a greater improvement in coronary (82 - 95%) than aortic flow (65 - 69%), although this was not significantly different. Doubling the adenosine and lignocaine concentrations improved CO recoveries to 75%. While the AL(200:1500 μM) group had significantly better recoveries of CO to 87% , there was an increased number and duration of post ischaemic arrhythmias throughout the 60 mins of reperfusion.

Improved cardiac function in the ALMI hearts over the AL hearts may be due to the higher AL(LowCa^{2+}:HighMg^{2+}) concentration stabilizing-effect on membrane polarization and temperature-dependent gating kinetics of the Na^+ fast channel (Makielski and Falleroni, 1991), thereby reducing Na^+ and Ca^{2+} loading during cold storage and rewarming. Improved cardiac function may also be due to the addition of insulin and it's effect to possibly facilitate myocardial glucose uptake during cold exposure (Orme and Kelly, 1977). Multiple cardioprotective properties of insulin have been described, such as stimulation of NO production via ischaemia-induced

protein kinase C-dependent phosphatidylinositol 3´-kinase-Akt-dependent signalling and inhibition of superoxide anion (O_2^-) (Fischer-Rasokat and Doenst, 2003; Ji *et al.*, 2010). Similarly, the addition of melatonin may have contributed to the improved protection seen with ALMI. The pleiotropic properties described for melatonin include: (i) its high solubility in both aqueous and lipid phases across cells and into mitochondria and nucleus (ii) its antioxidant and free radical scavenging properties including superoxide radical (O_2^-), hydroxyl radical (OH^-) and the lipid peroxyl radical, (iii) protection from mitochondrial permeability transition pore opening and subsequent collapse of mitochondrial membrane potential, (iv) protection of mitochondrial membranes by maintaining levels of inner membrane dimeric phospholipid cardiolipin which plays a key role in mitochondrial bionenergetics, and (v) stabilizing effect on components of the electron transport chain including preventing the loss of cytochrome c (Petrosillo *et al.*, 2009b). Further studies are required to investigate the mechanisms of action of both the melatonin and insulin in AL cardioplegia.

The higher return of left ventricular pump function in the AL preservation groups compared to that of the HTK (Custodial) and Celsior groups may have been associated with improved maintenance of cell membrane polarity during all phases of the heart transplantation protocol (Dobson, 2010). The diastolic membrane potential of AL hearts has been reported to be -80 mV (Dobson and Jones, 2004; Sloots and Dobson, 2010) and based on the Nernstian relation between membrane potential (E_m) and extracellular potassium where E_m (mV) = 26.23 ln [K^+ (mM)] – 123.44 (Masuda *et al.*, 1990), the membrane potential for HTK (Custodial) and Celsior hearts can be predicted to be around -63 mV for potassium levels of 10 mM

K^+. This agrees with the direct microelectrode membrane measurements of -60 ± 3.6 mV reported by Bretschneider and colleagues on sheep cardiac Purkinje fibres bathed in HTK (Custodial) solution (Krohn *et al.*, 1989).

7.4.2.1 ***Celsior vs HTK (Custodial) preservation solutions***

The finding in this study that Celsior hearts performed better than HTK (Custodial) hearts, has also been reported by a number of experimental and clinical investigators for solid organ transplantation in heart (Wieselthaler *et al.*, 1999);(Wildhirt *et al.*, 2000);(Vega *et al.*, 2001);(Ackemann *et al.*, 2002);(Michel *et al.*, 2002), lung (Warnecke *et al.*, 2002) and liver (Nardo *et al.*, 2005). Our results are consistent with the rat heart study of Gao and colleagues (2005) who reported cardiac output recoveries of 20% and less than 5% at 30 mins after 6 and 10 hours of cold storage (2-3°C) in Celsior. Celsior has also been reported to be clinically superior to the University of Wisconsin solution (Wildhirt *et al.*, 2000);(Mohara *et al.*, 1999; Michel *et al.*, 2002). Superior preservation in Celsior is believed to be due to the lower depolarising potassium levels (15 mM), low Ca^{2+}/high Mg^{2+}, impermeant lactobionate, and potent antioxidant properties of reduced glutathione (Menasche *et al.*, 1994);(Vega *et al.*, 2001);(Wieselthaler *et al.*, 1999);(Remadi *et al.*, 2002);(Garlicki, 2003). Celsior appears to be originally designed as a single solution platform that could be used during most of the successive steps of the preservation procedure. However, it was not designed as a normokalaemic, normothermic reperfusion solution where the heart may be challenged during the cold-to-warm transition (Menasche *et al.*, 1993).

A surprising finding in the present study was the low ventricular outputs from HTK (Custodial) hearts as they, like Celsior hearts, appeared healthy, soft to touch with no visual signs of ischaemia after 8 hours cold storage. As with Celsior hearts, the HTK (Custodial) hearts could not generate enough left ventricular output against a fixed pressure head to generate AF; that is, they could not perform adequate physical work (force x distance) against the 76 mmHg afterload. In 1993, Reichenspurner and colleagues evaluated HTK (Custodial) for myocardial preservation in cooperation with Eurotransplant and five heart transplant centers, and it was concluded that HTK (Custodial) provided good results as long as the ischaemic time did not exceed 4 hours (Reichenspurner *et al.*, 1993). The five main features of the HTK (Custodial) solution claimed to be useful for heart include: i) its low K^+, Na^+, and Ca^{2+} concentrations, ii) the presence of energy substrate alpha-ketoglutarate, iii) the presence of anti-oxidant tryptophan, iv) mannitol to reduce cell swelling, and v) a high histidine (198 mM) buffering to counter tissue acidosis during cold global ischaemia (Krohn *et al.*, 1989; Reichenspurner *et al.*, 1993). In 2010, Lee and colleagues using a rat transplant model reported superiority of HTK (Custodial) over Celsior based on lower serum creatine kinase levels and less macroscopic deterioration of the graft after 6 or 18 hours of cold storage (Lee *et al.*, 2010). However, no pump function was reported and, as the authors acknowledged, these hearts were not loaded and cannot be extrapolated to clinical conditions (Lee *et al.*, 2010).

In general, the functional recoveries of hearts following cold storage in HTK (Custodial) solution reported in the literature vary widely. In 2000, Saitoh and colleagues (Saitoh *et al.*, 2000) reported a return of 79.9% cardiac output after 8

hours cold- storage (4°C) in HTK (Custodial) compared to 4% in this study. The study by Saitoh and co-workers used male Wistar rats while this study used male Sprague Dawley and the working afterload was lower, 60 mmHg compared to 76 mmHg in our study. This group did not specify the calcium concentration of their HTK (Custodial) solution, which they perfused for 15 minutes of reperfusion in Langendorff mode at unspecified temperatures, compared to 5 min at 37°C in this study. Reperfusion measurement in the study from the Saitoh group involved working mode function for 25 minutes at unspecified temperatures compared to 60 mins and 37°C in this study. Elsewhere in their methods section, Saitoh and colleagues state that they reperfused for 20 mins in Langendorff and 40 mins in working mode after cold storage (Saitoh *et al.*, 2000). These differences and discrepancies make functional comparisons difficult. In an earlier 1992 study by Galinanes and colleagues, they reported a return of 22% CO in isolated rat hearts stored in HTK (Custodial) for 8 hours (4°C). In these experiments, hearts were subjected to 15 mins reperfusion followed by 20 mins working mode (Galinanes *et al.*, 1992b).

The low cardiac outputs in HTK (Custodial) (and Celsior) hearts after 8 hours in this study may have been due to the shorter 5 mins rewarming flush period (see Figure 7.1). Warm reperfusion in an oxygenated depolarised state may have promoted unnatural heterogeneous membrane voltage, ionic and metabolic imbalances, and possibly altered lipid membrane organization such as temperature-dependent phase transitions and increased fluidity, which are critical in maintaining the viability of cells before switching to working mode. Normokalaemic, polarising adenosine and lignocaine preservation solution was versatile at both profoundly hypothermic and 5

mins warm transitions immediately after 8 hours cold storage**.** Functional deficits of HTK (Custodial) hearts may also have arisen from lack of broad-spectrum antioxidant protection. Indeed, Schroder and colleagues (Schroder *et al.*, 2007) recently reported cold-induced mediated oxidant injury in isolated cells after HTK (Custodial) storage and they have proposed the addition of iron chelators (or other broad spectrum antioxidants) to the traditional HTK (Custodial) solution to bolster protection. Custodiol-N base solution (modified HTK-solution) is now being developed to address some of the concerns associated with the traditional HTK (Custodial) solution (Loganathan *et al.*, 2010).

7.5 Conclusion

Normokalaemic, polarising AL cardioplegia with low Ca^{2+}/high Mg^{2+} (0.22 mM/2.6 mM) returned up to 78% cardiac output, 100% heart rate and 90 to 105% developed pressures with no detectable troponin T release following 8 hours of cold storage. In contrast, HTK (Custodial) hearts failed to recover function with regaining 4% pre-arrest CO, 16% of pre-arrest HR and 11 to 17% developed pressures. The presence of troponin T (0.13 ug/ml) in the effluent during the 5 minute rewarm period indicated myocyte death. Celsior hearts, despite recovering 86% of pre-arrest HR, generated significantly less AF (17%) than the AL hearts and thereforesignificantly less CO (25%) when compared to the AL groups. Again, detectable troponin T (0.28 ug/ml) in the rewarm effluent after 8 hours indicated myocyte damage.

CHAPTER 8: GENERAL DISCUSSION

"It is clear……that a major advance is required to be able to translate the knowledge gained from animal experiments into clinical practice."
Fallouh, H.B 2009

8.1 Restatement of the Problem:

Heart transplantation, a successful treatment option for patients suffering chronic heart failure, is now facing unprecedented challenges and pressures from an aging population and donor organ shortages (Jamieson and Friend, 2008). These challenges are exacerbated by the use of organs from extended criteria donors and suboptimal preservation during the transplantation process and may lead to suboptimal post-transplant outcomes for recipients (Schmauss and Weis, 2008; Russo *et al.,* 2010; Iyer *et al.,* 2011). Despite over forty years of dedicated to research into strategies to improve hyperkalaemic preservation and outcomes following heart transplantation; safe ischaemic times for donor hearts remain limited to 4 – 5 hours (Kirklin *et al.*, 2004; Maria Rosa *et al.*, 2010).

Ischaemic times longer than 4 hours have been identified by the International Society for Heart and Lung Transplantation (ISHLT) as an independent predictor of primary graft failure following heart transplantation (Taylor *et al.*, 2005; Lima *et al.*, 2006). Several studies have shown that suboptimal preservation of the donor heart during

prolonged storage may result in damage to the myocytes, vascular smooth muscle and endothelium leading to the development of acute allograft failure (Bourge *et al.*, 1993; Jahania *et al.*, 1999) and chronic rejection or chronic allograft vasculopathy (CAV) (Schmauss and Weis, 2008). An improvement in safe preservation times for donor hearts could have a direct impact on acute and chronic outcomes for heart transplant recipients (Lima *et al.*, 2006).

8.2 Overall Hypothesis and Discussion Outline

The overall hypothesis was that a normokalaemic AL preservation solution could be developed from an existing AL cardioplegia which would provide superior protection for hearts when compared with hearts stored in hyperkalaemic preservation solutions, Celsior and HTK (Custodial).

Using the isolated working rat heart model a series of studies was designed to;

1) Establish the efficacy of AL cardioplegia for cold static storage;

2) Establish a new post-storage reperfusion and rewarming paradigm prior to heart implantation;

3) Modify the AL cardioplegia using low Ca^{2+}, high Mg^{2+} and higher AL concentrations for extended cold storage times;

4) Evaluate the new AL cold preservation solution with the addition of melatonin and insulin as a new approach towards therapeutic suspended animation.

The discussion will be set out under the following broad headings:

- AL solution's versatility for cold and warm preservation
- Development of a new AL reperfusion paradigm
- Modification of AL preservation solution for 8 hours of cold static storage
- Possible mechanisms of action responsible for AL cardioprotection
- Possible future studies and clinical applications: Donor harvest to donor patient and multi-organ harvest
- Cold and warm storage boxes and extracorporeal technologies
- Conclusions

8.3 General Discussion

8.3.1 AL solution's versatility for both cold and warm preservation

An important finding from studies in Chapter 4 was that isolated rat hearts stored in AL preservation solution showed superior functional recoveries at both cold (4ºC) and warmer temperatures (28 - 30ºC) over 6 hours compared with hyperkalaemic Celsior solution (Chapter 4; pgs 100-107). The main findings for *cold* and *warm* hearts were:

Cold storage (4ºC): Hearts arrested and stored in AL solution for 6 hours in cold static storage fully recovered pre-arrest HR and 68% of CO which was composed of 64% and 80% recovery of pre-arrest AF and CF respectively. This was in direct contrast to hearts arrested and stored in Celsior solution under the same conditions which showed statistically lower functional recoveries ($p<0.05$). This is the first time

AL cardioplegia has been shown to maintain arrest in the cold (4ºC) and reanimate in the warm. Previous studies have reported improved functional recoveries following 4 hours intermittent (28 - 30ºC) (Dobson and Jones, 2004) and 2 hours intermittent or continuous at 32°C (Sloots *et al.*, 2007).

Warmer storage (28 - 30ºC): In addition to AL's applicability to cold storage, AL stored hearts using warmer intermittent flushes (2 mins flush, every 18 mins) over 6 hours recovered 55% of pre-arrest CO (Table 4.3 and Figure 4.3), which was significantly higher ($p<0.05$) than hearts flushed with Celsior which failed to recover aortic or coronary flow and developed contracture. Furthermore, functional recoveries of the warm AL hearts were significantly improved by the addition of 1mM L-pyruvate with 75% recovery of pre-arrest CO and full recovery of HR ($p<0.05$) (Chapter 4; Figure 4.3, Table 4.3 and 4.4). Pyruvate studies were not continued in this thesis because it was discovered upon further reading that pyruvate had stability problems in cardioplegic solutions and may not be suitable for clinical applications (Woo *et al.,* 2004; Mallet *et al.,* 2005).

Overall the data in Chapter 4 highlights the versatility of AL cardioplegia in both cold and warm over a 6 hour preservation time, and indicated that the AL solution may be applicable for preserving donor hearts in cold static storage or emerging new machine perfusion preservation technologies (see Section 8.4.3 pg 221). The mechanisms of improved arrest and reanimation at low and cold temperatures are not known but may relate to polarised arrest (Sloots and Dobson, 2010; Dobson, 2010) and/or normokalaemic linked improvement of calcium handling in the myocyte and coronary vessels (endothelium and smooth muscle) (See section 8.3.3; pg 216).

8.3.2 Development of a new AL reperfusion paradigm

In addition to improved functional recovery following both cold and warm storage techniques, a major finding of Chapter 5, was the importance of AL arrest during rewarming and re-oxygenation of the isolated rat heart in early reperfusion and implantation following 6 hours of cold static storage. This result may be important for donor heart preservation because it is well known that early reperfusion and implantation are times of high vulnerability for the donor heart.

The reperfusion studies showed that rewarming with oxygenated AL cardioplegia (physiological levels of Ca^{2+} and Mg^{2+} with no additions) significantly improved early functional recoveries in hearts after cold AL storage, and interestingly after storage in depolarising Celsior solution (See Figure 5.2, 5.3 and 5.4; Table 5.1 and 5.2). Rewarming with oxygenated AL solution following AL storage was associated with higher myocardial oxygen consumption during the rewarm period. This increase in myocardial oxygen consumption may be attributed to higher maintenance metabolism during the AL rewarm which is consistent with earlier observations of Allen and colleagues (1986c). While mechanisms were not examined in this thesis, it would be interesting to investigate these different storage and reperfusion states using ^{31}P NMR (ATP, PCr, pH and free Mg^{2+}), metabolic (glycogen, lactate, intracellular pH and Ca^{2+}), electrophysiological (membrane potential) and isolated mitochondrial studies to tease apart the differences in basal metabolism found for AL stored hearts compared with Celsior hearts with and without the AL arrest/reperfusion strategy.

It was concluded that the warm oxygenated AL arrest/reperfusion strategy may offer a new reperfusion strategy for donor hearts and could become one of the controlled reperfusion strategies including temperature control; haemodynamic control (unloaded, non-working mode); pressure control and reperfusate control (Jahania *et al.*, 1999; Stoica *et al.*, 2001; Bethea *et al.*, 2003; Osaki *et al.*, 2006). The new addition would be *membrane potential control* using normokalaemic, oxygenated polarising AL rewarm strategy.

8.3.3 Modifying the AL preservation solution for 8 hours cold static storage

The final studies combine the findings of the previous chapters to examine the effect of the addition of melatonin and insulin to the modified AL preservation solution for extended storage (8 hours). Recoveries were compared to two currently used storage solutions Celsior and HTK (Custodial). Augmenting the doubled AL, low calcium high magnesium solution with 100 μM melatonin and insulin resulted in 78% recovery of CO, 90-100% return of developed pressures and 101 – 104 % recovery of HR following 8 hours of cold storage with no troponin T detected in the coronary effluent during the rewarm period. In contrast hearts stored in Celsior and HTK (Custodial) could not generate more than 11% of left ventricular function (AF).

8.3.4 Possible mechanisms of action responsible for AL cardioprotection

Improvement in functional recoveries of rat hearts stored in nomokalaemic AL preservation solution may be related to the solution's physiological level of potassium and the ability of AL to arrest the heart at or near the resting membrane potential for the myocyte, coronary endothelium and smooth muscle (polarised arrest) (Dobson, 2010; Sloots and Dobson, 2010). Polarised arrest has been

associated with improved ionic balance and improved intracellular calcium regulation (Snabaitis *et al.*, 1997a; Sunamori *et al.*, 2001; Jilkina *et al.*, 2003); Chambers, 2003) as opposed to hyperkalaemic depolarising Celsior solution (Chapter 4; Dobson, 2010). Future studies are required to examine the effect of temperature on the cell voltage differences and intracellular calcium levels during heart preservation with AL and Celsior solutions.

As mentioned earlier, during cardioplegic arrest at resting diastolic membrane potentials of the myocyte, fewer open membrane channels, pores and exchangers are operating compared with depolarised states (Snabaitis *et al.*, 1997b; Snabaitis *et al.*, 1997a; Chambers, 2001; Dobson, 2004; Sloots and Dobson, 2010). An interesting finding from Chapter 6 was that lowering the Ca^{2+} and increasing the Mg^{2+} concentrations of the AL preservation solution during arrest and storage resulted in significantly improved functional recoveries during early reperfusion (Figures 6.6 and 6.8) compared with those hearts stored in AL with physiological levels of calcium and magnesium. Yet, during reperfusion the opposite occurred, rewarming in AL with physiological levels of calcium and magnesium was superior to rewarming in AL with Low Ca^{2+} and High Mg^{2+} or KH alone. This finding demonstrates the importance of keeping physiological levels of Ca^{2+} and Mg^{2+} in the warm oxygenated AL arrest/reperfusion strategy, but not in the AL normokalaemic preservation solution.

The beneficial effects of keeping calcium low has been shown for extended storage in hyperkalaemic solutions and these have been reviewed by Chen (Chen, 1996); these benefits appear to be largely due to prevention of calcium accumulation and

disruption of intracellular calcium compartmentalisation during ischaemic storage (Sunamori *et al.,* 2001). It would be of interest to investigate the extent of polarised arrest and subsequent free and total calcium accumulation during cold and warm AL arrest and reanimation strategy. Sunamori and colleagues (2001) reported a reduction in calcium concentration of myocytes following arrest and storage in a non-depolarising solution containing low sodium concentration (60 mmol/L) and lignocaine (1mmol/L). This group also reported that the non-depolarised arrest, measured via micro-electrodes in guinea pig purkinje fibres not whole hearts, persisted for only the first 30 minutes of a 6 hour arrest. Therefore knowledge of both the persistence of polarised arrest and calcium accumulation during extended storage in AL preservation solution with normal and altered calcium and magnesium concentrations would further inform the design of the AL preservation solution.

8.3.5 Study Limitations

The isolated rat heart perfusion model in working mode is ideally suited for the investigation of preservation solutions for heart transplantation and the advantages and disadvantages of this model are discussed in the Materials and Methods section. The rat heart model of cold static storage and rewarming used in these studies differs from heart transplantation in a number of ways. While both rat and human hearts are empty and non-working in the physical sense and are perfused via the coronary vessels following storage, the rat heart in working mode has preload and afterload that are pre-set during normothermic reperfusion. In contrast, during weaning off cardiopulmonary bypass the preload and afterload of the human donor heart are dynamic variables where preload is determined by a balance of blood volume between the patient and oxygenator, and afterload is dependent on the status of

peripheral vascular resistance and cardiac output. However, the rat model is still clinically relevant in these studies because myocardial and coronary vascular injury does occur in the human graft during early rewarming and reperfusion.

The absence of ECG recordings during arrest, storage and reperfusion of the the isolated rat hearts also presents a limitation of these studies, both in the determination of mechanical and electrical arrest and in the visualisation of current of injury during reanimation. Another limitation of these studies involves the sensitivity of measurements of tissue viability, the end points are largely functional and do not assess regional ischaemia/reperfusion injury. More thorough investigation of tissue preservation by the use of histological and immuno-histological studies is certainly an aim for future studies.

When attempting to extrapolate these results to humans it should be recognised that there are considerable species differences. In particular the availability of collateral flow, Scaper (2000) demonstrated that necrosis occurred rapidly in animals which have little or no collateral flow such as the rabbit, rat and pig, and more slowly in animals with moderate levels of collateral flow such as dog and cat. Necrosis does not occur in animals with extensive collateral flows such as guinea pig (Schaper, 2000). It has been speculated that younger human hearts are more like the pig in terms of collateral flow and older hearts are more closely related to that of a dog.

The rat heart also differs from the human in energetics, metabolic rate, presence of xanthine oxidase and the expression of ion channels, transporters and receptor subtypes. Receptors were initially defined based on their responses (inhibition or stimulation) to agonists and antagonists, there are subtle interspecies differences in

their structure and therefore responses to agonists and antagonists may differ (Mubagwa and Flameng, 2001). These disadvantages are outweighed by the benefits of the technique which allows a unique balance between low cost, quantity and quality of data for heart preservation for transplantation research.

8.4 Possible Future Studies and Clinical Applications

The work from this thesis may have wider applications for protecting the donor heart during all stages of heart transplantation:

- prior to and during harvest;
- during transportation;
- during the immediate reperfusion rewarming, flush period and implantation

The use of AL to protect the donor organs in the period between brain death and harvest of donor organs is an area of unmet need, which warrants exploration.

8.4.1 From donor heart to donor patient

AL may be applicable for protecting the donor organ prior to harvest. Organ procurement is hampered by the hormonal surges following brain death which result in a "catecholamine storm", an up-regulation of inflammatory factors, rapid expression of cytokines and adhesion molecules, and increase the organ's immunogenicity, thereby accelerating the evolution of acute and chronic rejection in the recipient (Wilhelm *et al.*, 2000; Rosendale *et al.*, 2002). It is for this reason that certain donor hearts for transplant may be discarded (Rosendale *et al.,* 2002). To date the use of aggressive donor management has had some effect on transforming a

significant number of potential donors into acceptable and successful heart donors (Zaroff *et al.*, 2002).

The AL non-arrest solution has proven cardioprotective in an *in vivo* model of regional ischaemia, demonstrating not only a reduction in infarct size but also a reduction of post-ischaemic arrhythmias (Canyon and Dobson, 2004; Canyon and Dobson, 2005). Adenosine is also known to have anti-adrenergic effects (Dobson and Jones, 2004) and more recently AL has been shown to exhibit potent anti-inflammatory effects (Shi *et al.,* 2011) and these may also be exploited in the donor patient.

Thus, in future studies, an AL pre-treatment (non-arrest solution) could be adjusted to pre-treat donor rats prior to organ harvest. In preliminary studies, pre-treating male Sprague Dawley rats with a bolus of AL at non-arresting concentrations (2.4 mg/Kg adenosine and 5.0 mg/Kg lignocaine) 15 minutes prior to organ retrieval resulted in improved functional recoveries (AF and CO) of isolated rat hearts after 2 hours of cold storage in AL preservation solution when compared to no pre-treatment. Interestingly, the AL pre-treatment results were comparable to hearts pre-treated with ischaemic preconditioning (3 x 1 minute bouts of pre-arrest ischaemia). It would be of interest to expand on these pilot studies and include pre-treatment of rats prior to longer ischaemic times.

8.4.2 From donor heart to multi-organ harvest

Another area of clinical application for AL solution may be multi-organ harvest including lung, kidney, liver and pancreas. Although improvements in surgical techniques and perioperative care have improved outcomes for lung transplant the

ISHLT reports that the 1 and 5 year survival are 80% and 50% respectively, much lower than those for heart or liver (Okada and Kondo, 2009). Primary graft dysfunction still remains the primary cause of death in lung transplant recipients, and the pathogenesis appears to be related to vascular injury during the transplantation process. Lung injury has also been related to hyperkalaemic hypothermic lung preservation and flush solutions which can lead to vasospasm and reflex vasoconstriction (Okada and Kondo, 2009). The AL solution has been shown to be protective of coronary vascular function (Dobson, 2004; Sloots *et al.*, 2007) possibly due to adenosine's reported anti-neutrophil effects and down regulation of inflammatory cytokines, (Vinten-Johansen *et al.*, 1995). The new non-depolarising AL paradigm may find utility in harvest of other organs for transplantation.

In an effort to extend AL preservation studies from heart to long-term kidney preservation a study has commenced in collaboration with Imperial College in London. Modification of the AL preservation solution for use in porcine kidneys involved increasing adenosine, lignocaine and magnesium concentrations, and the addition of an oncotic for prevention of graft swelling. The use of an oncotic was not required for long term rat heart preservation (Chapter 4), however, preliminary findings from the pig kidney studies showed that an oncotic was important for preservation of the porcine kidney. Of course this finding is not new for kidney and was identified by Belzer and Southard in 1988, where they emphasised the importance of a colloid to prevent expansion of interstitial spaces during flushing of donor kidneys. During pilot studies porcine kidneys were harvested from Australian Yorkshire pigs and flushed with HTK (Custodial) or AL preservation solution containing varying combinations and concentrations of albumin, dextran and

lactobionate. Initial kidney weights and weights prior to and following 12 hours of cold static storage were recorded. Following storage kidneys were processed for histological analysis. The results of these studies will be compared to similar studies conducted using a perfusion box and 12 hours of preservation. This work is on-going, however, the preliminary results look promising for a role of AL in preserving the kidney.

8.4.3 Cold and warm storage boxes and extracorporeal technologies

The new AL arrest, storage and reperfusion strategy may find utility in machine perfusion at both hypothermic and normothermic conditions and it would be interesting to study these in rat and porcine hearts before a pilot human trial. Currently there is no evidence-based cardioplegia or preservation solution recommended for storing and preserving donor hearts at higher temperatures.

Early work in the preservation of organs for transplantation not only involved the development of suitable preservation solutions but also perfusion pumps for isolated organ perfusion at both hypothermic and normal body temperatures (Boettcher *et al.*, 2003; Birnbaum, 2004). While warm intermittent flush techniques similar to the method employed in Chapter 4 or continuous low-pressure infusion delivery during cold-storage, are not widely practiced today, they are generating increasing interest in the field of organ preservation. Preclinical data demonstrating their superiority has been gathered experimentally for the last 20 years (Wicomb *et al.*, 1992; Nickless *et al.*, 1998; Jahania *et al.*, 1999; Ozeki *et al.*, 2007). Hypothermic machine perfusion, for example, has been used successfully with kidneys (Southard and Belzer, 1995; McLaren and Friend, 2003) and similar techniques have been employed in

experimental heart transplantation (Toledo-Pereyra *et al.*, 1979; Qayumi *et al.*, 1991; Rosenbaum *et al.*, 2007).

Donor organ perfusion preservation has been shown to increase metabolism through the preservation of cellular energetics and this has been demonstrated by reduced oxygen consumption, lactate release, and coronary vascular resistance (Poston *et al.*, 2004; Peltz *et al.*, 2005; Rosenbaum *et al.*, 2007). Perfusion preservation may also offer the opportunity to assess the viability of the donor heart and provide longer time frames for better donor and recipient matching (Poston *et al.*, 2004; Rosenbaum *et al.*, 2007; Garbade *et al.*, 2008); (Garbade *et al.*, 2009).

8.4.3.1 AL preservation solution for use with intemittent flush or continuous perfusion devices at both cold (4℃) and warmer temperatures (32 - 35℃)

Studies using the isolated rat heart perfusion model are ideal for investigating the use of warm flush and continuous flow methods for extended preservation of hearts. Of interest would be a study to investigate the performance of AL during arrest and storage with either intermittent flush or continuous flow for extended storage periods of 10, 15 and 24 hours at both cold (4°C) and warm (32-35°C) temperatures using new extracorporeal perfusion boxes. Both intermittent flush and continuous perfusion techniques have been associated with myocardial oedema, therefore the use of oncotic agents such as lactobionate, albumin and dextrans and their appropriate concentrations would need investigation (Cobert *et al.*, 2010).

8.4.3.2 Use of AL solution in perfusion pumps for the resuscitation of non-heart-beating donor organs

In the last decade there has been increased interest in preserving marginal hearts including hearts from non-heart-beating donors, and the potential applications of new extracorporeal perfusion technologies. However, there has been slow uptake of the new perfusion technologies; which may be due to logistical complexity, cost and adequate outcomes following static storage. This unmet need in heart preservation was recognised by Hassanein and colleagues (1998) and others who developed a prototype portable perfusion apparatus for use in normothermic donor human beating heart preservation which allows for continuous or intermittent perfusion (Wicomb *et al.*, 1985; Hassanein *et al.*, 1998).

The benefits of reperfusion with the oxygenated AL arrest reperfusion strategy reported in this thesis may offer a solution for resuscitation of hearts from non-heart-beating donors (NHBD). Other studies have demonstrated resuscitation and tissue repair through the use metabolic enhancement of preservation solutions with metabolic substrates, antioxidants (Allen *et al.*, 1986b; Bolli *et al.*, 1989) and genetic or immune-modulation through the use of blocking antibodies against specific inflammatory markers such as ICAM-1(Chen *et al.*, 1999) or tissue factor (Matsuyama *et al.*, 2003) in an effort to reduce ischaemia/reperfusion injury (McLaren and Friend, 2003; Ozeki *et al.*, 2007). The use of extracorporeal perfusion technologies is an area of active and ongoing investigation and it will be interesting to see if the AL solution can play a role in it's further development.

8.5 General Conclusion

Studies described in this thesis map the development of a new heart preservation paradigm in the isolated working rat heart based on an adenosine and lignocaine cardioplegic solution. The solution showed significantly improved functional recoveries following warm and cold arrest after 6 hours. In addition, a new warm reperfusion strategy with oxygenated AL arrest solution was developed that may prove useful when used immediately following cold static storage. This new reperfusion strategy showed efficacy for hearts stored in AL and interestingly, following storage in traditional hyperkalaemic solutions as shown in Chapter 5.

Maintaining cardioplegic arrest at or near the resting membrane potential of the myocyte, vascular smooth muscle and endothelial cells, may limit calcium loading during prolonged arrest. This new cardioprotective mechanism could be further augmented by lowering of calcium and increasing magnesium (Low Ca^{2+}:Mg^{2+}) in the storage solution. Rewarming by maintaining a normokalaemic polarised arrest using AL arrest/reperfusion strategy may also further protect the heart from reperfusion injury.

In an attempt to extend cold storage times to 8 hours, the addition of melatonin and insulin provided superior protection of the donor rat heart with recoveries of 104% of HR, 91% of SP, 103% of DP, 76% of AF, 86% CF and 78% of cardiac output. In direct contrast, hearts stored under identical conditions in FDA approved hyperkalaemic solutions Celsior and HTK (Custodial) returned 11% or less of ventricular function.

Translation of this novel lower potassium polarising preservation and reperfusion strategy into human hearts may offer extended safe ischaemic time for donor hearts beyond 4 – 5 hours. In an era of donor shortages and expanded criteria donors, longer timeframes with a reduced dependence on storage temperature may expand the use of organs from marginal beating and non-beating donor organs, allow for donors to be sourced from regional, rural and remote sites, allow for more extensive donor and recipient matching, gathering of the more experienced transplant team and thereby allowing for better use of the very limited precious donor organ resource. Finally, the new arrest, storage and reperfusion paradigm may find utility in the preservation of multiple solid donor organs such as kidney, lung, liver and pancreas; particularly for use with new portable perfusion storage modalities.

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APPENDIX A

Composition of Reagents and Solutions

Perfusion buffer, cardioplegic and reperfusion solutions

All solutions were made fresh daily. The cardioplegic (arrest and storage) and reperfusion solutions were filtered using 0.2 μm filters (Pall, Australia). Perfusion buffer and cardioplegic solutions were maintained at 37°C. The same solution was then used for storage of hearts and following heart immersion the solution is cooled to 4°C. The cardioplegic (arrest and storage) solutions were not actively bubbled with 95% O_2/5% CO_2; hence they had a higher pH. The average pO_2 of the cardioplegic solutions were 140mmHg and the pCO_2 was 5-10 mmHg. The reperfusion solutions were the same as the cardioplegic solutions described above except that they were actively bubbled with 95% O_2/5% CO_2 to achieve a pO_2 greater than 600 mmHg and not recirculated. This was to facilitate slow warming and oxygenation during early reperfusion of the hearts. Components used in the preparation of the perfusion buffer and cardioplegic solutions are listed in Table A1 below.

Table A1: Arrest and Perfusion Buffer Components
Components used for preparing Krebs Henseleit solution, Adenosine and Lignocaine (AL), Celsior and Custodial solutions. All compounds are of analytical grade purity.

Compound	Catalogue Number	Source
Adenosine	A9251	Sigma Aldrich (St Louis, Mo)
Alpha-Ketoglutarate	K1750	Sigma Aldrich (St Louis, Mo)
Calcium Chloride ($CaCl_2$)	C3881	Sigma Aldrich (St Louis, Mo)
Glucose	G7520	Sigma Aldrich (St Louis, Mo)
Glutamate	G1251	Sigma Aldrich (St Louis, Mo)
Glutathione-reduced	G6529	Sigma Aldrich (St Louis, Mo)
Heparin		Lyppards, Australia
Histidine	H8000	Sigma Aldrich (St Louis, Mo)
Histidine-HCl	H8125	Sigma Aldrich (St Louis, Mo)
Insulin (Caninsulin)		Lyppards, Australia
Lactobionate	L2398	Sigma Aldrich (St Louis, Mo)
Lignocaine-HCl		Lyppards, Australia
Magnesium Chloride ($MgCl_2$)	M0250	Sigma Aldrich (St Louis, Mo)
Mannitol	M9546	Sigma Aldrich (St Louis, Mo)
Melatonin	M5650	Sigma Aldrich (St Louis, Mo)
Potassium chloride	P5405	Sigma Aldrich (St Louis, Mo)
Sodium Chloride (NaCl)	S7653	Sigma Aldrich (St Louis, Mo)
Sodium Hydroxide	1823	Univar Ajax Laboratory Chemicals
Sodium pyruvate	P8574	Sigma Aldrich (St Louis, Mo)
Sodium phosphate (monobasic)	S8282	Sigma Aldrich (St Louis, Mo)
Tryptophan	T0254	Sigma Aldrich (St Louis, Mo)

Table A2: Modified Krebs-Henseleit Solution (containing glucose).

Compound	Concentration (mM)
Sodium	145
Potassium	5.9
Chloride	124.5
Magnesium	0.5 (free Mg^{2+} = 0.5mM)
Calcium	1.12 (free Ca^{2+} = 1.07mM)
Glucose	10
Phosphate	1.2
pH	7.4
Osmolarity	300mOsm/L

Table A3: Modified Krebs-Henseleit Solution (containing glucose) with Low Ca^{2+}/High Mg^{2+}.

Compound	Concentration (mM)
Sodium	145
Potassium	5.9
Chloride	124.5
Magnesium	2.6 (free Mg^{2+} = 2.5mM)
Calcium	0.22 (free Ca^{2+} = 0.21mM)
Glucose	10
Phosphate	1.2
pH	7.4
Osmolarity	300mOsm/L

Table A4: Adenosine and Lignocaine Solution
Components used for preparing adenosine and lignocaine solution (AL). All compounds are of analytical grade purity.

Compound	Concentration (mM)
Sodium	145
Potassium	5.9
Chloride	124.5
Magnesium	0.5
Calcium	1.12
Glucose	10
Phosphate	1.2
Adenosine	0.2
Lignocaine	0.5
pH	7.7
Osmolarity	300mOsm/L

Table A5: Adenosine and Lignocaine Solution with Low Ca^{2+}/High Mg^{2+}
Components used for preparing Adenosine and Lignocaine Solution. All compounds are of analytical grade purity.

Compound	Concentration (mM)
Sodium	145
Potassium	5.9
Chloride	124.5
Magnesium	2.6
Calcium	0.22
Glucose	10
Phosphate	1.2
Adenosine	0.2
Lignocaine	0.5
pH	7.7
Osmolarity	300mOsm/L

Table A6: Adenosine (200µM) and Lignocaine (1000µM) Solution
Components used for preparing adenosine and lignocaine solution (AL). All compounds are of analytical grade purity.

Compound	Concentration (mM)
Sodium	145
Potassium	5.9
Chloride	124.5
Magnesium	0.5
Calcium	1.12
Glucose	10
Phosphate	1.2
Adenosine	0.2
Lignocaine	1.0
pH	7.7
Osmolarity	300mOsm/L

Table A7: Adenosine (200µM) and Lignocaine (1500µM) Solution
Components used for preparing adenosine and lignocaine solution (AL). All compounds are of analytical grade purity.

Compound	Concentration (mM)
Sodium	145
Potassium	5.9
Chloride	124.5
Magnesium	0.5
Calcium	1.12
Glucose	10
Phosphate	1.2
Adenosine	0.2
Lignocaine	1.5
pH	7.7
Osmolarity	300mOsm/L

Table A8: Adenosine (400µM) and Lignocaine (1000µM) Solution
Components used for preparing adenosine and lignocaine solution (AL). All compounds are of analytical grade purity.

Compound	Concentration (mM)
Sodium	145
Potassium	5.9
Chloride	124.5
Magnesium	0.5
Calcium	1.12
Glucose	10
Phosphate	1.2
Adenosine	0.4
Lignocaine	1.0
pH	7.7
Osmolarity	300mOsm/L

Table A9: Adenosine and Lignocaine Solution with Low Ca^{2+}/High Mg^{2+} plus cyclosporin A
Components used for preparing AL (Low Ca^{2+}/High Mg^{2+}) +CYA. All compounds are of analytical grade purity.

Compound	Concentration (mM)
Sodium	145
Potassium	5.9
Chloride	124.5
Magnesium	2.6
Calcium	0.24
Glucose	10
Phosphate	1.2
Adenosine	0.2
Lignocaine	0.5
Cyclosporin	0.00002
pH	7.7
Osmolarity	300mOsm/L

Table A10: 2 x ALMI Solution
Components used for preparing ALMI Solution (ALMI). All compounds are of analytical grade purity.

Compound	Concentration (mM)
Sodium	145
Potassium	5.9
Chloride	124.5
Magnesium	2.6
Calcium	0.24
Glucose	10
Phosphate	1.2
Adenosine	0.4
Lignocaine	1.0
Melatonin	0.1
Insulin	0.01IU/ml
pH	7.7
Osmolarity	300mOsm/L

Table A11: Celsior Solution
Components used for preparing Celsior Solution. All compounds are of analytical grade purity.

Compound	Concentration(mM)
Sodium	100
Potassium	15
Chloride	41.5
Calcium	0.25
Magnesium	13
Glutamate	20
Mannitol	60
Lactobionate	80
Histadine	30
Reduced Glutathionine	3
pH	7.3
Osmolarity	360mOsm/Kg

Table A12: Histidine Tryptophan Ketoglutarate Solution (HTK or Custodial)
Components used for preparing Custodial Solution. All compounds are of analytical grade purity.

Compound	Concentration(mM)
Sodium	15
Potassium	9
Chloride	32.0
Calcium	0.015
Magnesium	4
Alpha-Ketoglutarate	1
Tryptophan	2
Mannitol	30
Histidine	180
Histidine-HCl	18
pH	7.2
Osmolarity	310mOsm/Kg

APPENDIX B