The Role of the Mitochondrial Permeability Transition Pore in Human Myocardial Protection

Thesis submitted by

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ABSTRACT

Background:

Coronary artery disease is set to be the world's leading cause of mortality by 2020. Hence novel treatment strategies are urgently required to protect the human myocardium against ischemia-reperfusion injury. This thesis examines the role of the mitochondrial permeability transition pore (MPTP) as a novel target for myocardial protection by interventions applied solely at the time of reperfusion, which can protect the human heart against lethal reperfusion injury.

Methods and Results:

Using human atrial tissue (harvested at the time of routine cardiac bypass surgery), subjected to simulated ischemia and reperfusion injury model, we have demonstrated that the opening of the MPTP at the time of reperfusion is a critical determinant of cardiomyocyte death. We also show that inhibiting MPTP opening, by administering known pharmacological inhibitors of MPTP at the onset of reperfusion, is cardio-protective. Using experimental models in adult human atrial trabeculae, we demonstrate that inhibiting MPTP opening at the time of reperfusion improves myocardial contractile function. Also using human atrial cardiomyocytes we demonstrate that inhibiting MPTP opening at the time of reperfusion improves cellular viability. Finally using the human atrial cardiomyocyte model for inducing and detecting the MPTP opening, we demonstrate the opening of MPTP and also the inhibitory effect of known MPTP inhibitors on MPTP opening.

Conclusion:

We find that MPTP opening does occur in the human atrial cardiomyocyte following ischemia-reperfusion injury, and that inhibiting the opening of the MPTP at the time of reperfusion, provides a potential target for human myocardial protection, when the intervention is applied at the time of reperfusion. Therefore, interventions, which target and inhibit MPTP opening, at the time of reperfusion, can protect the myocardium from lethal reperfusion injury and may improve morbidity and mortality from coronary artery disease. This is useful in the clinical settings of ischemia-reperfusion injury such as thrombolysis following an acute myocardial infarction, heart surgery and percutaneous transluminal coronary angioplasty.

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Declaration

I confirm that the work presented in this thesis is my own.

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LIST OF ABBREVIATIONS

The following is a list of abbreviations used in this thesis.

%	percentage
ACE-I	angiotensin-converting enzyme inhibitor
ADP	adenosine diphosphate
AIF	apoptosis-inducing factor
Akt	cellular Akt/ protein kinase B
AMISTAD	Acute Myocardial Infarction STudy of ADenosine
AMP	adenosine monophosphate
ANOVA	analysis of variance
ANT	adenine nucleotide translocase
APAF-1	apoptosis protease-inducing factor-1
ATP	adenosine triphosphate
ATPase	ATP synthase
Bad	Bcl-X_L/Bcl-2-associated death promoter
Ca^{2+}	calcium ion
Caspase	cystein aspartate specific proteases
CK	creatine kinase
CsA	cyclosporin-A
Da	Dalton
DIABLO	direct IAP-binding protein with low pI
DMSO	dimethyl sulphoxide
ETC	electron transport chain
F_0F_1-ATPase	ATP synthase
GUARDIAN	Guard During Ischemia Against Necrosis
HMG-CoA	hydroxyl-3-methylglutaryl-co-enzyme A
H^+	hydrogen ion/proton
IMAC	inner membrane anion channel
IMM	inner mitochondrial membrane

IPC	ischemic preconditioning
K^+	potassium ion
K_{ATP}	ATP-sensitive potassium channel
kDa	kilodalton
LDH	lactate dehydrogenase
LIMIT-2	Second Leicester Intravenous Magnesium Intervention Trial
Mg^{2+}	magnesium ion
MAPK	mitogen activated protein kinase
MEK	MAPK/Erk kinase
MMC	mitochondrial megachannel
MMP	mitochondrial membrane permeabilisation
MPTP	mitochondrial permeability transition pore
Na^+	sodium ion
NADH	nicotinamide adenine dinucleotide
NHE	Na^+-H^+ exchanger
NO	nitric oxide
OMM	outer mitochondrial membrane
pH	pH
P_i	inorganic phosphate
PKC	protein kinase C
PPIase	peptidyl prolyl transisomerase
ROS	reactive oxygen species
SfA	sanglifehrin-A
SMAC	second mitochondrial activator of caspases
SWOP	second window of protection
TK	tyrosine kinase
TMRM	tetramethyl-rhodamine methyl ester
2-DOG	2-deoxyglucose
VDAC	voltage-dependent anion channel

LIST OF PUBLICATIONS

The following is a list of publications arising from the thesis.

ABSTRACTS:

1. Inhibiting Mitochondrial Permeability Transition Pore Protects the Human Myocardium from Reperfusion Injury.

   Shanmuganathan S, Hausenloy DJ, Duchen M, Yellon D.
   Circulation. 2004 Oct; 26; Vol 110; 17 (Suppl).

2. Inhibiting mitochondrial permeability transition pore opening protects the human heart from lethal reoxygenation injury.

   Shanmuganathan S, Hausenloy DJ, Duchen M, Yellon D.
   Cardiovasc J S Afr. 2004 Jul;15(4 Suppl 1):S11.

PRESENTATIONS:

1. "Inhibiting Mitochondrial Permeability Transition Pore Opening Protects the Human Myocardium from Ischemia-Reperfusion Injury";

   S Shanmuganathan, DJ Hausenloy, M Duchen, D Yellon – Oral presentation at Scientific Sessions 2004, American Heart Association, New Orleans.

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   S Shanmuganathan, DJ Hausenloy, M Duchen, D Yellon – Poster presentation at UCL Cardiovascular Science and Medicine Symposium, May 2004. (**First prize**).

3. "Inhibiting Mitochondrial Permeability Transition Pore Opening Protects the Human Myocardium from Ischemia-Reperfusion Injury ";

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PUBLICATIONS:

3. Mitochondrial permeability transition pore as a target for cardio-protection in the human heart.

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Chapter One: GENERAL INTRODUCTION

1.1 Coronary Artery Disease

Coronary artery disease (CAD) is the leading cause of death in the world. About 3.8 million men and 3.4 million women die of this disease every year[1]. The global burden, in terms of disability-adjusted life years (DALY) or "healthy years of life lost" is projected to rise from around 47 million DALY globally in 1990 to 82 million DALY in 2020. CAD ranges from sub-clinical coronary artery disease, angina on exertion, unstable angina and myocardial infarction, congestive cardiac failure and finally death.

CAD is caused by the build up of atherosclerotic plaques in the coronary arteries, known as atherosclerosis. This leads to limitation of the coronary blood flow to the myocardium resulting in oxygen and nutrient deprivation, termed ischemia. The unstable atheromatous plaque can rupture resulting in the formation of a thrombo-embolic plug occluding the major coronary arteries. This acute occlusion leads to acute myocardial infarction. Salvaging the myocardium in this situation of acute myocardial infarction is by the timely restoration of coronary blood flow to the ischemic myocardium by - thrombolysis, primary percutaneous coronary artery intervention or coronary artery bypass graft (CABG) surgery. Data from the British Heart Foundation (BHF) indicates that CAD in the UK is the most common cause of mortality and accounts for more than 125,000 deaths per year. Thankfully, the majority of these are non-fatal at the time of presentation. A large proportion of patients presenting with CAD benefit from revascularisation either using percutaneous coronary intervention or CABG surgery.

To salvage viable myocardium following an acute coronary artery occlusion, reperfusion is mandatory. However, the restoration of blood flow paradoxically leads to the death of cardiomyocytes which were viable just before the ischemic episode. This myocardial injury is termed lethal '*reperfusion injury*'[2] and which in itself can induce cardiomyocyte death and increase infarct size. This could possibly explain the increase in the incidence of death after an acute myocardial infarction reaching 10% and the incidence of cardiac failure after an acute myocardial infarction reaching up to 25% despite optimal management.

Therefore myocardial protection against this lethal reperfusion injury is required and novel treatment strategies that can be applied *after* the onset of ischemia but at the time of onset of reperfusion need to be identified.

One of the most powerful mechanisms for protecting the myocardium *before* the acute coronary artery occlusion / ischemia is to ischemically precondition (IPC) the myocardium. This powerful phenomenon was first described in the seminal study by Murry and colleagues in 1986[3]. IPC refers to the ability of short periods of ischemia to make the myocardium more resistant to a subsequent lethal ischemic insult.

Cardioprotection has been shown to be achieved by applying the brief episodes of non-lethal ischemia and reperfusion to an organ or tissue remote from the heart. This phenomenon has been termed remote ischemic 'conditioning'[4,5]. Remote ischemic conditioning can offer widespread systemic protection to other organs which are susceptible to acute ischemia–reperfusion injury such as the brain, liver, intestine or kidney. Ischemic myocardial injury can also be reduced by another mechanism called ischemic post conditioning, which is achieved by interrupting myocardial reperfusion[6].

Although the above mentioned protective strategies are separated by timing of the intervention and the conceived mechanisms of action, all these cardio-protective strategies, may converge on the mitochondrial permeability transition pore (MPTP) which is the main subject of this thesis. This was first described in the late 1970's by the pioneering studies by Haworth and Hunter[7].

The aim of this thesis is to explore the critical role of MPTP in ischemia-reperfusion injury and examine its relation and contribution in the salvation of the human myocardial viability and function.

1.2 Myocardial Ischemia-Reperfusion Injury

Myocardial reperfusion injury was first postulated by Jennings et al in 1960[8]. In their histological description of reperfused ischemic canine myocardium, they reported cell swelling, contracture of myofibrils, disruption of the sarcolemma, and the appearance of intra-mitochondrial calcium phosphate particles. Reperfusion injury to the heart causes four recognised types of cardiac dysfunction i.e. reperfusion induced arrhythmias; myocardial stunning; reversible microvascular obstruction; and cell death[9].

The concept of lethal reperfusion injury as an independent mediator of cardiomyocyte death distinct from ischemic injury is debatable. Some suggest that reperfusion only exacerbates the cellular injury that was sustained during the ischemic period[9]. To show the existence of lethal reperfusion injury as a distinct mediator of cardiomyocyte death, it is important to show that the size of a myocardial infarct can be reduced by an intervention used at the beginning of myocardial reperfusion[10].

The main causes of reperfusion injury are increased intracellular calcium and reactive oxygen species (ROS) which is initiated during ischemia and amplified during reperfusion and targeting the mitochondria[11-13].

1.3 Myocardial Protection by Ischemic Preconditioning

In 1986 Murry et al made a seminal discovery of an endogenous mechanism offering profound protection which they termed ischemic preconditioning[14]. In their seminal paper they showed that four cycles of 5 minutes of ischemia with intermittent reperfusion were shown to limit infarct size by 75%. This cardio-protection is termed as classical or early preconditioning.

IPC has been demonstrated in all animal species studied to date including chicken, dog, mouse, pig, rabbit, rat, and sheep. The preconditioned state is very transient and lasts for only 1-2 hours in anesthetized animals[14,15] and is lost between 2-4 hours in conscious rabbits. Although the initial protection is transient a delayed protection reappears within 24 hours of the preconditioning stimulus and is termed second window of protection (SWOP)[16].

The original end point used as the assessment for protection from the IPC was infarct size and this was expressed as a percentage of the risk zone[3]. Though stunning has been used as an end point of assessment, doubts do exist regarding IPC effect on stunning. Post-ischemic recovery of contractile function is a commonly used end point for IPC[17,18]. IPC has been reported to alter the incidence of reperfusion induced arrhythmias in dogs and rats.

1.4 Preconditioning the Human Myocardium

Human ventricular cardiomyocytes and atrial trabeculae studies have been ingeniously used as a surrogate to study the effects of ischemic preconditioning. The evidence is reviewed below

1.4.1 In Vitro studies

Ikonomidis et al, were the first to demonstrate the effect of preconditioning in human ventricular cardiomyocytes in cell culture and they used trypan blue exclusion and metabolic end points of injury[19]. Arstall et al, showed that in addition to classical preconditioning, human ventricular cardiomyocytes exhibited delayed cardioprotection 24 hours after a short period of simulated ischemia[20].

Our group were the first to investigate the preconditioning phenomenon in the human atrial muscle obtained from the patients undergoing coronary artery bypass surgery[18]. They were able to precondition the human muscle and used post-ischemic recovery of mechanical function as the end point. This has been confirmed by other authors[21]. Ghosh et al, using sections from the right atrium, have demonstrated both early and delayed preconditioning using both simulated ischemia as well as pharmacological means and used leakage of creatine kinase as the index of injury[22].

1.4.2 In Vivo studies

Exercise induced angina

Some patients are able to exercise to the point of angina, take rest and continue further with no more symptoms and this phenomenon is termed as warm-up or first effort angina[16]. The

above phenomenon was initially thought to be mediated by the coronary vasodilatation and recruitment of collateral vessels but some investigations suggests other mechanisms might be involved. Okazaki et al and Williams et al, reported a reduction in the severity of angina and the degree of S-T segment depression during the second period of myocardial ischemia and these changes were not accompanied by the recruitment of collateral vessels and this was evidenced by the similar coronary and great cardiac vein blood flow measurements[23]. Stewart et al and Tomai et al, showed that the duration of the warm-up angina is 1-2 hours after the first period of exercise, a time course that closely parallels classical ischemic preconditioning[24,25]. However studies examining the cellular mechanism have given conflicting results[26,27].

Pre-infarction Angina

Kloner et al, in their retrospective analysis of the TIMI-4 trial, showed that the presence of pre-infarct angina was associated with smaller infarct size and showed lower creatine kinase release, improved left ventricular function with reduced incidence of congestive heart failure and shock and reduced mortality[28]. Patients experiencing angina before acute myocardial infarction (MI) seem to have reduced occurrence of life-threatening arrhythmias[29] and a lower mortality[30,31]. Reports from TIMI-9B investigators[31] and studies by Ishihara et al[30] and Yamagishi et al[32] indicate that angina is only effective if it occurs within 24-72 hours of MI.

Angioplasty studies

Studies have shown that when the first balloon inflation of the coronary artery is between 60-90 seconds, indicators of myocardial ischemia such as chest pain severity, ventricular regional wall motion abnormality, S-T segment elevation, Q-T depression, ventricular ectopic activity, lactate production and release of markers such as Creatinine Kinase MB (CKMB) are attenuated during subsequent balloon inflations and thus providing evidence for myocardial adaptation induced by the first period of inflation[33-35].

Surgical studies

Yellon et al were the first to report that, inducing global ischemia by intermittently cross-clamping the aorta and pacing the heart (2x3 minutes cycle) followed by 2 minutes of reperfusion before a 10 minute period of global ischemia and ventricular fibrillation, ATP levels were better preserved (ventricular biopsy – ATP content) during the subsequent global ischemia period[36]. Myocardial necrosis was also used as end point where serum levels of troponin-T were studied and Jenkins et al showed that patients subjected to the preconditioning protocol suffered significantly less myocardial necrosis[37]. Illes et al showed that preconditioning the human heart just before the cardioplegic arrest resulted in a significant improvement in postoperative cardiac index and reduced inotropic requirement[38]. Isoflurane has been shown to have beneficial effects during coronary artery bypass surgery by Tomai et al[39] and also shown to act as a direct preconditioning stimulus[40].

1.5 Myocardial Protection by Intervening at Reperfusion

Re-establishing coronary blood flow to the ischemic myocardium, following an acute myocardial infarction, can itself result in cardiomyocyte death, a phenomenon termed lethal reperfusion injury[3].

The existence of lethal reperfusion injury as an entity separate from ischemic injury is controversial and some commentators have suggested that reperfusion only exacerbates the cellular injury sustained during the ischemic period[9]. Reperfusion following a fixed period of ischemia can exacerbate the necrotic component of cell death as evidenced by an extension in infarct size as shown by some authors[41].

1.5.1 Protecting the Human Myocardium Against Ischemia-Reperfusion Injury

Glucose-insulin-potassium (GIK) offers cardio-protection in response to ischemia-reperfusion injury and was first introduced in 1962 by Sodi-Pallares and colleagues[42]. In the late 1990's, the Diabetes Mellitus Insulin-Glucose Infusion in Acute Myocardial Infarction (DIGAMI) clinical trial demonstrating a 29% significant reduction in mortality[43]; as well as the ECLA (Estudios

Cardiológicos Latinoamérica) clinical trial which reported a 66% reduction in mortality in diabetic patients reperfused following an acute myocardial infarction[44]. GIK therapy has also been shown to be cardio-protective in the setting of coronary artery bypass graft surgery[45].

Clinical studies with magnesium therapy have produce mixed results with the Second Leicester Intravenous Magnesium Intervention Trial (LIMIT-2) trial[46] reporting a benefit but the larger fourth International Study of Infarct Survival (ISIS-IV) trial[47] reporting no benefit.

The Acute Myocardial Infarction STudy of ADenosine (AMISTAD) demonstrated that the administration of adenosine as an adjunct to thrombolysis resulted in a 67% relative reduction in infarct size in patients with anterior myocardial infarction[48].

Na^+-H^+ exchanger as an adjunct to primary PTCA in patients with an acute myocardial infarction reported positively attenuating CKMB release, improved ejection fraction and improved regional left ventricular wall motion[49]. Subsequent larger study, ESCAMI trial, however reported no attenuation in CKMB release in patients given the Na^+-H^+ exchanger inhibitor, eniporide as an adjunct to reperfusion therapy for an acute myocardial infarction[50]. The GUARDIAN trial[51] reported that cariporide produced a reduction in the combined end-point of death and MI, only in the sub-group of patients awaiting cardiac surgery.

Intravenous diltiazem administered within three hours of acute MI (90 patients) has been shown to have beneficial effects on the infarct size, residual viability and recovery of regional function by Pizzetti et al[52].

The multicenter DIGAMI trial was performed in diabetic patients who were experiencing an acute MI and admitted to the coronary care units (CCUs) of 16 Swedish hospitals between January 1990 and December 1993. 1,240 patients were potentially eligible for the study, but 620 (50%) of the patients were immediately excluded. The remaining 620 patients were then randomized to either a control group (314 patients) or an insulin infusion group (306 patients). The study was to evaluate the effect of aggressive initial insulin therapy continued for 3 months on patients' morbidity and mortality following an acute MI. The modest sample size and lack of positive findings in other endpoints made this finding less robust. The ECLA group reports the largest prospective, randomized trial of GIK for the treatment of AMI ever performed and the only such trial done in the era of thrombolytic therapy. Remarkable 66% reduction in the relative in-hospital mortality risk was observed when GIK was added to

reperfusion (95% of those reperfused had thrombolysis) (252 patients). Survival benefit persisted during a 1-year follow-up period in the group that received high-dose GIK plus reperfusion as AMI treatment. Despite its being the largest prospective randomized trial of GIK to date, the ECLA study is still only 1 trial of GIK, and it is small in comparison with contemporary randomized trials of various thrombolytic agents for AMI. Even though this sub-group was prospectively stratified and represented 62% of the total number of patients (307 patients) studied, a conclusion based on a subgroup result is not as convincing as a result from the entire study population. In LIMIT-2 trial sixty patients out of 260 patients were randomized to magnesium sulphate (8 mmol IV over 15 minutes and 65 mmol over 24 hours) or placebo within 12 hours of clinically diagnosed middle cerebral artery stroke. Thirty percent of magnesium-treated and 40% of placebo-treated patients was dead or disabled (Barthel Index score <60) at 3 months (*P*=0.42). There was a decrease in the number of early deaths in the magnesium-treated group (*P*=0.066, log-rank test). Further trials are required to determine efficacy. The AMISTAD trial was a prospective, open label trial of thrombolysis with randomization to adenosine or placebo in 236 patients within 6 hours of infarction onset. There was a 33% relative reduction in infarct size (p=0.03) with adenosine. There was a significant reduction in infarct size in patients with anterior infarction but no reduction in patients with infarcts located elsewhere and the data supported the need for further clinical trials. ESCAMI trial was an international, prospective, randomized, double blind, placebo-controlled phase 2 trial in patients undergoing thrombolytic therapy or primary angioplasty for acute ST-elevation myocardial infarction where the effect of eniporide on infarct size and clinical outcome was studied. In stage 1, administration of eniporide resulted in smaller infarct sizes especially in the angioplasty group. In contrast, in stage 2 there was no difference in the enzymatic infarct size between the groups. Overall there was no effect of eniporide on clinical outcome and in this large study administration of the NHE-1 inhibitor eniporide, before reperfusion therapy in patients with acute ST elevation MI, did not limit infarct size or improve clinical outcome. A total of 11,590 patients with unstable angina or non–ST-elevation myocardial infarction (MI) or undergoing high-risk percutaneous or surgical revascularization were randomized to receive placebo or 1 of 3 doses of cariporide for the period of risk. The trial failed to document benefit of cariporide over placebo on the primary end point of death or

MI assessed after 36 days. Lower doses had no effect, whereas a high dose was associated with a 10% risk reduction and benefit was limited to patients undergoing bypass surgery. No significant benefit of cariporide could be demonstrated across a wide range of clinical situations of risk. The trial documented safety of the drug and suggested that a high degree of inhibition of the exchanger could prevent cell necrosis in settings of ischemia-reperfusion.

1.6 Myocardial Protection by Ischemic Post-conditioning (IPost)

Studies published in the mid-1980s first established that ischemic myocardial injury could be reduced, if the myocardial reperfusion process was modified to a staged or gradual form of myocardial reperfusion i.e. selective low-pressure (40 to 50 mm Hg) coronary reperfusion with normal blood for 20 minutes at 30 ml/min before the occlusion was relieved completely[6]. In 2003 Vinten-Johansen et al[53], by interrupting myocardial reperfusion with three cycles of 30 s coronary artery re-occlusions, were able to show several beneficial effects including a 44% reduction in infarct size, less myocardial oedema, less neutrophil accumulation, reduced apoptotic cell death and improved endothelial function.

The ability of IPost, an intervention that can be applied at the onset of myocardial reperfusion to reduce myocardial injury, also provided confirmatory evidence for the existence of lethal myocardial injury as an independent mediator of cardiomyocyte death. IPost has been shown to target many of the properties of lethal reperfusion injury such as oxidative stress, calcium accumulation, inflammation, and mitochondrial permeability transition pore (MPTP) opening[54,55]. The mechanism underlying IPost has been intensively investigated, and that a signal transduction pathway similar to that recruited by IPC mediating the protection has been elicited by post-conditioning. Our group recently discovered common signaling pathways that are shared by both IPC and Ipost[56-58]. In this respect a variety of diverse pharmacological post-conditioning agents including inhalational anesthetics, G-protein coupled receptor ligands such as opioids, adenosine and bradykinin, growth factors such as insulin and erythropoietin, natriuretic peptides, adipocytokines, and ‘statins’ have been linked to the activation of the reperfusion injury salvage kinase (RISK) pathway, a critical component

of this signaling pathway[59]. Ipost can be applied as a cardioprotective strategy in patients presenting with acute myocardial infarction.

1.6.1 Ischemic post-conditioning in clinical arena

For patients presenting with an acute myocardial infarction (AMI), IPost can be clinically applied at the time of myocardial reperfusion as it can for example in those receiving percutaneous intervention (PCI). Studies reporting the clinical application of IPost in patients presenting with an AMI were published in 2005[60,61]. Following PCI and stent deployment in the infarct-related coronary artery, myocardial reperfusion was interrupted with four-1 min low pressure inflations and deflations of the coronary angioplasty balloon and this improved myocardial reperfusion, reduced myocardial infarct size both acutely and at 6 months, and improved left ventricular ejection fraction at 1 year[60,61]. Jacob Lønborg et al showed a tendency toward a better outcome with IPost, by occluding the coronary artery with a balloon under low pressure 4 times with each occlusion lasting 30 seconds followed by 30 seconds of reperfusion suggesting that IPost is beneficial in achieving complete ST-segment elevation (STR), improving New York Heart Association class, reducing infarct size and a better ejection fraction at 15 months[62]. The beneficial effect of Ipost in terms of improved left ventricular ejection fraction and myocardial perfusion grade were shown by Garcia et al even after three years of intervention[63].

Studies like Post-conditioning in ST-Elevation Myocardial Infarction (POSTEMI) study are under way. POSTEMI is a prospective, randomized, open-label clinical trial with blinded endpoints on final infarct size, TIMI myocardial perfusion grade, resolution of ST-segment elevation, release of markers of ischemia and left ventricular function[64].

Recent clinical studies have been investigated regarding the potential of Ischemic post-conditioning (Ipost) in patients undergoing elective cardiac surgery[65]. However, like IPC, this particular cardioprotective strategy requires an invasive protocol of aortic clamping and de-clamping. In children undergoing repair for Tetralogy of Fallot (congenital condition), Luo et al applied the post-conditioning protocol during the time of de-clamping after the operation. This comprised re-clamping the aorta for 30 s and de-clamping it for 30 s, twice just before

de-clamping the aorta after the finish of the operation. They have been shown to reduce myocardial injury as evidenced by less peri-operative troponin-T and CK-MB release and smaller inotrope requirements post-surgery[65]. A similar protocol has also been demonstrated to reduce myocardial injury in adult patients undergoing valve surgery and in children undergoing corrective surgery for congenital heart disease using cardioplegia[66]. Wanjun Luo et al showed a reduction in creatine kinase-MB and troponin I, in children scheduled for surgical correction of congenital ventricular septal defect, by post-conditioning the heart which consisted of 30 seconds of ischemia and 30 seconds of reperfusion achieved by clamping and unclamping the aorta, repeated three times over 3 minutes immediately after cardioplegic arrest[67]. Clinical application of this could be restricted because of the invasive nature of this interventional treatment strategy and the inherent risks of thromboembolism associated with aortic clamping and de-clamping.

1.6.2 Pharmacological post-conditioning

Pharmacological post-conditioning means that a variety of pharmacological agents can be used to reduce myocardial infarct size when administered at the point of myocardial reperfusion. Many of these cardioprotective pharmacological agents exert powerful cardioprotection by targeting the reperfusion injury salvage kinase (RISK) pathway at the onset of myocardial reperfusion[59] and by targeting MPTP, a possible end target for protecting the heart at the time of myocardial reperfusion[68].

Several clinical studies have reported beneficial effects using drugs such as adenosine[69] GLP-1[70,71] and atrial natriuretic peptide[72] acting on the RISK pathway or cyclosporine-A[73] which is known to inhibit MPTP opening. Two recent meta-analyses have reported the potential benefits of inhalation anesthetics in the setting of cardiac surgery and demonstrated better LV function, less troponin release, less inotrope use, shorter ventilation time and shorter hospital stay[74,75].

1.7 Myocardial Protection by remote Ischemic conditioning (RIC)

By applying brief episodes of non-lethal ischemia and reperfusion to an organ or tissue remote from the heart it has been shown that cardioprotection can be achieved. This phenomenon has been termed remote ischemic 'conditioning'. By applying remote ischemic conditioning widespread systemic protection can be offered to other organs, which are susceptible to acute ischemia–reperfusion injury such as the brain, liver, intestine or kidney. In 1993, Przyklenk et al[76] showed that by applying short episodes of myocardial ischemia and reperfusion to the left anterior descending coronary territory, they were able to subsequently reduce the size of a myocardial infarct generated in the circumflex coronary artery territory and this phenomenon was termed 'regional ischemic preconditioning'.

Subsequently came the exciting 'remote ischemic preconditioning' (RIPC), where the preconditioning protocol could be applied to organs and tissues distant or remote from the heart such as the kidney or small intestine[77,78]. The underlying mechanism remains unclear though many of the signaling pathways underlying myocardial preconditioning and post-conditioning have been implicated in RIPC. It has been proposed that substances released from the preconditioned organ or tissue such as adenosine, bradykinin or opioids stimulate local afferent nerves pathways, which then activate efferent nerve pathways, which then terminate on the myocardium. Another suggestion is that a substance or humoral factor is carried in the blood stream to the heart where it manifests its protective effect.

Kerendi et al's experimental studies suggest that remote ischemic conditioning can be effective even if applied after the onset of myocardial ischemia—a phenomenon which has been termed remote IPost[79]. Using an anaesthetized rat, they demonstrated that applying a 5 min episode of renal ischemia followed by 5 min of renal reperfusion after the onset of myocardial ischemia and immediately before myocardial reperfusion was capable of reducing myocardial infarct size, through a mechanism which required binding to an adenosine receptor[79].

Subsequent experimental studies have confirmed the presence of RIPost as a viable cardioprotective intervention in the rabbit[80] and porcine hearts[81] using limb ischemia as the RIPost stimulus.

Schmidt et al showed a reduction in myocardial infarction size by applying short periods of limb ischemia during the time of well established myocardial ischemia and called it as “remote perconditioning”[82]. Recently Loukogeorgakis et al have reported improvement in endothelial function in patients with stable coronary artery disease, using upper limb ischemia as the remote post-conditioning stimulus and contra lateral upper limb endothelial function as the indicator of protection[4]. Very recently our group has reported a decrease in the incidence of Acute Kidney Injury (AKI) in non-diabetic patients undergoing elective CABG surgery in a retrospective analysis where RIPC consisted of three 5-minute cycles of right forearm ischemia, induced by inflating a blood pressure cuff on the upper arm to 200 mm Hg, with an intervening 5 minutes of reperfusion[83].

1.7.1 Remote ischemic preconditioning in clinical arena

By applying electrical stimulation to the lower limb skeletal muscle of rabbits[84] Birbaum et al found that remote ischemic preconditioning could be elicited and this was pivotal in terms of the clinical application of this cardioprotective phenomenon. Though in a small clinical study comprising only eight patients undergoing CABG surgery, RIPC using lower limb ischemia was found not to be cardioprotective[85].

Pioneering work by MacAllister and colleagues characterized a non-invasive RIPC protocol in human volunteers which involved an automated cuff placed on the upper arm to induce brief episodes of forearm ischemia and reperfusion and endothelial function was measured as the end-point[86]. Subsequent clinical studies by our laboratory and others have demonstrated reduced myocardial injury in terms of cardiac enzyme release in the settings of both cardiac surgery[87].

A small clinical study examined the role of RIPC in patients undergoing low-risk elective PCI and paradoxically, they reported myocardial injury to be increased[88]. However, a larger and robust clinical study with 242 patients undergoing elective PCI, elicited a reduction in troponin-I in patients receiving the RIPC protocol[89].

A recent meta-analysis comprising of 22 clinical trials and 933 patients[90] reported fewer ventricular arrhythmias, smaller inotrope requirements and shorter stays on the intensive care unit in patients treated with RIPC.

1.8 The Mitochondrial Permeability Transition Pore (MPTP)

The mitochondrial permeability transition pore (MPTP) is a voltage dependent proteinaceous pore of the inner mitochondrial membrane (IMM) and on opening mediates the mitochondrial permeability transition (MPT). This describes the abrupt increase in permeability of the inner mitochondrial membrane which takes place in response to various inducing factors like high matrix calcium, oxidative stress, high phosphate and low adenine nucleotides[91-93]. The idea of an actual inner mitochondrial membrane proteinaceous pore (MPTP) was first described in the late 1970's by Haworth and Hunter[91-93] as a "Ca^{2+}-Induced Membrane Transition in Mitochondria.

Opening of MPTP allows free passage of molecules <1.5 kDa disrupting the permeability barrier of the IMM. This allows the free movement of proton across the IMM causing uncoupling of oxidative phosphorilation and eventually hydrolysis of ATP. The mitochondrial pores are either fully open or closed and once they are fully opened the mitochondria swell extensively[94].

1.8.1 Characterisation of the Mitochondrial Permeability Transition Pore

After nearly two decades of studies on mitochondria, the following various phenomena were discovered separately and considered as independent events: Swelling of mitochondria in isotonic medium in the presence of calcium causing – uncoupling, inhibition of ATPase activity, swelling and inhibition of respiration, as well as protection by EGTA or phosphate against uncoupling and swelling and loss of nucleotides, Krebs cycle intermediates and Coenzyme A. Later it was recognised that the above phenomenon were manifestations of the same process that is MPTP[95].

By 1980, Haworth and Hunter characterised many of the key features of the MPTP [7,91-93]. According to them the MPTP was unselectively permeable to H^+, Ca^{2+} Mg^{2+}, choline, glucose, sucrose, and NAD(P)H and that the MPTP opening was a reversible process. On determining the size of the pore they used dextrans of different molecular weight and postulated the molecular cut-off to be <1500 Da[91]. Subsequent studies by Crompton's group examining the permeability of the MPTP to [^{14}C] solute fluxes, calculated that the MPTP had a diameter of 2.0-2.6 nm[96,97]. They proposed that the adenine nucleotide translocase (ANT) might be part of the MPTP based on the finding that the MPTP could be opened by atractyloside and closed by bongekrekic acid both known to act on the ANT, and that MPTP opening was sensitive to ADP[7,91-93]. They also postulated that the components of MPTP might form at contact sites between the inner and outer mitochondrial membranes.

A number of inducers and inhibitors of MPTP opening have been described and the following provides the important known inducers and inhibitors:

1.8.1.1 Inducers of MPTP Opening

1.8.1.1.a Calcium and Phosphate

The rise in intracellular Na^+ is due to he increased generation of protons during ischemia, via Na^+ - H^+ exchanger (NHE)[98] and also due to Na^+ entering the cell during ischemia[99]. This results in the reversal of Na^+ - Ca^{2+} exchanger (NCX) leading to the rise in cytosolic Ca^{2+}[100]. Studies have shown that a rise in cytosolic free calcium concentration ($[Ca^{2+}]_i$) preceded irreversible myocardial injury and that drugs that reduced or delayed this rise in cytosolic $[Ca^{2+}]_i$ also reduced or delayed myocardial death[98,100-102]. Though some studies have reported that a rise in cytosolic calcium was not important[103], the majority of recent studies have shown that a rise in $[Ca^{2+}]_i$ precedes irreversible injury and that blockage of this rise in $[Ca^{2+}]_i$ can attenuate or delay the onset of irreversible injury[101,104,105].

MPTP is triggered by calcium overload especially when this is accompanied by oxidative stress and these two parameters appear critical determinants of the recovery of isolated heart cells from simulated ischemia and is believed that calcium induces MPTP opening by binding to two activating sites on the matrix side of the pore[91,106]. Ca^{2+}-induced

MPTP opening can be facilitated in the presence of MPTP inducers such as P_i, a weak acid that allows the accumulation of enormous amount of mitochondrial Ca^{2+} up-take by providing a mechanism for reducing matrix alkalinisation. P_i must enter the matrix to exert its effect, which might be due to its ability to buffer the matrix pH or by causing a reduction in the matrix ADP. In fact the extent of recovery of the cell has been reported to correlate inversely with mitochondrial matrix calcium concentrations at the end of ischemia[107,108] and to be triggered by the rise in ROS during reperfusion[103]. However, more recent data from Bernardi's laboratory has suggested that Pi actually inhibits (rather than activates) pore opening. They also suggested that this effect is overcome by the presence of CyP-D, accounting for the protective effects of CsA in the presence of Pi and hence argued against a role for the PiC in the MPTP. These authors assayed the calcium retention capacity (CRC) of energised liver mitochondria from wild-type and CyP-D knockout mice[109,110].

1.8.1.1.b Oxidative Stress

The role of reactive oxygen species (ROS) in ischemia-reperfusion injury has been extensively studied and ROS has been measured using electron spin resonance (ESR)[111,112] and by using fluorescent indicators[113,114]. During reperfusion, with the return of oxygen, a large burst of ROS generation have been consistently demonstrated by several studies[114,115]. This increase in ROS during ischemia and reoxygenation is thought to be due to damage to electron transport chain components resulting in inefficient transfer of electrons, generating superoxide. Kim et al, have reported that ROS generated during early reperfusion is the primary activator of the MPTP and cardiomyocyte death[103].

Mitochondria are thought to be both a major source of ROS as well as a major target for ROS damage. Mitochondrial electron transport is one of the primary sources of ROS production in the cell and ROS are generated during electron transport at complexes I and III. The low levels of ROS that are generated during ischemia can damage the electron transport chain[116]. Inhibition of electron transport (particularly complex I) reduces ROS generation during ischemia and mitochondrial uncouplers have been shown to reduce ischemia-reperfusion injury[116].

Opening of the MPTP can be induced by: (1) Endogenous ROS - Reactive oxygen species (ROS) generated by the mitochondrial electron transport chain, xanthine oxidase, and NADPH oxidase; (2) Exogenous sources of ROS; and (3) Oxidising agents such as t-butylhydroperoxide. Oxidative stress induces MPTP opening by oxidising critical thiol groups of the inner mitochondrial membrane (IMM) to form thiol cross-linkage. Thiol agents such as phenylarsenine oxide, hydroperoxides and diamide induce MPTP opening by cross-linking inner membrane thiol groups. Halestraps' group have postulated that these agents induce MPTP opening by cross-linking Cys160 and Cys257 of the ANT[117].

1.8.1.1.c *Thiol Reagents*

Bernardi's group confirmed the contribution of thiol groups to MPTP opening, showing both the protective (at low concentration) and the inducing (at higher concentration) effects of N-ethylmaleimide (NEM)[118].

1.8.1.1.d Fatty Acids

Fatty acids diffuse into the mitochondria as the protonated electroneutral form and act as classical uncouplers, inducing MPTP opening by decreasing the transmembrane potential[119]. Petronilli et al also showed a possible direct interaction of fatty acids with the MPTP[120].

1.8.1.1.e Adenine Nucleotide Translocator Ligands

ANT ligands can act either as MPTP inducers or inhibitors. Atractyloside and carboxyatractyloside, which stabilise the ANT in the 'c' conformation (with the adenine nucleotide binding site facing the cytosol) favour MPTP opening[121] and bongkrekate, which stabilise the ANT in the 'm' conformation (with the adenine nucleotide binding site facing the matrix) inhibit MPTP opening[91].

1.8.1.1.f Acyl-CoA

The effect of acetyl-CoA on MPTP opening is reported to depend on their side of attachment to ANT, that is by acting either from the outer or inner side of the membrane[122]. Bernardi et al

proposed an alternative explanation for the acyl-CoA's action and accounted for by the variations in membrane surface potential[94].

1.8.1.1.g Arachidonic acid

In isolated mitochondria arachidonic acid stimulates MPTP opening and in intact cell it causes cell death due to intracellular calcium overload, most likely reflecting mPTP opening 121[123].

1.8.1.2 Inhibitors of MPTP Opening

1.8.1.2.a Cyclosporin-A

Following the lead by Fournier and colleagues in late 1980s[124], Crompton's group[125] and Pfeiffer's groups[126] reported that cyclosporine-A (CsA), the immunosuppressive agent, strongly prevented the opening of MPTP at sub-micro molar concentrations by inhibiting mitochondrial Ca^{2+} efflux and allowing mitochondria to accumulate large amounts of Ca^{2+}. Nazareth et al[127] demonstrated protection with CsA using an isolated cardiac myocyte model of anoxia and reoxygenation, and, subsequently, this has been confirmed by others[128,129]. The protective effect of CsA in the Langendorff-perfused heart model of reperfusion Injury was shown by Halestrap's group[130,131]. In the presence of CsA, the recovery of haemodynamic function during reperfusion was greatly improved, as reflected by higher left ventricular developed pressure (LVDP) and lower end diastolic pressure (EDP), and reduced release of intracellular lactate dehydrogenase. Using a chromatography affinity binding column, Halestrap's group[132] demonstrated that a fusion protein between cyclophilin D and glutathione-*S*-transferase (GST) bound to ANT in response to the thiol, diamide, and the binding was inhibited by CsA, suggesting that CsA inhibited MPTP opening by preventing the binding of cyclophilin D to ANT[132]. The effect of CsA on the MPTP is described as 'desensitization' by Bernardis group and the inhibitory effect of CsA can be overcome by increasing Ca^{2+} load[94,131,133]. Lemaster's group proposed the existence of an unregulated CsA-insensitive form of MPTP opening[134].

Recently in a proof-of-concept clinical study, a single bolus of cyclosporine-A (CsA) administered to patients presenting with an acute MI just prior to primary coronary

angioplasty, was reported to reduce myocardial infarct size by 30–40%[135]. Of interest, the recent studies using an *in vivo* porcine model of ischemia reperfusion injury have produced mixed results where the cardioprotective effect of isoflurane is abolished by the combined usage of isoflurane and CsA[136]. Following cardioplegic arrest in a neonatal rabbit model, Leung et al by demonstrating CsA-sensitive MPTP opening at the time of myocardial reperfusion suggested that application of CsA or another MPTP inhibitor may be beneficial in terms of reducing perioperative myocardial injury in neonates undergoing corrective cardiac surgery[137]. Cour and colleagues using a rabbit model found improved survival, reduced myocardial necrosis and inhibited MPTP opening in isolated cardiac mitochondria following administering either intravenous CsA (5 mg·kg-1) or N-methyl-4- isoleucine cyclosporin (NIM811) (2.5 mg·kg-1) at the time of reperfusion following a 15 min period of primary asphyxia *in situ* cardiac arrest[138].

CsA, as well as inhibiting MPTP opening, can inhibit the protein phosphatase *calcineurin* through its binding to cyclophilin A. This has resulted in the use of CsA analogues such as N-methyl 4-valine CsA and N-methyl-4-iosleucine-CsA (NIM811), which inhibit MPTP opening without inhibiting calcineurin. A new MPTP inhibitor called sanglifehrin-A has been described which also inhibits MPTP opening without inhibiting calcineurin[139].

1.8.1.2.b Sanglifehrin-A

Using the langendorff-perfused heart model, Halestrap's group have characterised a newer MPTP inhibitor called sanglifehrin[139,140], an immunosuppressant which differs from CsA in that it does not inhibit calcineurin[141,142]. The mechanism of MPTP inhibition by SfA also differed from that of CsA. SfA was demonstrated to bind to and inhibit the PPIase activity of cyclophilin D (K_i of about 2.2 nmol/l) but unlike CsA, SfA did not prevent the binding of cyclophilin D with ANT[139]. The concentration-dependence of SfA's inhibition of the MPTP is sigmoidal compared to the linear dependence of CsA. Using the isolated perfused rat heart Halestrap's group demonstrated that inhibiting MPTP opening using SfA (at 1.0 µmol/l) protected the heart against ischemia-reperfusion injury. They demonstrated using above experiment an attenuated LDH release and improved recovery of left ventricular contractile function[139].

Limitation in the use of CsA and SfA as inhibitors of the MPTP is that both CsA and SfA fail to inhibit MPTP opening when mitochondria are exposed to a sufficiently strong stimulus[139,143] such as elevated matrix calcium, oxidative stress, and adenine nucleotide depletion that accompany reperfusion after a period of ischemia[11,144].

1.8.1.2.c Bongkrekic acid and ubiquinone derivatives

It has been demonstrated that bongkrekic acid (BKA) is a potent inhibitor of the MPTP in isolated mitochondria and also been reported of its use to inhibit pore opening in cultured cells[145,146] including cardiac myocytes[147,148]. Bongkrekic acid acts by inhibiting the export of ATP from the mitochondria to the cytosol and hence its action has been questioned.

Bernardi's group have demonstrated that substrates which increase electron flow through the complex I of the electron transport chain compared to complexes II and IV, sensitise the MPTP to Ca^{2+}, and therefore ubiquinone analogues which act to inhibit electron flow through complex I can inhibit MPTP opening.

1.8.1.2.d Divalent and trivalent Cations

Divalent cations such as Mg^{2+}, Sr^{2+}, Mn^{2+}, Ba^{2+} can inhibit MPTP opening by competing with Ca^{2+} for its activating site on the MPTP and Sr^{2+} and Mn^{2+} have been shown to exert an inhibitory effect at a matrix-side site. Alternatively, these ions may inhibit MPTP opening by binding to an alternative site on the cytosolic side of the MPTP[149].

1.8.1.2.e Pyruvate and Propofol

Reducing oxidative stress by the use of free radical scavengers has been shown to offer some protection against reperfusion injury[150]. Radical scavengers may directly inhibit the opening of the MPTP by preventing oxidative cross linking of critical cysteines on the ANT[146] or by indirect effect on the MPTP opening, as oxidative stress is known to inhibit plasma membrane ion pumps, leading to perturbation of ionic homeostasis and calcium overload.

Halestrap's group have investigated the effects of two free radical scavengers on MPTP opening, namely pyruvate[151] and the anesthetic propofol[152] and showed improved functional recovery of hearts during reperfusion. They also demonstrated that all the ''open''

mitochondria subsequently resealed as the reperfusion continued[151]. The ability of pyruvate to protect hearts and other tissues may be mediated by its free radical scavenging activity[153]. Also unlike glucose or glycogen, pyruvate does not require ATP for activation before its metabolism. Cross et al, using nuclear magnetic resonance (NMR) studies, showed that pyruvate can lead to a decrease in pH[154] and hence inhibit MPTP. Propofol has been shown to protect the heart against reperfusion injury, in a langendorff-perfused rat heart model,[155] and also against damage caused by H_2O_2-induced oxidative stress[156]. Halestraps group using the 3H-DOG technique has confirmed that the protection offered by propofol is accompanied by less opening of the MPTP[152]. Moreover to match the clinical practice, Halestrap's group used an in vivo pig model of cardiopulmonary bypass with warm blood cardioplegia, and showed that propofol could improve functional recovery of the heart, reduce troponin-I release, and maintain higher tissue ATP levels[157].

1.8.1.2.f Local anaesthetics and Phenothiazines

The phenothiazine, trifluoperazine was originally thought to inhibit MPTP opening by competing with Ca^{2+} for its binding site on the MPTP[149]. However, it also has many non-specific effects, which include inhibition of calmodulin, and it also inhibits the F_1F_0ATPase. Later studies have suggested that it may modify the voltage sensitivity of the MPTP[158]. Local anesthetics agents bind to phospholipid Ca^{2+} binding sites on biological membranes, displacing Ca^{2+} in a competitive manner.

1.8.1.2.g Adenine Nucleotides

The evidence of the involvement of the ANT in MPTP formation comes from the effects of oxidative stress and thiol reagents to enhance MPTP opening by acting both by decreasing adenine nucleotide binding and inhibition of MPTP opening and by increasing CyP-D binding[159]. Halestrap's group have showed that inhibition of MPTP opening by high membrane potential works by increasing matrix adenine nucleotide binding to the ANT[159]. The binding of ATP (to the cytosolic side of the ANT) and the binding of ADP (to the matrix side of the ANT) inhibits MPTP opening. Binding of ADP competes for the Ca^{2+}-activating site on the

MPTP[91]. Novgorodov et al confirmed that the affinity of calcium for the activating site was decreased by ADP[106].

1.8.1.2.h Oligomycin

The protective effect of oligomycin could be due to either an increase in matrix ADP content[160] or an increase in the transmembrane potential attributed to an inhibition of the proton leaks through the ATP synthetase[161].

1.8.2 The Molecular Identification of the MPTP

The all-or-nothing character of the MPTP convinced Hunter and Haworth that the permeability transition resulted not from the degenerative process but from the opening of a bona-fide pore MPTP[91-93]. Following their work, Crompton and co-workers showed that the permeabilisation process could be immediately interrupted by EGTA[162-164]. Crompton et al proposed that the pore of each mitochondrion opened or closed with high degree of synchronisation[96]. Definitive evidence for the involvement of a bona-fide pore in the permeabilization process came from electrophysiological patch-clamp studies in 1989 where a large 1.3 nS (in 150 mmol/l KCl) multi-state channel in the inner mitochondrial membrane was identified[165] as MPTP. It was estimated that the MPTP diameter would be 2-3 nm. The actual molecular composition of the MPTP is currently unknown[166] though several models have been proposed.

1.8.2.1 ***Possible Components of the MPTP***

1.8.2.1.a Adenine Nucleotide Translocase

In 1979, Haworth and Hunter first proposed that the ANT might be part of the MPTP, based on their findings that MPTP opening was sensitive to ADP, and to the ANT inhibitors atractyloside and ANT in MPTP formation and proposed that MPTP inducers such as atractyloside, carboxyatractyloside, a high NADP/NADPH ratio, and fatty acids stabilised the ‘c’ conformation (where the nucleotide-binding site is on the cytosolic side) of the ANT and MPTP inhibitors such as bongkrekic acid and matrix ADP stabilised the ‘m’ conformation

(where the nucleotide-binding site is on the matrix side)[167]. Halestrap and co-workers proposed that ANT adopts an open pore conformation upon binding with Calcium and cyclophilin and that binding of both Calcium and cyclophilin is required for MPTP opening. They suggested that the ANT was the sesitisator of MPTP to calcium by oxidative stress and the vicinal thiol reagent phenylsarsine oxide (PAO) [159]. Halestrap's group first proposed and have subsequently demonstrated that cyclophilin D induces the conformational change in ANT to form the MPTP in a CsA sensitive manner[117,131,168].

However the requirement of ANT as an essential component has been seriously questioned by the results obtained from mice lacking ANT[169]. They found that CsA inhibition of MPTP in mice lacking ANT could still be detected but it required a larger Ca^{2+} load. Therefore they proposed that ANT might play a regulatory role and is not essential to be a component of MPTP and also ANT is not the receptor for cyclophylin D[169]. They also found that MPTP in ANT-null mitochondria was not sensitive to opening by atractyloside and to closure by ADP. One of the possible explanation for this, is that ANT could be the normal membrane component of MPTP but the other less abundant members of the mitochondrial carrier family can fulfil this role in the absence of ANT[170]. LeMasters group proposed an alternative possibility in that ANT is the most abundant proteins in the unfolded proteins that form the MPTP[134]. Also Dejean et al showed that the up-regulation of the uncoupling protein-3 induced sensitization of the MPTP to calcium, suggesting that inner membrane transporters other than the ANT may also regulate mitochondrial permeability transition[171].

1.8.2.1.b Cyclophilin D

It has been generally accepted that cyclophilin D, a peptidyl-prolyl cis-trans isomerase, facilitates the calcium triggered change in conformation of an inner mitochondria membrane component[172]. Crompton et al were the first to suggest the role of CyP-D in the interaction of MPTP as they discovered that cyclosporin A acted as a potent inhibitor of pore opening[125]**.** Different CsA analogues were also shown to inhibit MPTP opening, thereby correlating their ability to inhibit the peptidyl prolyl cis-trans isomerase activity within the matrix[168,173] and this was subsequently identified as CyP-D[174,175]. Using CyP-D knockout mice, matrix peptidyl-prolyl cis-trans isomerase activity of cyclophilin D was subsequently confirmed using CsA and

SfA to inhibit MPTP opening by some authors[176,177]. Mitochondria have been demonstrated to contain a CsA-sensitive PPIase, which has been purified and cloned[176,177]. Lemasters et al proposed a chaperone like property to CyP-D and proposed that MPTP would be formed by clusters of misfolding proteins in a process prevented by CyP-D[134].

Recently our group reported that the CYP-D component of the MPTP is critical to IPC protection as it is required to generate mitochondrial ROS, which in turn activates the important mediatory protein kinases, Akt, and Erk1/2[178]. Murphy's group showed that Cys-203 residue of CyP-D is necessary for redox stress-induced activation of MPTP using mouse embryonic fibroblasts[179].

1.8.2.1.c Voltage-Dependent Anion Channel

In 1994 Zoratti et al[180], proposed that the Voltage activated anion channel (VDAC, also known as porin) might be involved in the formation of the MPTP. The MPTP has been proposed to form at contact sites between the inner mitochondrial membrane (IMM) and outer mitochondrial membrane (OMM)[181] and VDAC has been shown to form at these contact sites[182,183]. In 1998 Crompton et al showed the involvement of VDAC in MPTP formation[184] by suggesting that CyP-D can bind strongly to both VDAC and ANT. This cyclophilin D-VDAC-ANT complex was incorporated into liposomes and demonstrated MPTP activity in response to Ca^{2+} and P_i which was blocked by CsA[184].

Bernardi's group in their initial report suggested that VDAC might be the locus for inhibition by ubiquinone analogues[185], however recently the liver mitochondria from mice lacking VDAC1 exhibited normal MPTP opening and inhibition by ubiquinone analogues[186] suggesting that VDAC1 could be unlikely to be a component of MPTP. Moreover Baines's experiment have raised doubts regarding VDACs involvement in MPTP, as the three VDAC isoforms knockouts continue to express MPTP activity[187]. However, Marco Colombini has demonstrated that closure of VDAC actually increases Ca2+ flux[188] which would suggest that closure of VDAC should actually promote opening of the MPTP. This was supported by Tikunov et al who utilized G3139, a phosphorothioate blocker of VDAC, to cause VDAC closure and opening of MPTP[189]. However VDAC continues to be purported as part of the

MPTP mechanism in normal cells and were even proposed to be directly responsible for cytochrome c release[190-193].

1.8.2.1.d The mitochondrial phosphate carrier (PiC)

Halestraps group have recently proposed a model for the MPTP in which the pore forming component is the PiC rather than the ANT[194]. PiC can be inhibited by ubiquinone analogues and by N-ethylmaleimide (NEM), which is a potent inhibitor of MPTP opening at low concentrations. PiC undergoes a calcium-triggered conformational change which is facilitated by the peptidyl–prolyl cis–trans isomerase activity of CyP-D and this allows for MPTP opening even in the absence of CyP-D or the presence of CsA but at a much higher calcium load that was observed[131,195-197]. This calcium induced conformational change might be enhanced by an interaction of the PiC with the "c" but not the "m" conformation of the ANT[177,178,194]. Kokoszka et al have demonstrated pore opening in mitochondria containing no ANT1 or ANT2, but with reduced sensitivity to calcium and no sensitivity to ligands of the ANT[169]. Further evidence comes from Krämer and colleagues, where they demonstrated that the PiC of yeast mitochondria can be converted into a non-specific anion channel[198].

However, more recent data from Bernardi's laboratory has suggested that Pi actually inhibits (rather than activates) pore opening. They also suggested that this effect is overcome by the presence of CyP-D, accounting for the protective effects of CsA in the presence of Pi and hence argued against a role for the PiC in the MPTP. These authors assayed the calcium retention capacity (CRC) of energised liver mitochondria from wild-type and CyP-D knockout mice[109,199,200].

1.8.3 The MPTP opens during reperfusion and not during ischemia

Halestarp's group in 1995, demonstrated that the MPTP was kept firmly closed in the heart under normal physiological conditions but opened upon reperfusion following a period of ischemia[143]. They used a technique that involved the entrapment of [^{3}H]-2-deoxyglucose in the mitochondria that has undergone transition[143]. Our group, by measuring the MPTP opening using fluorescent microscopy in isolated cardiac myocytes, showed that the MPTP opens upon reperfusion in a setting of ischemia and reperfusion[201]. Opening of MPTP at

reperfusion is also supported by Lemaster's group[103] and they demonstrated MPTP opening occurring at the time of reoxygenation, in hepatocytes subjected to periods of simulated ischemia. In this model MPTP opening was indicated by the entry of the membrane-impermeant fluorescent dye calcein and mitochondrial membrane depolarisation[202]. Di Lisa's group[203] demonstrated the same finding using the CsA-sensitive loss of mitochondrial NAD+, that occurred during the first few minutes of reperfusion to indicate MPTP opening.

Very recently Duchen et al devised an approach that delivers Ca^{2+} directly to the matrix of mitochondria independently of uptake and therefore independently of potential ($\Delta\psi m$) and this allows direct study both of the Ca^{2+} efflux pathway and of the specific sensitivity of MPTP to Ca^{2+} in intact and in permeabilized cells[204]. Duchen et al achieved this using the photolytic release of Ca^{2+} by flash photolysis of caged Ca^{2+} using compounds, such as o-nitrophenyl EGTA, introduced into the cell as the acetoxymethyl (AM) ester (NP-EGTA, AM)[204].

During ischemia the low pH (<7), a powerful inhibitor of MPTP, keeps the pore closed and the return of pH to normal during reperfusion induces pore opening[205]. It is the low pH accompanying ischemia that prevents the MPTP opening despite the conditions where MPTP inducers are present[151]. Hence, maintaining a low pH during the initial phase of reperfusion following an ischemia is shown to protect the heart from reperfusion injury[206].

During ischemia, the rise in cytosolic Ca^{2+} begins when about two-thirds of cellular ATP has been depleted and this rise in cytosolic Ca^{2+} results in mitochondrial Ca^{2+} loading and MPTP opening, suggesting that the mitochondrial [Ca^{2+}] is a critical determinant of MPTP opening and cell death[207]. During reperfusion the rapid energisation of the mitochondria will lead to electrogenic calcium uptake into the mitochondria from the cytosol inducing MPTP opening[12]. However, experiments carried out in isolated cardiac myocytes showed that it is the mitochondrial calcium that was responsible for MPTP opening[108]. Furthermore the surge of ROS production during reperfusion is one of the most important factors determining the outcome of reperfusion.

Recently our group demonstrated that the MPTP can reversibly open in adult cardiomyocytes under basal conditions, and that this form of MPTP opening is augmented by hypoxic preconditioning[178].

1.8.4 Consequences of MPTP Opening

Along with increased matrix swelling MPTP opening results in major modifications of mitochondrial function and structure that ultimately affect the maintenance of the cell viability. The immediate effect of the MPTP opening is the collapse of the mitochondrial membrane potential and opening of MPTP results in both apoptotic and necrotic cell death.

1.8.4.1 MPTP opening and Necrotic Cell Death

Once the pore opens it allows free passage of protons across the inner membrane leading to a dissipation of the membrane potential and pH gradient comprising the proton motive force. Maintaining the relative impermeability of the inner mitochondrial membrane is critical to ATP production, as it permits the generation of this electro-chemical gradient - the chemiosmotic hypothesis first described by Mitchell & Moyle[208]. Therefore, the abrupt permeation of the IMM allows entry of protons into the mitochondrial matrix, which dissipates the proton-mediated mitochondrial membrane potential. This halts ATP production and results in ATP hydrolysis as the F_0F_1-ATPase complex (reverse operation) breaks down ATP in an attempt to maintain the mitochondrial membrane potential leading to ATP depletion[209,210].

When the tissue ATP levels become severely compromised and, left unchecked, these will lead to major perturbations in the ionic and metabolic homeostasis of the cell leading to necrotic cell death through the activation of phospholipases, nucleases and proteases[12,166,210]. Opening of the MPTP is associated with loss of matrix Ca^{2+}, depletion of reduced glutathione, depletion of NADPH, and the hypergeneration of superoxide anion. More recent data have begun to suggest that necrosis may be ‘‘programmed’’ and is not a default ‘‘accidental’’ pathway[211-213].

The importance of the MPTP in the necrotic death using MPTP inhibitors such as cyclosporin A and sanglifehrin A have been demonstrated in the heart, brain and liver[130,139,202,214]. More recently, it has been shown that CyP D dependent MPTP plays an important role in some forms of necrotic cell death. Nakagawa and Baines groups demonstrated that CyP D-deficient embryonic fibroblasts (MEFs) show significantly increased resistance to H_2O_2- induced necrosis[195,197] and CyP D-deficient hepatocytes showed

resistance to necrosis induced by a Ca^{2+} ionophore (A23187) or by H_2O_2[195,197]. Interestingly when necrosis was inhibited by CyP D deficiency, apoptosis did not occur as an alternative death mechanism[197] suggesting that the H_2O_2/Ca^{2+}- induced apoptotic signalling pathways are somehow blocked in these types of cells.

1.8.4.2 Apoptotic Cell Death and MPTP

Another consequence of MPTP opening is mitochondrial matrix swelling and this may lead to apoptotic cell death under some conditions. Swelling of the mitochondria occurs as the MPTP is permeable to all solutes of sizes <1.5 kDa and the non-protein components of the mitochondrial matrix will rapidly equilibrate across the IMM. However, the matrix proteins remain inside the mitochondria and their higher concentrations exert a colloidal osmotic pressure causing the matrix to swell. This unfolds the cristae in the IMM without breaking the IMM, but the outer membrane ruptures allowing the contents of the inter membrane space to be released including cytochrome c and other proapoptotic proteins such as Smac/Diablo and Apoptosis Inducing Factor (AIF). Thus, even if MPTP opening is insufficient to deplete ATP levels and cause necrosis, apoptosis may result[12,166]. However, the release of pro-apoptotic proteins from the inter membrane space is normally mediated by specific permeabilisation of the outer membrane through the action of pro-apoptotic members of the Bcl-2 family such as Bax[215,216].

Caspases activation is thought to be a major mechanism of apoptotic cell death and their role in ischemia-reperfusion injury has been debated[217,218]. Authors have reported that inhibition of caspases can only result in a modest reduction in infarct size[218]. However, a large number of studies demonstrated that addition of caspase inhibitors reduced infarct size, suggesting an important role for caspase activation in ischemia-reperfusion injury[219,220]. Caspase 9 are activated during ischemia, whereas caspases 8 and 9 are activated during reperfusion[220]. It has also been suggested that inhibition of caspases promotes necrotic cell death[221].

Inhibiting mitochondrial membrane permeability (MMP) using MPTP inhibitors such as cyclosporine-A or bongkrekic acid have been demonstrated to inhibit apoptotic cell

death[222,223]. At low doses Bax was demonstrated to induce permeabilisation of the OMM only, though at higher doses it also induced the permeabilisation of the IMM. MPTP opening in this setting will result in ATP depletion, which would be expected to halt the energy-dependent apoptotic process, so it appears counter-intuitive to have MPTP opening in apoptotic cell death.

Furthermore, mitochondrial swelling and OMM rupture does not always occur in apoptotic cells, and mitochondrial cytochrome C release can occur independent of MPTP opening[224,225]. Therefore, MMP may occur by a direct action of pro-apoptotic protein on the OMM, independent of the MPTP. Studies have shown that over expression of CyP D, inhibits apoptosis induced by over expression of caspase-8 (but not Bax) or by exposure to arsenic trioxide[226,227]. There is a possibility that these forms of apoptosis are mediated by the MPTP and they are somehow affected by CyP D over expression. Baines et al showed that cardiac myocytes isolated from transgenic mice with myocardial expression of CyP D did have a tendency to undergo mitochondrial swelling and spontaneous death suggesting that the effects of CyP D expression might be cell type-specific[195]. Crompton and colleagues showed that a neuronal cell line over expressing CyP-D, was hypersensitive to necrotic cell death induced by Ca^{2+} and oxidative stress, but was more resistant to apoptosis induced by nitric oxide or staurosporine[228].

1.8.5 The Role of the MPTP in Reperfusion Injury

The conditions that prevail during ischemia (drop in ATP/ADP, adenine nucleotide depletion and the build-up of lactic acid, and drop in intracellular pH) are exactly those that favour MPTP opening[170]. ATP levels get severely compromised and calcium accumulates inside the cell. Upon reperfusion, this Ca2+ enters the re-energised mitochondria, and the replenished oxygen supply leads to the formation of oxygen free radicals and this sets the scene for pore opening.

Using the ‘Hot-DOG’ (DOG is 2-deoxyglucose) technique, Halestrap’s group have confirmed that MPTP opening does not occur during ischemia, but does occur after approximately 2 min of reperfusion when the pH has returned to normal[170]. They were also

able to demonstrate that a significant proportion of the mitochondria that do open initially during the reperfusion period, do subsequently close again, and that the recovery of the heart correlates best with the extent of this closure[151].

1.8.5.1.1 Inhibition of the MPTP protects hearts against ischemia reperfusion injury

The following strategies can be used to inhibit MPTP opening and there by protecting the heart against ischemia-reperfusion injury.

1.8.5.1.2 Targeting CyP-D

In 1991, Nazareth et al demonstrated that CsA could protect isolated cardiac myocytes from reoxygenation injury[127]. They demonstrated that cyclosporine-A at a concentration of 0.2 µmol/l protected the cell against necrotic cell death (assessed by Trypan-blue exclusion and LDH release). They showed that the best protective effect could be achieved at concentrations of CsA between 0.2-0.4 µmol/l[127]. Subsequently Griffiths and co-workers showed protection from reperfusion injury in the Langendorff-perfused heart[130]. These findings were confirmed by our group (reduction in the infarct size of hearts subjected to ischemia - reperfusion injury with CsA added only at reperfusion) and others using both global and regional models of ischemia and reperfusion using enzyme release, infarct size and haemodynamic function as end points[68,229]. Furthermore, genetic ablation of CyP-D has been demonstrated to protect against ischemia reperfusion injury of the heart[195,197] and brain[230] with CyP-D knockout mice showing a substantial decrease in infarct size. Thus targeting the MPTP has proven potential as a pharmacological target for the reduction of reperfusion injury following stroke, coronary thrombosis and heart surgery. However, CsA also inhibits calcineurin, which is a calcium-sensitive protein phosphatase, by binding to cytosolic cyclophilin-A. This has direct effects on heart function[231] and also undesirable immunosuppressive activity[232]. However, protection by CsA against MPTP opening has been reported in human clinical trials and this confirmed reduced infarct size following CsA treatment of patients undergoing PCI treatment following a coronary thrombosis[135].

To overcome the issue of CsA, SfA can be used which are inactive against calcineurin but inhibit MPTP opening and are as effective as CsA even when given at the time

of reperfusion[139,143,233,234]. Our group have shown that CsA and SfA can reduce the infarct size of hearts when a coronary artery is occluded and then re-opened to mimic the clinical treatment of a coronary thrombosis[68,229].

1.8.5.1.3 Targeting other components of the MPTP and using other strategies

Targeting another component of the MPTP would be an attractive strategy for cardioprotection as MPTP opening may not be totally dependent on CyP-D. When CyP-D is ablated genetically or when fully inhibited with CsA, it has been shown that the permeability transition of MPTP can be induced with a higher calcium load or oxidative stress[159,196]. The MPTP can be inhibited by targeting the ANT with BKA and this can protect cells from MPTP-induced cell death in some systems[146,210]. As ANT is vital for the generation of cytosolic ATP via oxidative phosphorylation to energise the contractile cycle, this form of cardioprotection is not appropriate.

Popofol can directly inhibit MPTP opening in isolated mitochondri[235] by scavenging free radicals. Javadov et al, using the "Hot-DOG" technique, has demonstrated the inhibition of the MPTP by propofol in the intact heart[152] and in vivo pig model of cardiopulmonary bypass with warm blood cardioplegia[157]. Other anaesthetic agents such as isoflurane and desflurane have also been shown to offer protection and are associated with less ROS formation and calcium overload[236,237].

Inhibition of the Na^+/H^+ exchanger with amiloride derivatives (cariporide) protects the heart from reperfusion injury, by preventing sodium and therefore calcium overload during ischemia and maintaining low pH during reperfusion[238,239].

Pyruvate, free radical scavenger, also maintains a low intracellular pH and is an excellent respiratory substrate to replenish ATP during reperfusion. Direct measurement with the "Hot-DOG" technique has confirmed that pyruvate not only reduces MPTP opening at reperfusion but closes those pores that were open initially[151].

Verapamil and ruthenium red can protect the hearts from reperfusion injury by reducing cytosolic and mitochondrial calcium overload[240].

1.8.5.2 Inhibiting MPTP Opening During Ischemia-Reperfusion: the pH Paradox

The rapid restoration of physiologic pH during myocardial reperfusion after ischemic episode contributes to lethal reperfusion injury. This phenomenon is termed the pH paradox[241]. Studies In neonatal rat cardiomyocytes have shown that reoxygenation with acidic buffer is cardioprotective[242] and this effect may be mediated by the inhibition of MPTP opening[103].

1.8.5.3 The MPTP - a target for preconditioning and postconditioning

Finding of IPC by Reimer and Jennings in 1986, has generated a huge research effort to elucidate the signal transduction pathways. Current proposal is that the IPC stimulus generates autocoids such as adenosine, bradykinin, and opioids, which in turn activate their specific G-protein coupled receptors (GPCR) on the cardiomyocyte plasma membrane. A complex network of intracellular signalling pathways are recruited many of which converge on the mitochondria. This results in the production of mitochondrial reactive oxygen species (ROS) activating PKC, Akt and Erk1/2 which convey the cardioprotective signal and the 'memory-effect' of IPC to the unidentified end effector[95,243].

Our group demonstrated for the first time that IPC elicited its cardioprotective effect by targeting and inhibiting the opening of the MPTP using diazoxide, a purported opener of the ATP-sensitive mitochondrial potassium (mitoKATP) channel in adult rat cardiac mitochondria[229].

Subsequent to its original description in 2003 by Vinten-Johansen's group[53] our research group and others have provided evidence that IPost is able to activate cell-surface receptors such as adenosine, bradykinin and opioids, which then recruit signal transduction pathways such as the PI3KAkt, MEK1/2-Erk1/2, cGMP-PKG kinase cascades, which are known to terminate on the mitochondria. Ovize's research group[244] were able to link IPost with the inhibition of MPTP opening using rabbit hearts.

Several mechanisms have been proposed through which IPC and Ipost inhibits the opening of the MPTP at the time of myocardial reperfusion but the actual mechanism is currently unresolved.

1.9 Summary and Main Objectives of the Thesis

Mitochondrial permeability transition pore opening occurs just after reperfusion under pathological conditions such as ischemia-reperfusion injury, where conditions' predisposing for MPTP opening prevails. These include a high mitochondrial [Ca^{2+}] and [P_i], ATP depletion and oxidative stress. Opening of the MPTP disrupts normal mitochondrial function and depletion of energy sources. Hence opening of MPTP is a critical determinant of cell death - both apoptotic and necrotic cell death. Therefore, inhibiting MPTP opening during ischemia-reperfusion injury offers a powerful target for protecting the heart against the reperfusion injury.

The main aim of this thesis is to demonstrate that MPTP opening does occur at reperfusion in human cardiomyocytes and that inhibiting the MPTP opening occurring at the time of reperfusion, following a lethal period of ischemia, is an important mechanism for cardio-protection in **humans,** by applying agents at the time of reperfusion.

Chapter Two

HYPOTHESIS

Overall hypothesis:

The mitochondrial permeability transition pore (MPTP) is a target for cardioprotection in the human heart

Aim and objectives:

1. ***To determine whether pharmacologically inhibiting MPTP opening at the onset of myocardial reperfusion / reoxygenation improves recovery in contractile function in human atrial muscle following a period of sustained simulated ischemia.***
2. ***To determine whether pharmacologically inhibiting MPTP opening at the onset of myocardial reoxygenation improves the survival of human atrial cardiomyocytes subjected to a period of sustained simulated ischemia.***
3. ***To demonstrate that MPTP opening occurs in human atrial cardiomyocytes subjected to oxidative stress, and that MPTP opening can be inhibited by known pharmacological MPTP inhibitors.***

In Chapter 4, we examine the effect of pharmacologically inhibiting MPTP opening with known inhibitors during the first few minutes of post-ischemic reperfusion (the time-period when MPTP opening has been demonstrated to occur) in Human atrial trabeculae and its contractile functional recovery. In Chapter 5 and 6, we examine the effects of pharmacologically inhibiting MPTP opening by known MPTP inhibitors at the onset of reperfusion, on isolated Human myocardial cellular viability and also on the percentage of apoptosis and necrosis, in a setting of hypoxia and reoxygenation model. In chapter 7, we examine the opening of MPTP secondary to oxidative stress and the direct inhibitory effect (delayed opening of MPTP) on the opening of MPTP by the known pharmacological MPTP inhibitors, by applying solely at reperfusion, in an isolated Human myocyte using oxidative stress model.

Chapter Three: GENERAL METHODS

3.1 Patients

All the experiments were performed on the tissues harvested from the human right atrial appendage. Specimens of human right atrial appendages were obtained from the right atrial venous cannulation site at the time of cardiac bypass operation from patients, at the Heart hospital, London, undergoing either elective coronary artery bypass graft surgery or elective aortic valve replacement, just before they went onto cardio-pulmonary bypass. The patients either had coronary artery disease or stenotic disease of the aortic valve with good left ventricular function.

The procedure was explained in detail to all the patients along with the information sheet and was given enough time to read, understand and ask questions about the same. They were compos mentis while signing the form and were in full agreement for providing their right atrial sample for the present study. Prior ethical approval had been obtained from the Middlesex Hospital Clinical Investigations panel (00/0275).

3.2 Exclusion criteria

The exclusion criteria were: Patients with

- Arrhythmias
- Poor left or right ventricular function
- Age ≥ 80 years
- Recent myocardial infarction (within two weeks)
- Emergency surgery
- Unstable angina
- Bacterial endocarditis
- Patients with anti-arrhythmic drugs, oral hypoglycaemics and Insulin
- Combined cardiac surgical procedures

3.3 Preparation of Adult Human right atrial trabeculae

3.3.1 Isolation of Trabeculae

The advantages of isolated human right atrial muscle (trabecula) preparations are:

(1) Avoiding the problems of collateral circulation experienced with in-vivo models

(2) The confounding effects of alterations in pre-load and after-load

(3) The measurement of contractile function in the whole heart during simulated ischemia and reperfusion is complicated by alterations in the geometry of the cavity associated with ischemic contracture, tissue oedema and the effects of changes in coronary perfusion pressure

(4) Free from neuro-humoral effects

(5) It was available and because sampling was part of the routine cardiac procedure.

(6) The specimens make stable preparations and are generally disease free.

The disadvantages of isolated human right atrial muscle (trabecula) preparations

This does not truly represent the ventricular muscle and hence translation into clinical scenario is not strictly representative.

There are several clinical studies suggesting that preconditioning occurs in humans,[28,34,36,245-247] but the early direct evidence comes from in vitro studies. Ikonomidis et al[19] used human ventricular cardiomyocyte cell cultures and showed that these isolated myocytes could be protected against a 90-minute period of SI by a preconditioning protocol. Our department[18] were able to show that human atrial trabeculae can be preconditioned and that had a significantly better postischemic recovery of contractile function than non-preconditioned trabeculae. There are differences between atrial and ventricular tissue, and results from one may not be applicable to the other; however, early experiments in our laboratory (unpublished data, 1995) suggest that an identical preconditioning effect can be induced in ventricular tissue if a shorter ischemic insult is used (60 minutes instead of 90 minutes).

In place of "true" ischemia, the present study used a period of hypoxic superfusion in combination with rapid pacing to simulate ischemia, and there is a great deal of evidence in animal models of both regional and global hypoxia and in cell culture that hypoxia is as effective as ischemia in inducing preconditioning[248-251].

We know that preconditioning causes improved recovery of contractile function as well as reduced infarct size. Jenkins et al,[252] *m*easured both infarct volume and functional recovery after ischemia and showed that the improved recovery of global left ventricular function produced by preconditioning is proportional to a reduction in infarction. Cohen et al[253] and Przyklenk et al[76] were able to correlate improved recovery of systolic shortening with reduced infarct size in vivo models of regional ischemia.

Though there is no direct evidence in atrial tissue that recovery of global function is proportional to infarct volume it can be safely assumed that this is likely to be the case. It is clear from the evidence that the enhanced recovery of contractile function due to preconditioning, following a prolonged period of ischemia, is due to a reduction in infarct size and is not due to a reduction in stunning. This model uses a 90-minute period of SI as the "ischemic insult," a period much more likely to lead to cell death than stunning, which is induced by shorter periods of 5 to 15 minutes of ischemia suggesting that our model is indeed one of preconditioning. However, although unlikely, we cannot entirely exclude the possibility that our model involves stunning.

3.3.1.a Oxygenation of isolated muscle preparation

In the isolated muscle preparation, delivery of a satisfactory amount of oxygen to all of the cells is determined by the rate of oxygen utilisation of the cells, the distance between the muscle surface and the core and the oxygen tension of the solution that is surrounding the muscle. The core tissue of the isolated muscle preparation may be inadequately oxygenated if the diffusion distance for the oxygen is high even in the presence of 95% O_2 in the superfusate. However the muscle preparations with a small diameter and adequately perfused with well oxygenated buffer can remain viable without arterial perfusion. In theory for maximum contractility, the muscle specimen must be small enough for the free diffusion of substrates (oxygen, glucose and pyruvic acid) and metabolites and that their production can

match their diffusion. The diffusion of oxygen to the muscle is the main determinant of their contractility[254].

There is some dispute among various investigators about what is an appropriate maximum diameter of the superfused contracting isolated muscle in pertaining to adequate oxygenation. One of the early investigators was A V Hill and he determined the formula from biophysical principles to calculate the maximum diameter for adequate oxygenation of an isolated muscle preparation[255]. Prasad and Callaghan using the formula calculated that a muscle of less than 0.932 mm diameter would be adequately supplied by oxygen by simple diffusion in an atmosphere of 95% O_2 and 5% CO_2 at 37°C and the pacing was at 1 Hz[256]. Page and Solomon showed that a superfused cylindrical cat papillary muscle with a diameter of 1.12 mm would be adequately oxygenated[257]. The method of superfusing isolated specimens of cardiac muscle is well recognised and there are published studies to support this technique in human ventricle obtained from explants, where the sample diameter was 1-2 mm[258,259].

The human right atrial trabeculae used in this study ranged between (of diameter 1-2 mm and length $\geq$2 mm). The thinnest of the trabeculae were always used when possible with no branches. It was assumed that the muscles were adequately oxygenated as supported by the studies above. It might have been possible that the larger muscles have undergone some core hypoxia and if that is the case it would equally affect all the groups almost to the same degree. The experiments conducted in this study would be still valid as all the atrial trabeculae in the groups were similar at baseline.

3.3.1.b Method of isolation of muscle

This method has been well established and shown to work in our laboratory[260-262]. Specimens of right atrial appendages were transported to the laboratory in ice cold oxygenated modified Tyrode's buffer containing (in mM): NaCl 118.5, KCl 4.8, $NaHCO_3$ 24.8, KH_2PO_4 1.2, $MgSO_4.7H_2O$ 1.44, $CaCl_2.2H_2O$ 1.8, glucose 10.0 and pyruvic acid 10.0. It was ensured that the transport time of the specimen from theatre to laboratory was limited to less than 15-20 minutes. Once in the laboratory the samples were transferred to and well bathed in a Petri dish containing oxygenated modified Tyrode's buffer at room temperature. The specimen was

immobilised with pins taking great care and the trabeculae (of diameter 1-2 mm and length ≥ 2 mm) were harvested with the help of surgical loops (Keeler, 3.5 x magnification). From one to three trebaculae were harvested when possible from the each specimen and each of the trabeculae were subjected to individual experiments only. The trebeculae were gently separated and isolated using fine forceps from the surrounding tissue. The ends of the trabeculae were tied with 5/0 silk sutures taking care not to cut the trabeculae. The trabeculae were cut from the surrounding tissue i.e. the atrial wall, beyond the silk sutures. They were then transferred to an organ bath which is superfused with oxygenated modified Tyrode's solution at 37°C. The trabeculae were suspended horizontally in the organ bath, by tying (snared) one end of the trabeculae to a fixed post in the bath and the other end of the trabeculae to the force transducer (Gould Statham UCT2, Ohio, USA) that is calibrated before each experiment. The organ bath was continuously superfused with oxygenated (95% O_2 / 5% CO_2, British Oxygen Company, London, England) modified Tyrode's buffer and the temperature was maintained constant at 37°C using a heat exchanger (Techne Circulator C 85-A, Cambridge, UK) and was monitored by a thermocouple in the bath. The pH was maintained between 7.35 – 7.45; pO_2 between 50 – 60 kPa and pCO_2 between 4.0 – 6.0 kPa. During simulated ischemia (hypoxia) pH was maintained between 7.24 – 7.34 and pO_2 <7 kPa.

3.3.2 Force transducer

The force transducer was attached to a micromanipulator (Prior, Cambridge, England) which allowed the force transducer to be manoeuvred with precision. This allowed the manipulation of trabeculae on the horizontal position and with precision. The whole setup was mounted on a low vibration table.

3.3.3 Organ bath

The organ bath was made of non-toxic Perspex and it had a volume of 4 ml and the flow through the bath was regulated at about 8 ml / min. The bath was designed with a well at the entry point of the buffer to damp the pulsatile flow of the buffer delivered through the pump (Watson-Marlow, Cornwall, England). The buffer was constantly removed from the bath through pump and so a fresh, constant and steady flow of buffer was ensured to the trabeculae. The inflow of buffer was matched with the outflow of the organ bath. At all time point it was ensured that the trabeculae were well immersed in the buffer to ensure adequate oxygenation. Glass cover slips were used to cover the bath to minimize the gas exchange with the atmosphere. Two platinum electrodes were sited by the side of the bath, which enabled the trabeculae to be stimulated at required hertz. Once suspended, the trabeculae were paced individually by field stimulation at 1 Hz using an isolated stimulator (Digimeter DS2, Hertfordshire, UK) triggered by a computerized clock.

The baths were connected to the water-jacketed glass reservoirs for holding buffers, by means of either polythene or silicone tubing. The water-jacketed glass reservoirs were connected to the heat exchanger to maintain the temperature at 37°C. The buffers in the reservoirs were bubbled constantly with the appropriate gas mixture where necessary and were covered with tin foils to minimise heat exchange to the atmosphere and to keep the buffers dust free. Three way taps were used to alternate the buffers to the reservoir between protocols. The buffers in the reservoir were bubbled with appropriate gas mixtures for a minimum of 20 minutes to allow them to be mixed well and this also allowed the buffers to reach and maintain the temperature at 37°C.

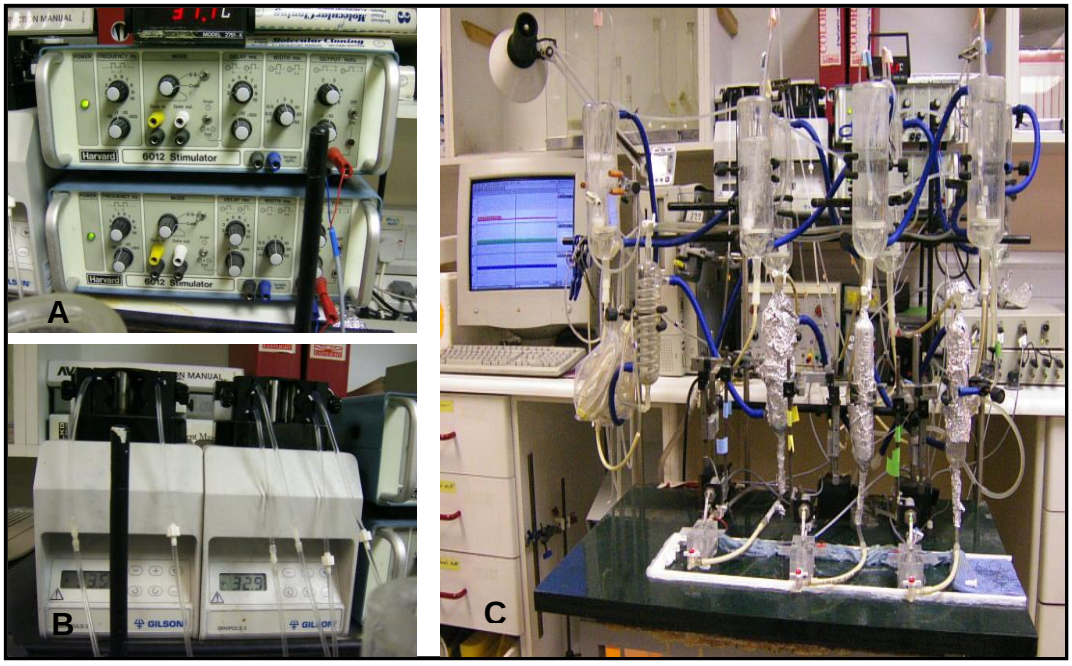

Figure 3-1 A to C: **Figure showing (a) stimulator, (b) pump for constant flow of buffer and (c) the whole rig including water jacket and the computer recording.**

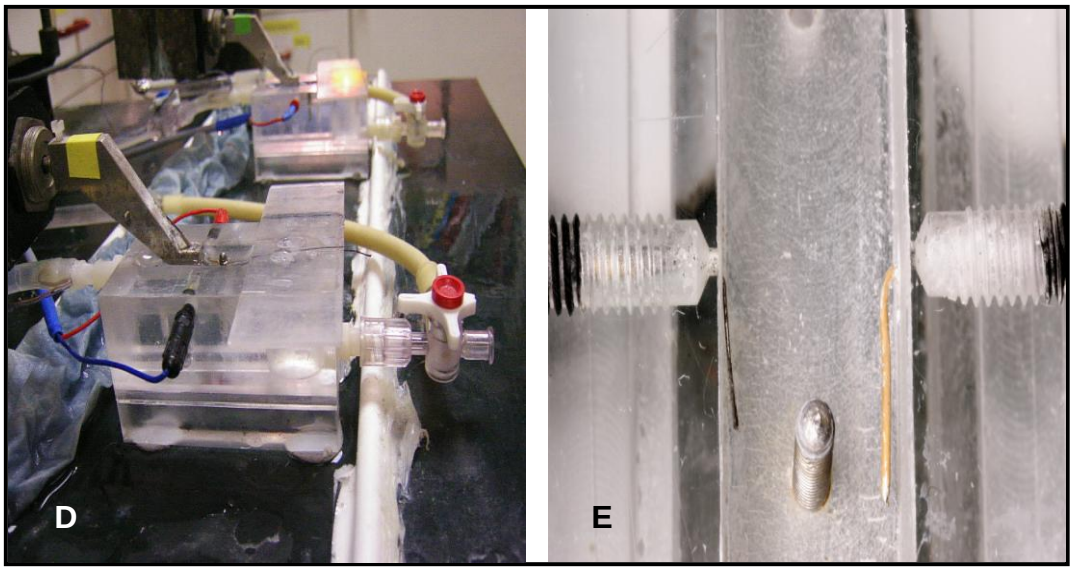

Figure 3-2 D and E: **Figure showing force transducer and organ bath with electrodes on either side mounted on a low vibration table.**

3.3.4 Stabilisation of the trabeculae

All the trabeculae were suspended in the organ bath and paced at 1 Hz, unstretched, for 30 minutes. This was the equilibration phase where the trabeculae were allowed to equilibrate with the temperature and the buffer. The trabeculae were then gradually stretched in a stepwise manner over a period of 15 minutes duration and the resting tension was set at the length that produced the maximum degree of force of contraction. The gradual stretch was done using the help of the micromanipulator. The stretching process was stopped when there was no further increase in the developed force in relation to the length of the trabeculae. Once this maximum developed force is achieved the trabeculae were allowed to stabilise for further 40-45 minutes (stabilisation phase). The trabeculae were excluded if the maximum developed force were $\leq$1.0 g. All the groups eventually underwent a period of simulated ischemia (SI) and reperfusion which consisted of 90 minutes of hypoxia, when the trabeculae were superfused with the substrate free modified Tyrode's solution and rapid pacing at 3 Hz, followed by reperfusion for 120 minutes with normal modified Tyrode's solution at 1 Hz. A one-minute allowance was made for the change over between different buffers in the bath and the connecting tubes.

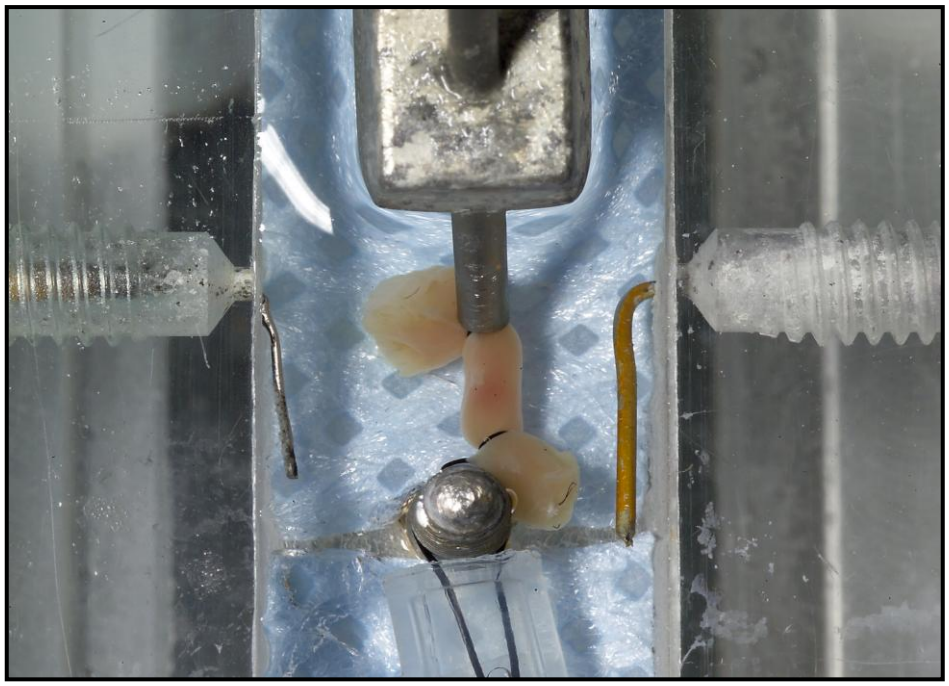

Figure 3-3: Figure showing Human right atrial trabecula attached to transducer from the pole and perfused in the organ bath.

3.3.5 Measurement of force

The trabeculae were suspended, as described above, one end to the fixed pole and the other end to the force transducer. The platinum pacing electrodes were positioned on either side of the trabeculae in the organ bath and were stimulated at the frequency 1 Hz. The pulse width was fixed at 5 ms and the pulse amplitude was set at twice threshold required by each individual trabecula for satisfactory pacing (6-8 V).

The frequency response of the force transducer and its mechanical linkage was known to be flat to 50 Hz. The range of the transducer, as per the manufacturer, was ± 30 g with a displacement of ± 0.006 mm at the upper ends of this force range. The excitation voltage for the force transducer was provided by a DC bridge amplifier (Digimeter, Neurolog recorder amplifier NL 107). The output of the force transducer was amplified and recorded using PowerLab / 8s (AD Instruments, Australia). The PowerLab recording unit together with the chart application program provides a multi-purpose data recording and analysis system and was used with a computer.

The force transducer was calibrated before every single experiment. The chart is opened and all the channels were set to zero. The chart was allowed to run while the transducer was attached to the known weights (0.5 & 1 g) and calibrated according to the known weights. While the baseline is calibrated to zero the upper limit of the weight were calibrated to either (0.5 or 1 g) depending on the weight used (mostly 0.5 g). The weights were attached to the force transducer with the help of a pulley so that the direction of the pull of the weight would be horizontal as the trabeculae would be. The known weights were weighed on a calibrated electronic balance, accurate to 5 figures (Mettler AJ50, Leicester, England).

At the end of the reoxygenation period, the contractile function, which is expressed as a percentage of the baseline force of contraction, was determined for each atrial trabecula. In addition, the width and length of each atrial trabecula was measured and all specimens were then weighed. The cross-sectional area was calculated from the measured diameter of the atrial trabecula (assuming the trabeculae were cylindrical).

3.3.6 Materials

(1) *Modified Tyrode's solution:* The modified Tyrode's solution contains (in mM): NaCl 118.5, KCl 4.8, $NaHCO_3$ 24.8, KH_2PO_4 1.2, $MgSO_4.7H_2O$ 1.44, $CaCl_2.2H_2O$ 1.8, glucose 10.0 and pyruvic acid 10.0.

(2) In the substrate free modified Tyrode's solution, choline chloride - 7mM was substituted for glucose and pyruvic acid to maintain constant osmolarity. All reagents were Analar grade (BDH Chemicals, Poole, England) except for pyruvic acid (Sigma Chemicals, Poole, England).

3.3.7 Experimental protocols

3.3.7.1 Control group

Group perfused with either normal buffer or buffer with 0.005% ethanol or 0.01% DMSO vehicle control.

3.3.7.2 protocol

The preconditioning protocol consisted of perfusing the trabeculae with 3 minutes of hypoxic substrate-free superfusion of modified Tyrode's with rapid pacing at 3 Hz followed by 7 minutes of reperfusion with oxygenated modified Tyrode's with pacing at 1 Hz. Subsequently these trabeculae were subjected to the simulated ischemia and reperfusion protocol. This ischemic preconditioning group was included as a positive control to verify that cardio-protection could be demonstrated in this atrial trabeculae model of SIRI. This hypoxic preconditioning protocol has been previous demonstrated in this atrial trabeculae model to improve the recovery of myocardial contractile function[18].

3.3.7.3 Drug groups

The human atrial trabeculae allocated to the drug groups Cyclosporin (CsA) (0.2 μM) and Sanglifehrin (SfA) (1.0 μM) received their corresponding drugs for the first 30 minutes of the reperfusion period.

1. Cyclosporin-A (CsA, Sigma Chemicals, Poole, Dorset) was dissolved in 50% ethanol and added to the buffer such that the final ethanol concentration was 0.005%.
2. Sanglifehrin-A (SfA, Novartis Pharma AG, Basel) was dissolved in dimethyl sulphoxide (DMSO, Sigma Chemicals, Poole, Dorset) and added to the buffer such that the final DMSO concentration was 0.01%.

Each trabecula was used for one protocol only and they were randomly assigned to the different groups studied. These concentrations of CsA and SfA have been demonstrated to inhibit MPTP opening in the isolated perfused rat heart [68,130,139,229].

3.4 Isolation of Adult Human Right Atrial Cardiomyocytes

Isolated adult human right atrial cardiomyocytes provide cells, which are free from the neuro-hormonal influence in an environment, which can be controlled. Human right atrial cardiomyocytes were isolated by enzymatic dissociation using protease and collagenase digestion, from the right atrial appendages harvested from the patients undergoing elective cardiac surgery, just before going on to cardio-pulmonary bypass[263]. Once isolated the right atrial samples were immediately transported to the laboratory (in less than 20 minutes) in the ice cold oxygenated medium-calcium (MC) solution comprising (in mM): NaCl 120, KCl 5.4, $MgSO_4$ 5, pyruvate 5, glucose 20, taurine 20, HEPES 10 and Ca^{2+} 0.05 (pH 7.4). All buffers were made from autoclaved distilled water. In the laboratory, the samples were weighed before cardiomyocyte isolation. The right atrial appendage was then freed of any fat and scar tissue while still bathed in the ice-cold oxygenated medium-calcium (MC) solution. The right atrial appendage was then cut into cubes of 1-2 millimetres using fresh sharp razor blades, taking care not to cause shearing action (to prevent crushing the cells) in the oxygenated medium calcium (MC) solution at room temperature. The sliced atrial pieces were then transferred to 25 ml of Low calcium (LC) solution in a sterile pot and oxygenated with 100% O_2 while stirring the solution gently for 3 minutes. At the end of 3 minutes the cubes were filtered through prolene mesh (300 µm) and were transferred to 25 ml of fresh Low calcium solution in a second sterile pot and the above process was repeated a further 3 times in

separate sterile pots each time at 37°C (4x3 minutes in total). The filtrate was discarded each time. At the end of 12 minutes (3x4 times) the atrial slices were filtered once more through the mesh, scraped off and placed into a falcon tube containing 10 ml of the enzyme solution (ES) containing protease (Type XXIV, Sigma, USA) and were gently agitated in the agitator / shaker in a circular motion at 37°C for 45 minutes, with gentle oxygenation without bubbling. Fifteen minutes before the first protease digestion was finished 10 ml of enzyme solution was added to the first collagenase in a separate falcon tube and this solution was warmed and oxygenated. At the end of 45 minutes of protease digestion the sample was filtered once again through the mesh and transferred into 10 ml of enzyme solution containing collagenase (Type V, (Clostridium Histolyticum) Sigma, USA) and was again gently agitated in the shaker at 37°C for 45 minutes. The sample was filtered once again and the filtrate from the digestion was spun in a centrifuge at 1000 rpm for 2 minutes at 37°C. The supernatant was then removed carefully by pipetting out the solution and the pellet was re-suspended into 10-12 ml of the enzyme solution in a fresh sterile falcon tube and transferred to the incubator. After isolation, cell viability was assessed using light microscopy and routinely averaged ~30%.

3.4.1 Materials

(1) *Medium-calcium (MC) solution:* comprising (in mM): NaCl 120, KCl 5.4, $MgSO_4$ 5, pyruvate 5, glucose 20, taurine 20, HEPES 10 and Ca^{2+} 1-2 μM (pH 7.4).

(2) *Enzyme Solution* (mM): pH 7.4, Sodium Chloride (NaCl) 120, Potassium Chloride (KCl) 5.4, Magnesium Sulphate ($MgSO_4$) 5, Pyruvate 5, Glucose 20, Taurine 20, HEPES 10, Calcium Chloride ($CaCl_2$) 35 μM.

(3) Protease 4 U/ml (Type XXIV, (Bacterial) Sigma, USA)

(4) Collagenase 1 mg/ml (Type V, (Clostridium Histolyticum) Sigma, USA)

3.5 Preparing Human right atrial Cardiomyocytes for Hypoxic chamber

Once the cardiomyocytes were isolated, as described above (section 3.4), they were stabilised in oxygenated medium calcium (MC) solution at 37°C in an incubator (CO_2 Incubator, CO28IR, New Brunswick Scientific, USA) in an atmosphere of 95% air/ 5% CO_2 for

50-60 minutes. Then the cells were transferred on to six well plates and subjected to experiments in the hypoxic chamber.

3.5.1 Subjecting cells to hypoxia and reoxygenation

Cells were subjected to lethal Simulated Ischemia (SI) as follows:

After stabilising the human atrial cardiomyocytes in the enzyme solution for 50-60 minutes in the incubation chamber the cells were transferred to the six well plates for the experiments. The cell suspension in the enzyme solution (falcon tube) was divided into 2 ml solutions in eppendorffs ((a) for the control, (b) for hypoxia and reperfusion alone, (c) for IPC group and (d) for hypoxia and drugs). These cells were centrifuged at 1000 rpm for 1-2 minutes at 37°C and re-suspended into the appropriate solutions i.e.

1. The control group (time control): cells was re-suspended back in the enzyme solution into a well of the plates and transferred back to the incubator to be kept at 37°C.
2. Ischemic preconditioning group (IPC): the cells were re-suspended in the modified Esumi buffer, pH 6.5 (mM / L): Sodium Chloride (NaCl), 137, Potassium Chloride (KCl), 12, Magnesium Chloride ($MgCl_2$) 0.49, Calcium Chloride ($CaCl_2$) 0.9, HEPES 4, Lactate 20) and transferred into the six well plater and subjected to three minutes of hypoxia in the hypoxic chamber followed by seven minutes of reoxygenation (*still in Esumi buffer*) by exposing cells to oxygenation in the incubator followed by hypoxic reoxygenation protocol.
3. Hypoxic group: the cells subjected for hypoxic protocols (control) were re-suspended in the modified Esumi buffer and transferred into the wells of the six well plates and were subjected to 20 minutes of hypoxia followed by 50 minutes of reoxygenation in the enzyme solution.
4. Drug groups: The drug group were subjected to 20 minutes of hypoxia in the modified Esumi buffer like their counterpart hypoxic group in the hypoxic chamber followed by 50 minutes of reoxygenation in the enzyme solution containing drug (CsA) for the entire period of reoxygenation.

The cells were then placed in the plates. The cells designated as the time control were placed back in the incubator at 37°C. The plates for hypoxic protocol (both hypoxic group and drug group) were transferred to the hypoxic chamber along with 20 gm of sodium dithionate (placed separately in the hypoxic chamber). The hypoxic chamber was closed air tight and the temperature was maintained at 37°C. The air in the hypoxic chamber was evacuated using tubing connected to the cold-water tap (one way) for the first 5 minutes to produce a vacuum. For the next fifteen minutes the hypoxic chamber was flushed with argon gas (BOC) to replace any oxygenated air left in the chamber and to maintain hypoxia (combined 20 minutes of hypoxia) with an atmosphere of 0% O_2 – 5% CO_2 balanced with argon. The outlet for the argon gas from the hypoxic chamber was connected to an underwater seal to prevent air entering into the hypoxic chamber (creating a one way valve). The ischemia was simulated by combination of (1) Modified Esumi Buffer, (2) Sodium Dithionate and (3) Hypoxia in the chamber. During this hypoxic period the control cells were counted for their baseline viability count under light microscope and the result is taken from the average of five fields from the single well by an independent observer.

At the end of the twenty minutes of simulated ischemia the hypoxic chamber was opened and the drug Cyclosporin-A (CsA) was added to the cells that are subjected for the drug protocol. Now the cells are pippetted out into separate eppendorffs (hypoxic group and drug group) including the control and were centrifuged for 1-2 minutes at 1000 rpm. The cells from all the groups were re-suspended back in the Enzyme solution (The cells in the drug group received the drug CsA along with the Enzyme solution) in separate wells of the 6 well plates and were in turn incubated at 37°C (reoxygenation).

The percentages of viable cells were counted under a light microscope at three different time points of reperfusion, i.e. at 10, 30 and 50 minutes of the reperfusion period. The percentages of viable cells were taken as the average count of five fields for each well at any one-time point by an independent observer. The rod shaped cells were counted as the viable cells and the rounded ones as dead cells.

3.5.2 Experimental protocols for measurement of percentage viability, apoptosis and necrosis

Isolated atrial cardiomyocytes were randomly assigned to the following treatment groups:

1) Time control group: these cells were left in normoxic conditions at 37.0°C for the entire duration of the experimental protocol to act as time controls (positive).
2) Hypoxic control group: these cells were subjected to 20 minutes of hypoxia followed by 30 minutes reoxygenation with either normal buffer / buffer containing the vehicle controls (DMSO or 50% ethanol).
3) CsA group and SfA groups: these cells were subjected to 20 minutes of hypoxia followed by 30 minutes of reoxygenation with buffer containing either cyclosporin-A (0.2 µM) or sanglifehrin-A (1.0 µM) correspondingly.

At the end of the 20 minutes re-oxygenation period, the cells were incubated in the dark for the final 10 minutes with Annexin-V-Fluos (AV) (20 µM) (Roche, 8 Germany) i.e. the AV was added to the cells at the end of 20 minutes of re-oxygenation. Propidium iodide (PI) (3 nM) (Sigma) was then added to the cardiomyocytes that was already stained with AV at the end of 30 minutes of re-oxygenation (10 minutes following the addition of AV) and the samples were analyzed immediately using a fluorescent microscope.

Annexin V (also known as anxA5) has been shown previously to detect the early stages of apoptosis by binding to the phosphatidyl serine residues [264]. This imaging protocol is based on the facts that apoptotic cells externalize the negatively charged phospholipid phosphatidylserine (PS) and that the human protein annexin A5 (anxA5) binds to PS selectively and with a high affinity[265]. Cellular necrosis was determined using PI, which binds to the nuclei of cells whose plasma membrane has become permeable[266].

For each treatment group, the numbers of rod-shaped, AV stained, and PI stained cells were counted in 3 randomly chosen fields by an operator blinded to the treatment, and an average value taken. Results were then expressed as a % of the cells counted in the time control group (including the solvent group) and were assigned to 3 categories: (1) Live cells (AV-negative, PI-negative and rod-shaped); (2) Apoptotic cells (AV-positive, PI-negative); and (3) Necrotic cells (PI-positive).

3.6 Preparing Human Atrial Cardiomyocytes for Confocal Microscopy

3.6.1 Human atrial cardiomyocyte model for induction and detection of MPTP opening

Human cardiomyocytes were isolated as above and suspended in the enzyme solution. The cells were then seeded onto laminin-coated 25 mm diameter round cover slips placed inside the wells of 6 well plates. Once the cover slips were dry, 200 µl of laminin (1mg/ml diluted in 30 ml of distilled water, Sigma Chemicals, Poole, Dorset) was pipetted onto the centre of the cover-slip and left to dry for 60-90 minutes. 500 µl of the cell suspension was then carefully placed on the laminin residue in the centre of the cover slip. The cells were left to settle and seed onto the cover-slips for 50-60 minutes in an incubator (CO_2 Incubator, CO28IR, New Brunswick Scientific, USA) at 37°C in an atmosphere of 95% air/ 5% CO_2. After adequate seeding 1 ml of the enzyme solution was added to the cells in each of the wells of the 6 well plates. The cells were then subjected for experiments under confocal microscope. All the following procedures were conducted in a sterile positive-pressure Microbiological Safety Cabinet (Walker Safety Cabinets Ltd, Derbyshire, UK).

Opening of the MPTP in adult rat myocytes was induced and detected using a well-characterized cellular model of oxidative stress[201,267-270]. Seeded human atrial cardiomyocytes, were incubated with the fluorescent dye, tetramethylrhodamine methyl ester (TMRM, 3 µM) for 15 minutes at 37°C, and visualised using confocal fluorescence microscopy, as described below. TMRM, a lipophilic cation, accumulates selectively into mitochondria according to the mitochondrial membrane potential[271]. Laser illumination of the mitochondrial TMRM generates oxidative stress, which is used in this model to induce MPTP opening, which is detected by the loss of mitochondrial membrane potential, which in this model appears as an increase in TMRM fluorescence intensity. The relatively high concentration of TMRM in the mitochondria causes auto-quenching of fluorescence, such that the fluorescence signal becomes a non-linear function of dye concentration; therefore, mitochondrial depolarisation results in the loss of dye into the cytosol where the signal increases[272].

3.6.2 Experimental protocols

After loading with TMRM, the cells were randomly assigned to the following treatment groups:

(1) control group: incubation in Enzyme medium with the presence or absence of the DMSO and ethanol vehicle controls.

(2) CsA group: incubation with CsA (0.2 µM) in Enzyme medium for 15 minutes at 37°C.

(3) SfA group (n=10): incubation with SfA (1.0 µM) in Enzyme medium for 15 minutes 37°C.

3.6.3 Confocal Microscopy

All confocal imaging and analysis was conducted in the Mitochondrial Biology Group, the department of Physiology, University College London in collaboration with Professor Michael Duchen. The 25 mm round cover-slip containing the seeded cardiomyocytes was placed in a custom-made chamber with 1 ml of enzyme solution and mounted on the stage of a Zeiss 510 CLSM confocal microscope equipped with x40 oil immersion, quartz objective lens (NA 1.3). For measuring tetramethylrhodamine methyl ester (TMRM) fluorescence, the cells were illuminated using the 543 nm emission line of a henna laser. The fluorescence of TMRM was collected using a 585 nm long pass filter. For photosensitization experiments, all conditions of the confocal imaging system (laser power, confocal pinhole, optical slice and detector sensitivity) were identical to ensure comparability between experiments. Images were analysed using the Zeiss software (LSM 2.8) and also using Lucida (Kinetic Imaging, Wirral) to measure changes in mean and SD of the signals with time.

Figure 3-4: Confocal Microscope with Camera Attached

3.7 Statistical Analyses

All results are presented as group means ± standard error of the mean (SEM). For comparison between more than two groups, factorial one-way analysis of variance (ANOVA) was employed. For analysing data recorded over a period of time, ANOVA for repeated measures was employed. Results were considered significant when $P \leq 0.05$. All statistical analysis was carried out on a Power Macintosh computer, using Statview statistical software (Version 4.5, Abacus Concepts Inc.).

Chapter Four

THE MITOCHONDRIAL PERMEABILITY TRANSITION PORE:

A POTENTIAL TARGET FOR PROTECTING HUMAN MYOCARDIUM FROM LETHAL REPERFUSION INJURY AND ***IMPROVING FUNCTIONAL RECOVERY***

(Trabeculae model)

4.1 Introduction

In section 1.5.5, the role of the mitochondrial permeability transition pore (MPTP) during ischemia-reperfusion injury was reviewed. Opening of the MPTP in the first few minutes of reperfusion, following an episode of lethal ischemia is a critical determinant of cell death in ischemia-reperfusion. The conditions that prevail during the first few minutes of reperfusion mediate the opening of the MPTP. These conditions which include a high mitochondrial [Ca^{2+}] and [P_i], ATP depletion, oxidative stress, correction of the acidic pH, are known inducing factors for MPTP opening[7,91-93]. Halestrap's group in 1995, demonstrated that the MPTP was kept firmly closed in the heart under normal physiological conditions but opened upon reperfusion following a period of ischemia[143]. Di Lisa's group[203] demonstrated the same finding using the CsA-sensitive loss of mitochondrial NAD^+, that occurred during the first few minutes of reperfusion to indicate MPTP opening. Marban's group demonstrated a CsA-sensitive increase in mitochondrial Ca^{2+} (indicating MPTP opening), occurring at the time of reoxygenation, in cardiomyocytes exposed to a sustained period of simulated ischemia.

Opening of the MPTP results in both apoptotic and necrotic cell death[12,166,209] and the inhibition of its opening during ischemia-reperfusion injury has been demonstrated to confer protection in various experimental models. The importance of the MPTP in the necrotic death using MPTP inhibitors such as cyclosporin A and sanglifehrin A have been demonstrated in the heart, brain and liver[130,139,202,214]. Even if MPTP opening is insufficient to deplete ATP levels and cause necrosis, apoptosis may result[12,166,273]. Inhibiting MMP using MPTP inhibitors such as CsA or bongkrekic acid have been demonstrated to inhibit apoptotic cell death[223,274,275].

Studies in the isolated perfused rat heart have demonstrated that inhibiting MPTP opening using CsA restored the ATP/ADP ratio and AMP to pre-ischemic levels and improved the recovery of LV function following a prolonged length of ischemia[68,130,229]. Being a phosphatase, calcineurin acts to dephosphorylate BAD, thereby enhancing apoptosis. Therefore, by inhibiting calcineurin, CsA may mediate cardio-protection via this anti-apoptotic mechanism rather than inhibiting MPTP opening.

Recently, the novel immunosuppressant, sanglifehrin-A (SfA), has been shown, to also act as a potent inhibitor of MPTP opening[68,139,143,229,233,234]. Studies have examined the effect of inhibiting MPTP opening using CsA or SfA at the crucial time of reperfusion alone, when the MPTP has been demonstrated to open[68,139,143,229,233,234]. All these studies were carried out in various animal and cellular models.

The method of superfusing isolated specimens of cardiac muscle is well recognised and there are published studies to support this technique in human ventricle obtained from explants, where the sample diameter was 1-2 mm[258,259].

The atrial trabeculae model has been well established and shown to work in our laboratory[260-262]. The hypoxic preconditioning protocol has also been previous demonstrated in this atrial trabeculae model to improve the recovery of myocardial contractile function[18]. The concentrations of CsA and SfA have been demonstrated to inhibit MPTP opening in the isolated perfused rat heart[68,130,139,229].

However, to our knowledge there has been no study so far which has examined the effect of inhibiting MPTP opening using CsA and SfA at the time of reperfusion in the human atrial trabeculae model. Therefore the aim of this study was to determine whether pharmacologically inhibiting MPTP opening *at the time of reperfusion,* by using the above known MPTP inhibitors, protects the human heart against lethal reperfusion injury, using functional recovery of the atrial trabeculae as the measured end-point, in a setting of ischemia-reperfusion injury.

4.4 Hypothesis

Opening of the MPTP at the time of reperfusion is a critical determinant of cell death and inhibiting its opening is target for cardioprotection in the human heart.

In this study we set out to examine the role of the MPTP in mediating the cell death and in turn affecting its functional recovery, induced by lethal reperfusion injury in a human atrial trabeculae model. We used cyclosporin-A[68,127,229] to inhibit the MPTP opening that has been demonstrated to occur in the first few minutes of reperfusion. As cyclosporine-A can also

inhibit calcineurin, which may be protective by a different mechanism, it was important to use a different known inhibitor of MPTP opening that does not inhibit calcineurin. Therefore, we also investigated the effect of the newly described MPTP inhibitor, sanglifehrin-A, which does not inhibit calcineurin[139,143,233,234]. We administered these agents ***only at the time*** of reperfusion in order to demonstrate that the opening of the MPTP is an important mediator of lethal reperfusion injury, which in turn would affect the functional recovery. We used samples from the right atrium of humans undergoing cardio-pulmonary bypass, in a setting of ischemia-reperfusion / hypoxia-reoxygenation model to demonstrate the effect of known pharmacological inhibitors on MPTP opening at the time of reperfusion.

4.3 Aim

To determine whether pharmacologically inhibiting MPTP opening at the onset of myocardial reperfusion improves recovery in contractile function in human atrial muscle following a period of sustained simulated ischemia.

4.3.1 Materials

1. *Cyclosporin-A:* (Sigma Chemicals, Poole, Dorset) was dissolved in 50% ethanol and added to the modified Tyrode's buffer such that the final ethanol concentration was less than 0.005%.
2. *Sanglifehrin-A:* (Novartis Pharma AG, Basel) was dissolved in dimethyl sulphoxide (DMSO, Sigma Chemicals, Poole, Dorset) and added to the modified Tyrode's buffer such that the final DMSO concentration was 0.01%. All other reagents were of standard analytical grade.
3. *Modified Tyrode's solution:* The modified Tyrode's solution contains (in mM): NaCl 118.5, KCl 4.8, $NaHCO_3$ 24.8, KH_2PO_4 1.2, $MgSO_4.7H_2O$ 1.44, $CaCl_2.2H_2O$ 1.8, glucose 10.0 and pyruvic acid 10.0. In the substrate free modified Tyrode's solution, choline chloride - 7mM was substituted for glucose and pyruvic acid to maintain constant osmolarity. All reagents were Analar grade (BDH Chemicals, Poole, England) except for pyruvic acid (Sigma Chemicals, Poole, England).

4.3.2 Isolated Human right Atrial Trabeculae model

The human right atrial samples were taken from patients undergoing routine bypass operations and the trabeculae isolated from them were mounted on a perfused bath apparatus and subjected to 90 minutes of simulated ischemia (hypoxia) followed by 120 minutes of reperfusion (reoxygenation) (see section 3.3.1). At the end of the reperfusion period, the baseline functional recovery was determined (see section 3.3.4 and 3.3.5).

4.3.3 Experimental protocols

All the trabeculae were suspended in the organ bath and paced at 1 Hz, unstretched, for 30 minutes. The trabeculae were then gradually stretched in a stepwise manner over a period of 15 minutes duration until resting tension was set at the length that produced the maximum degree of force of contraction. Once this maximum developed force is achieved the trabeculae were allowed to stabilise fore 45 minutes (stabilisation phase). All the groups eventually underwent a period of simulated ischemia (SI) and reperfusion which consisted of 90 minutes of hypoxia, when the trabeculae were superfused with the hypoxic substrate free modified Tyrode's solution and rapid pacing at 3 Hz, followed by reperfusion for 120 minutes with oxygenated modified Tyrode's solution at 1 Hz. Each trabecula was used for one protocol only and they were randomly assigned to the different groups studied.

The experiment protocols for the functional recovery of the trabeculae studies are presented in Figure 4.1. The trabeculae were randomly assigned to one of the following treatment groups:

(1) ***Control Group:*** *trabeculae* were perfused with the vehicles 0.005% ethanol (n=3), or 0.01% DMSO (n=3) or Modified Tyrode's buffer alone (n=6) *during* the first 30 minutes of reperfusion following the simulated period of ischemia.

(2) ***Ischemic Preconditioning Group (IPC)***: trabeculae (n=6) were subjected to the preconditioning protocol which consisted of 3 minutes of hypoxic substrate free superfusion of modified Tyrode's buffer with rapid pacing at 3 Hz followed by 7 minutes of reperfusion with oxygenated modified Tyrode's with 1 Hz pacing.

Subsequently these trabeculae were subjected to the simulated ischemia and reperfusion protocol. This group that includes solvents group (Ethanol / DMSO) was used as a positive control for the further experiments.

(3) ***CsA-treatment***: trabeculae (n=6) were perfused with cyclosporin-A (0.2 μmol/l) *during* the first 15 minutes of reperfusion. This concentration of CsA has been demonstrated to provided the most potent inhibition of MPTP opening in adult rat myocytes[130].

(4) ***SfA-treatment***: trabeculae (n=6) were perfused with sanglifehrin-A (1.0 μmol/l) *during* the first 15 minutes of reperfusion. This concentration of SfA has been demonstrated to give the most potent inhibition of MPTP opening[139].

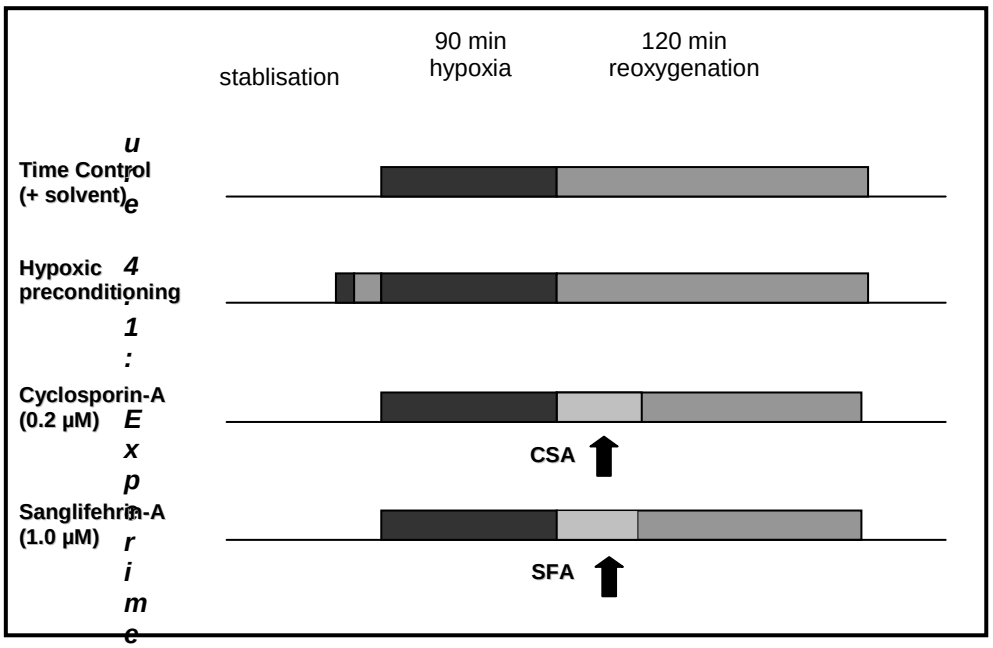

Figure 4.1: Experimental Protocols for Investigating the Effect of Inhibiting MPTP Opening on Functional Recovery of Human Right Atrial Trabeculae. CsA-cyclosporin-A, SfA-sanglifehrin-A. (Time control includes solvent group).

4.4 Results

4.4.1 Exclusions

Samples were obtained from 31 patients with stable ischemic heart disease (24 men and 7 women; age range 48-76; mean age 67 years). If two or three suitable trabeculae could be isolated from a single atrial appendage then each atrial trabeculae was allocated to one of

three experiment groups (three sets of apparatus were used simultaneously). 10 atrial trabeculae were excluded because of poor baseline contractile function.

4.4.2 Experimental Data and Results

Baseline characters such as the cross sectional area and length and weight (mass) of the trabeculae were similar in all the experimental groups (see table 4.1).

The percentage contraction of the trabeculae from their baseline at regular time intervals throughout the simulated hypoxia and reoxygenation is shown in the tables (see tables 4.2, 4.3) (Figure 4.2). The percentage contractile function were quite similar in all the groups at the end of the simulated ischemia (see table 4.2). During the reoxygenation period the recovery of the trabeculae in the IPC and in the drug groups were significantly ***(P<0.001)*** higher compared to the control group, even from the very beginning of the reoxygenation period of 15 minutes (see table 4.3). This recovery in the force of contraction of the trabeculae was maintained throughout the reoxygenation period up until 120 minutes in the IPC and drug groups (figure 4.2). Figure 4.3 shows the functional recovery of the human atrial trabeculae at the end of the experiment (30 minutes of hypoxia and 120 minutes of reperfusion). The functional recovery of the trabeculae in the IPC (48.7±4.3) and the drug groups (CsA = 48.7±2.2 and SfA = 46.4±2.2) (see table 4.3) were significantly (*P<0.001) higher than the control group (29.4±1.9).

Groups	Length (mm)	Mass (mg)	Area (mm^2)	Developed force (g)
1. Control	4.2±0.5	0.9±0.0	0.6±0.1	1.4±0.2
2. IPC	3.7±0.2	0.8±0.1	0.6±0.1	1.2±0.2
3. CsA	3.3±0.3	0.9±0.1	0.5±0.1	1.5±0.2
4. SfA	3.4±0.3	0.8±0.0	0.5±0.0	1.4±0.1

Values are mean ± SEM.

Table 4.1: Physical Characteristics of Human Atrial Trabeculae in Study Groups

Hypoxia (minutes)	Control	IPC	CsA	SfA
30 min	12.2±2.5	11.9±3.1	9.6±2.2	7.5±1.6
60 min	9.1±3.4	6.8±0.9	7.9±2.1	5.2±1.3
90 min	3.7±0.7	4.4±0.6	4.5±1.3	4.7±1.4

Values are mean ± SEM.

Table 4.2: Haemodynamic Data of Human Atrial Trabeculae in Study Groups during Ischemia (% of baseline contraction)

REOXYGENATION (minutes)	Control	IPC	CsA	SfA
15 min	17.9±3.6	32.4±7.2*	40.2±8.6*	34.4±9.2*
30 min	17.6±3.8	38.6±6.8*	35.9±6.6*	35.9±4.0*
45 min	22.5±3.6	46.7±7.6*	40.1±4.5*	38.5±4.3*
60 min	30.3±2.7	50.9±7.3*	44.6±3.5*	42.2±4.1*
75 min	24.6±2.3	51.6±6.6*	46.3±3.2*	43.6±4.1*
90 min	28.8±2.6	50.2±5.6*	47.5±3.0*	42.9±3.4*
105 min	29.9±2.4	50.2±4.9*	47.5±2.7*	44.3±3.0*
120 min	29.4±1.9	48.7±4.3*	48.7±2.2*	46.4±2.2*

Values are mean ± SEM.

Table 4.3: Contractile functional recovery of Human Atrial Trabeculae in Study Groups during Reperfusion / Reoxygenation (% of baseline contraction)

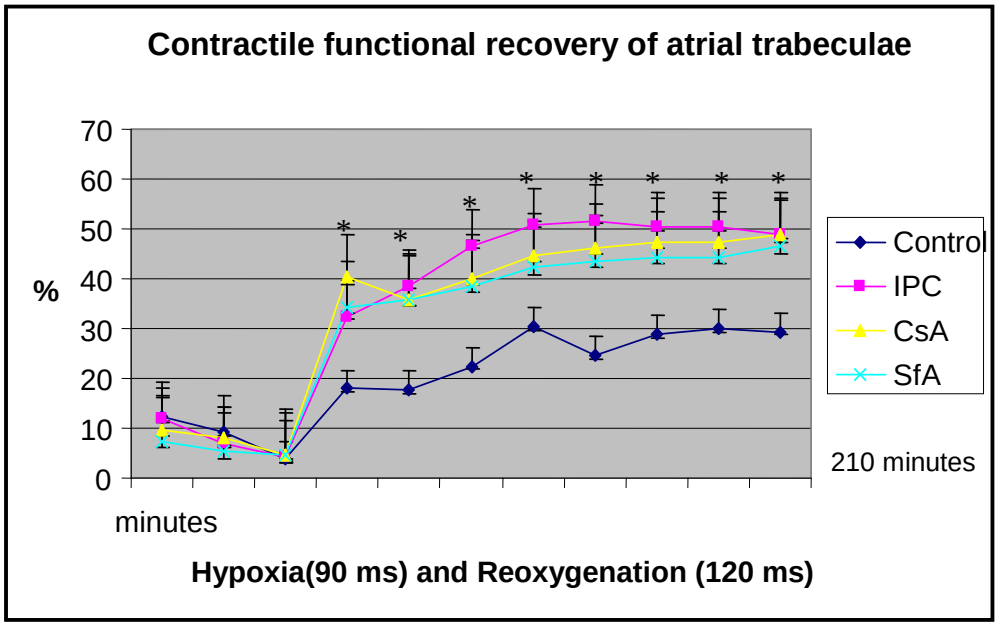

Figure 4.2: ***Inhibiting MPTP opening at the time of reperfusion improves the functional recovery of the human right atrial trabeculae and protects against lethal reperfusion injury***.
The functional recovery of the trabeculae in the IPC, CSA and SFA groups showed significant functional recovery compared to the control group from the very beginning of the reoxygenation period and was maintained throughout. *P<0.001.

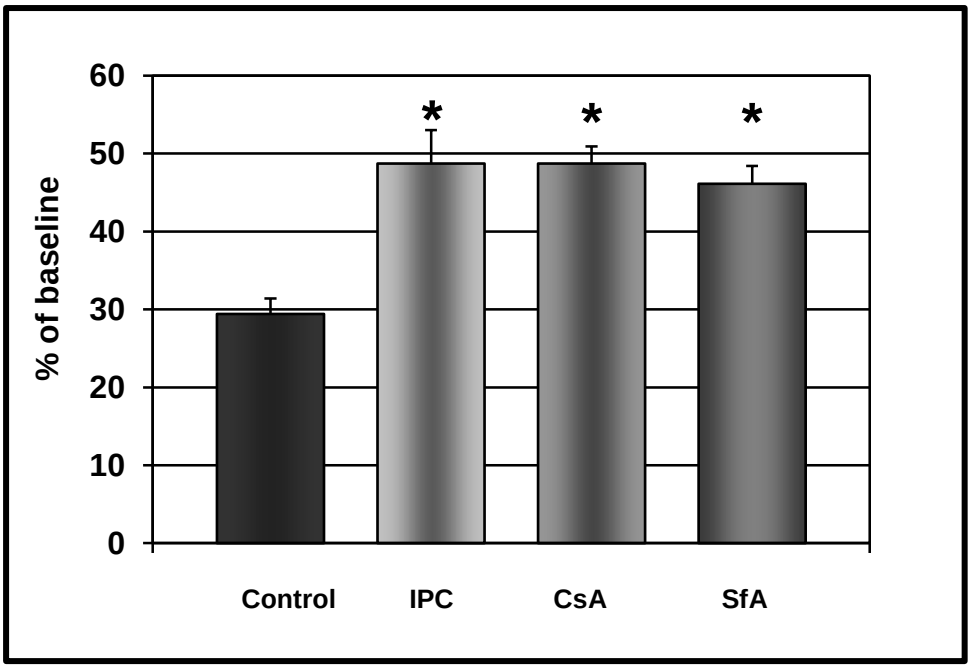

Figure 4.3 ***Inhibiting MPTP opening at the time of reperfusion protects the heart against lethal reperfusion injury and improves functional recovery***.
Pharmacologically inhibiting MPTP opening for the first 30 minutes of reperfusion using either cyclosporin-A (CsA) or sanglifehrin-A (SfA) improved the functional recovery of the trabeculae after 120 min reperfusion and so is cardioprotective. *P<0.001.

4.5 Discussion

In this part of the study we demonstrated that pharmacologically inhibiting MPTP opening for the first 30 minutes of reperfusion, following a lethal period of ischemia, using either cyclosporin-A (CsA) or sanglifehrin-A (SfA), protected the heart against lethal reperfusion injury, as evidenced by a increase in the recovery of the percentage contraction of the trabeculae from their baseline function. This is evident from the early recovery of the myocardial function even at 15 minutes of the beginning of the reperfusion period. The IPC group was used a positive control to show the effect of inhibition of MPTP with CsA and SfA. Importantly, the known MPTP inhibitors were only given at the time of reperfusion, to target the time period when MPTP opening has been demonstrated to occur[12,143,166,170]. Our experiments have shown that the opening of MPTP occurs during reperfusion and that by inhibiting their opening we could prevent the lethal effects of reperfusion injury and hence MPTP is a target for cardioprotection in human heart.

The improvement in the functional recovery induced by CsA at reperfusion was not due to its effect on calcineurin but was most likely due to its suppression of MPTP opening. In support of this, we also demonstrated protection against lethal reperfusion injury using sanglifehrin-A, which does not inhibit calcineurin[143]. Therefore, providing these agents were administered in a timely manner, we were able to inhibit MPTP opening despite the presence of the MPTP-inducing factors that prevail in the first few minutes of reperfusion.

4.6 Conclusion

In conclusion, we have demonstrated that pharmacologically inhibiting MPTP opening during early reperfusion protects the human heart against lethal reperfusion injury and improve its functional recovery. From the results of this part of the study, it appears that the opening of the MPTP at the time of reperfusion mediates lethal reperfusion injury. We used the known pharmacological MPTP inhibitors (CsA and SfA) with known concentrations that were proven to inhibit MPTP in various animal studies[68,130,139,202,214,229]. The early reperfusion therefore represent a ‘window of opportunity’ for interventions directed to attenuating lethal reperfusion injury, via inhibition of MPTP opening.

Chapter Five

THE MITOCHONDRIAL PERMEABILITY TRANSITION PORE:

A POTENTIAL TARGET FOR PROTECTING HUMAN MYOCARDIUM FROM LETHAL REPERFUSION INJURY AND ***IMPROVING CELLULAR VIABILITY***

(Cardiomyocyte model)

5.1 Introduction

Next we go on to show the effect, of inhibiting the mitochondrial permeability transition pore at reperfusion using the above known inhibitor Cyclosporin-A (CsA), on the viability of the human atrial cardiomyocytes after subjecting them to a period of SIRI.

Opening of the MPTP in the first few minutes of reperfusion, following an episode of lethal ischemia is a critical determinant of cell death in ischemia-reperfusion. Opening of the MPTP results in both apoptotic and necrotic cell death[12,166,170,209] and hence affects the viability of cells. Inhibition of MPTP opening during ischemia-reperfusion injury has been demonstrated to confer protection in various experimental models[130,139,202,214,223,274,275]. In myocytes subjected to lethal hypoxia-reoxygenation, the presence of CsA improved cell viabilit[68,127,229].

However, to our knowledge there has been no study so far to examine the effect of inhibiting MPTP opening using CsA at the time of reperfusion in human cardiomyocyte model.

Therefore the aim of this study was:

(1) To determine whether pharmacologically inhibiting MPTP opening *at the time of reperfusion,* protects the human heart against lethal reperfusion injury, using cell viability as the measured end-point, in a setting of ischemia-reperfusion injury.

(2) To characterise the model of ischemia / reperfusion in the human cardiomyocytes using hypoxic chamber to induce simulated ischemia / hypoxia simultaneously.

5.2 Hypothesis

Opening of the MPTP at the time of reperfusion is a critical determinant of cell death and inhibiting its opening is a target for cardioprotection in the human heart

In this study we set out to examine the role of the MPTP in mediating the cell death induced by lethal reperfusion injury in a human model. The aim of this study was also to characterise the model of ischemia / reperfusion (hypoxia / re-oxygenation) in the human cardiomyocyte

using the hypoxic chamber for simulating hypoxia / ischemia at the same time. We used cyclosporin-A (the archetypal MPTP inhibitor)[143] to inhibit the MPTP opening that has been demonstrated to occur in the first few minutes of reperfusion[202,203]. Cyclosporine-A can also inhibit calcineurin, which may be protective by a different mechanism. We only tested Cyclosporin-A to inhibit MPTP at reperfusion in this experiment as we were trying to establish the model of ischemia / reperfusion in human cardiomyocytes using hypoxic chamber to induce simulated ischemia, at the same time as we were trying our above hypothesis. We administered CsA ***only at the time*** of reperfusion in order to demonstrate that the opening of the MPTP is an important mediator of lethal reperfusion injury. We used samples from the right atrium of humans undergoing cardio-pulmonary bypass, in a setting of ischemia-reperfusion / hypoxia-reoxygenation model to demonstrate the effect of known pharmacological inhibitor (CsA) on MPTP opening at the time of reperfusion. We used the human cardiomyocyte model to demonstrate that inhibiting the opening of MPTP at reperfusion can be cardio-protective and increase the percentage of cellular viability.

5.3 Aim

To determine whether pharmacologically inhibiting MPTP opening at the onset of myocardial reoxygenation improves the survival of human atrial cardiomyocytes subjected to a period of sustained simulated ischemia.

5.3.1 Materials

1. *Cyclosporin-A:* (Sigma Chemicals, Poole, Dorset) were dissolved in 50% ethanol and added to the enzyme solution such that the final ethanol concentration was less than 0.005%. All other reagents were of standard analytical grade.
2. *Medium / Low-calcium (MC) solution:* comprising (in mM): NaCl 120, KCl 5.4, $MgSO_4$ 5, pyruvate 5, glucose 20, taurine 20, HEPES 10 and Ca^{2+} 1-2 μM (pH 7.4).

3. *Enzyme Solution* (mM): pH 7.4, Sodium Chloride (NaCl) 120, Potassium Chloride (KCl) 5.4, Magnesium Sulphate ($MgSO_4$) 5, Pyruvate 5, Glucose 20, Taurine 20, HEPES 10, Calcium Chloride ($CaCl_2$) 35 μM.
4. *Modified Esumi Buffer* (mM): pH 6.5, Sodium Chloride (NaCl), 137, Pottasium Chloride (KCl), 12, Magnesium Chloride ($MgCl_2$) 0.49, Calcium Chloride ($CaCl_2$) 0.9, HEPES 4, Lactate 20
5. *Sodium Dithionite (0.5 mM)*

5.3.2 Isolated Human right Atrial cardiomyocyte model

The human right atrial samples were taken from patients undergoing bypass operations and the isolated cardiomyocytes (section 3.4 and 3.5) were then placed on 6 well plates and subjected to 20 minutes of simulated ischemia (hypoxia) followed by 50 minutes of reperfusion (reoxygenation) (see section 3.5). During the reperfusion period, at three different time points (10, 30 and 50 minutes of reperfusion), the percentages of viable cells were determined.

5.3.2a Hypoxic Preconditioning Protocol

Cells were incubated in a hypoxic (PaO_2 < 3 KPa) chamber (Heraeus, Kendro Laboratory Products, Germany) for 3 minutes at 37°C in anoxic buffer with an intervening 7 minutes reoxygenation prior to undergoing hypoxic reoxygenation protocol. The anoxic buffer is a modification of that described by Esumi and colleagues[276]. The cells were in the modified Esumi buffer during the reperfusion period of seven minutes before the ischemia / reperfusion protocol, as changing the buffer for the seven minute period of reperfusion would have an additional variable to the cells i.e. centrifuging the cells for two more times and this was avoided.

5.3.2b Establishing the model of human atrial cell viability using hypoxic chamber

After stabilising the human atrial cardiomyocytes in the enzyme solution for 50-60 minutes in the incubation chamber the cells were transferred to the six well plates for the experiments. The cell suspension in the enzyme solution (falcon tube) was divided into 2 ml solutions into

eppendorffs ((1) for the control, (2) for hypoxia and reperfusion alone, (3) for IPC group and (4) for hypoxia and CsA + reperfusion). These cells were centrifuged at 1000 rpm for 1-2 minutes and re-suspended into the appropriate solutions i.e.

1) *The control group*: cells was re-suspended back in the enzyme solution into a well of the plates and transferred back to the incubator to be kept at 37°C.
2) *Ischemic preconditioning group*: the cells were re-suspended in the modified Esumi buffer and transferred into the six well plates and subjected to three minutes of hypoxia in the hypoxic chamber followed by seven minutes of reoxygenation (partial – as still in Esumi buffer) by exposing them to oxygenation in the incubator followed by hypoxic reoxygenation protocol.
3) *Hypoxic group*: the cells subjected for hypoxic protocols were re-suspended in the modified Esumi Buffer and transferred into the wells of the six well plates. This group consists of both the hypoxic group and the drug group as both were subjected for hypoxic protocol.

The plates for hypoxic protocol (both hypoxic group and drug group) were transferred to the hypoxic chamber along with sodium dithionite (separately in the hypoxic chamber). The hypoxic chamber was closed airtight and the temperature was maintained at 37°C. The air in the hypoxic chamber was evacuated using tubing connected to the cold-water tap (one way) for the first 5 minutes to produce a vacuum. For the next fifteen (15 minutes) minutes the hypoxic chamber was flushed with argon gas (BOC) to replace any oxygenated air left in the chamber and to maintain hypoxia (combined 20 minutes of hypoxia). The outlet for the argon gas from the hypoxic chamber was connected to an underwater seal to prevent air entering into the hypoxic chamber.

The ischemia was simulated by combination of (1) Modified Esumi Buffer, (2) Sodium Dithionate and (3) Hypoxia in the chamber. During this hypoxic period the control cells were counted for their baseline viability count under light microscope and the result was taken from the average of five fields from the single well by an independent observer.

At the end of the twenty minutes (of simulated ischemia the hypoxic chamber was opened and the drug Cyclosporin-A (CsA) was added to the cells that were subjected for the

drug protocol. The cells were then pippetted out into separate eppendorffs (hypoxic group and drug group) including the control and were centrifuged for 1-2 minutes at 1000 rpm. The cells from all the groups were re-suspended back in the enzyme solution (The cells in the drug group will get the drug CsA along with the enzyme solution) in separate wells of the 6 well plates, which were in turn incubated at 37°C (reoxygenation).

Next the percentage of viable cells was counted using a light microscope at three different time points of reperfusion, at 10, 30 and 50 minutes of the reperfusion period. The percentage of viable cells was taken as the average count of five fields for each well at any one-time point by an independent observer. The rod shaped cells were counted as the viable cells and the rounded ones as dead.

5.3.3 Experimental Protocols

The experiment protocols for the cellular viability of the atrial cardiomyocyte studies are presented in Figure 5.1.

The cells were assigned to one of the following treatment groups:

(1) ***Control Group:*** cardiomyocytes were stabilised in the enzyme solution perfused with the vehicles 0.005% ethanol (n=4), or 0.01% DMSO (n=4) *during* the whole experiment of 70 minutes (equivalent of 20 minutes of simulated hypoxia and 50 minutes of total reperfusion) This group was used as positive control for the further experiments.

(2) ***Hypoxic Group:*** *cardio*myocytes (n=7) were subjected to simulated hypoxia for 20 minutes in the hypoxic chamber in the modified Esumi buffer along with sodium dithionate at 37°C followed by 50 minutes of total reoxygenation in the enzyme solution in the incubator. This group was used as a positive control for the further experiments.

(3) ***Ischemic Preconditioning Group (IPC)***: cardiomyocytes (n=6) were subjected to the preconditioning protocol which consisted of 3 minutes of simulated hypoxia in the modified Esumi buffer followed by 7 minutes of reperfusion (oxygenated at 37°C in

the incubator but *still in the modified Esumi buffer*). Subsequently these cells were subjected to the simulated hypoxia and reoxygenation protocol.

(4) ***CsA-treatment***: cardiomyocytes (n=6) were perfused with cyclosporin-A (0.2 μmol/l) *during* the entire period of reperfusion. This concentration of CsA has been demonstrated to inhibit MPTP opening in the isolated perfused rat heart.

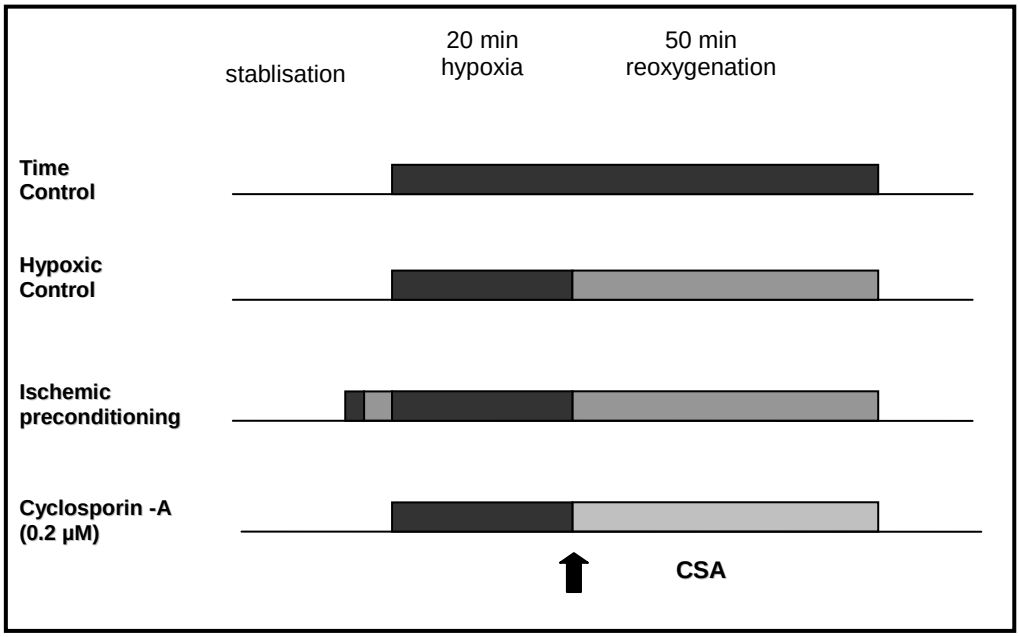

Figure 5.1: ***Experimental Protocols for Investigating the Effect of pharmacologically Inhibiting MPTP Opening on Viability of Human Right Atrial Cardiomyocytes*****.** CsA-cyclosporin-A.

5.4 Results

5.4.1 Exclusions

Samples were obtained from 29 patients with stable heart disease (20 men and 9 women; age range 48-76; mean age 67 years). When there were good yield of cells, it was possible to allocate the cells for the above four groups of experiments. 11 samples were excluded because of poor yield or no cells in the digest or dead cells (no live cells) at the beginning of the experiment.

5.4.2 Experimental Data and Results

The baseline percentage of viable cells from the time control ranged from 20% to 39% and the average was 28% (this was taken as 100% at baseline). The percentage of viable cells in the groups: hypoxic control, IPC and CsA were significantly lower compared to the time control all the way through the reperfusion period. The difference in the percentage of viable cells between the groups is evident from the beginning of reperfusion (Positive Control = 20.1±0.8, Hypoxic control = 11.1±0.9, IPC = 14.6±1.0 and CsA = 15.8±0.7) (see table 5.1). The percentage of live cells in the time control shows gradual decline from the baseline count to 50 minutes of reperfusion (from 20.1±0.8 to 14.2±1.4) (Figure 5.3). The percentages of viable cells were significantly higher in the time control compared to all other groups throughout the experiment. This can be explained by the cells in the other groups i.e. hypoxic, IPC and CsA groups undergoing stress during centrifuging (twice) and during simulated hypoxia / reoxygenation period. The viable cells in the groups IPC (12.3±1.1) and CsA (12.6±1.1) (see table 5.1) were significantly ($P<0.03$) higher in number compared to the viable cells in the hypoxic control (8.7±0.9) until 30 minutes of reperfusion Figure 5.2(a-b). At 50 minutes of reperfusion, though the cells in the groups IPC (8.8±0.6) and CsA (9.5±1.1) had more viable cells compared to their hypoxic counterpart (6.7±1.1) (see table 5.1), they were not statistically significant Figure 5.2c.

Groups	Number	10 min	30 min	50 min
1. Time Control	8	20.1±0.8**	16.9±1.1**	14.3±1.4**
2. *Hypoxic control*	7	11.1±0.9	8.7±0.9	6.7±1.1
3. IPC	6	14.7±1.0*	12.3±1.1*	8.8±0.6
4. CsA	6	15.8±0.7*	12.7±1.1*	9.5±1.1

Values are mean ± SEM.

Table 5.1: ***This table shows the average percentage of viable cells at 10, 30 and 50 minutes of reperfusion period.*** IPC – ischemic preconditioning; CsA – Cyclosporin A.

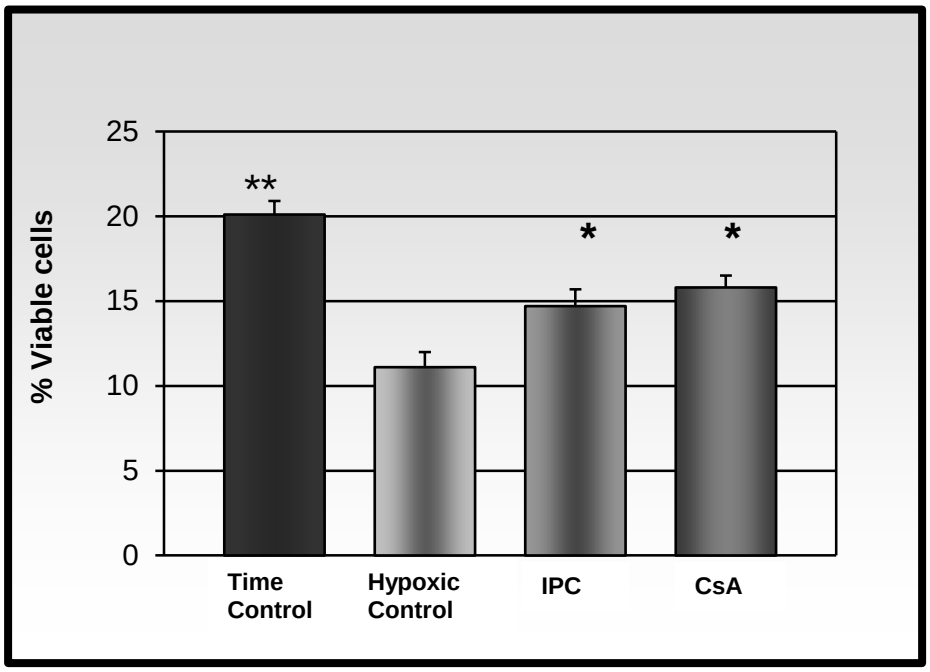

Figure 5.2.a: ***Graph showing the % of viable cells at <u>10 minutes</u> of reperfusion***. The % of viable cells in the IPC and CsA groups were statistically higher than their hypoxic control counterpart, *P<0.01; whereas the % of viable cells in the time control were statistically higher compared to the other groups, **P<0.002. IPC – ischemic preconditioning; CsA – Cyclosporin A.

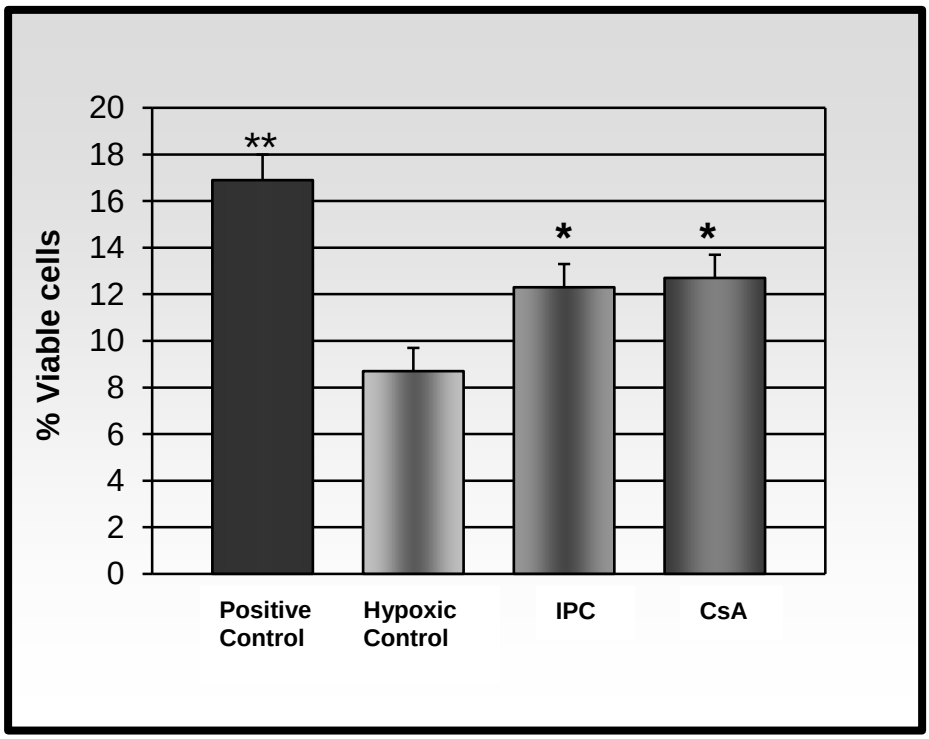

Figure 5.2.b: ***Graph showing the % of viable cells at <u>30 minutes</u> of reperfusion***. The % of viable cells in the IPC and CsA groups were statistically higher than their hypoxic control counterpart, *P<0.03; whereas the % of viable cells in the time control were statistically higher compared to the other groups, **P<0.0001. IPC – ischemic preconditioning; CsA – Cyclosporin A.

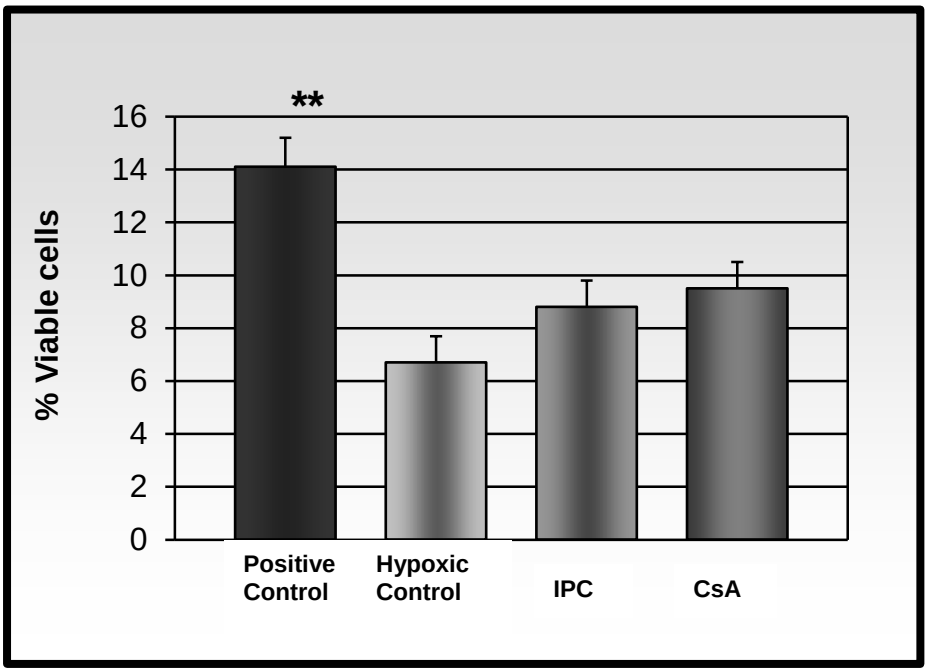

Figure 5.2.c: ***Graph showing the % of viable cells at <u>50 minutes</u> of reperfusion***. There was no statistical difference in the % of live cells between the hypoxic control, IPC and CsA groups. However the % of viable cells in the time control were statistically higher compared to the other groups, **P<0.0001. IPC – ischemic preconditioning; CsA – Cyclosporin A.

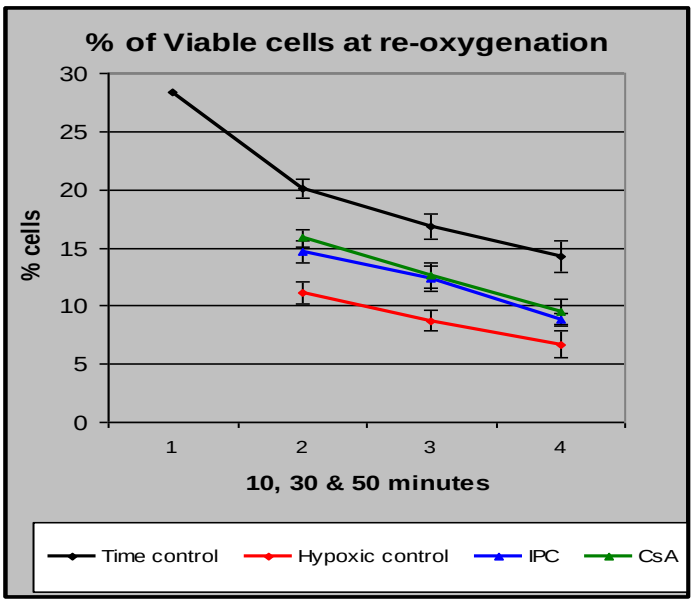

Figure 5.3: ***This graph shows the % of viable cells at reperfusion between Time control, Hypoxic control, IPC and CsA groups***. Pharmacologically inhibiting MPTP opening at reperfusion using cyclosporin-A (CsA) improved the % viability of the myocytes and so is cardioprotective. IPC – ischemic preconditioning; CsA – Cyclosporin A. *P<0.03.

5.4 Discussion

In this part of the study we demonstrated that pharmacologically inhibiting MPTP opening for the first 30 minutes of reperfusion, following a lethal period of hypoxia / ischemia, using cyclosporin-A (CsA), protected the heart against lethal reperfusion injury, as evidenced by an increase in the percentage of live cardiomyocytes. This is evident from the early recovery of the myocardial cell viability even at 10 minutes of the beginning of the reoxygenation / reperfusion period. The IPC group was used as a positive control to show the effect of inhibition of MPTP with CsA. Importantly, the known MPTP inhibitor were only given at the time of reoxygenation / reperfusion, to target the time period when MPTP opening has been demonstrated to occur[12,166,170]. Our experiments have shown that the opening of MPTP occurs during reperfusion and that by inhibiting their opening we could prevent the lethal effects of reperfusion injury and hence MPTP is a target for cardioprotection in human heart.

The cells in the IPC group were left in the Esumi buffer during their reoxygenation period for seven minutes following the index hypoxia / ischemic period of three minutes to avoid an additional variable (centrifuging X 2) to this cell group from others. This might have possibly not provided full oxygenation to the cells during the index reoxygenation / reperfusion period of seven minutes. Yet the cells in the IPC group showed a significant percentage of viable cells (12.3±1.1) compared to the percentage of viable cells in the hypoxic group (8.7±0.9) at the end of 30 minutes of reperfusion period. This suggests that reoxygenation was possible for preconditioning the cells though they were in hypoxic buffer (Esumi) for three minutes.

Therefore, providing CsA was administered in a timely manner, we were able to inhibit MPTP opening despite the presence of the MPTP-inducing factors that prevail during reperfusion.

5.6 Conclusion

In conclusion, we have demonstrated that pharmacologically inhibiting MPTP opening during reperfusion protects the human heart against lethal reperfusion injury and improve its cellular viability. From the results of this part of the study, it appears that the opening of the MPTP at the time of reperfusion mediates lethal reperfusion injury leading to necrosis and apoptosis. We used the known pharmacological MPTP inhibitor CsA with known concentrations that were proven to inhibit MPTP in various animal studies[68,130,139,202,214,229]. The first few minutes of reperfusion therefore represent a 'window of opportunity' for interventions directed to attenuating lethal reperfusion injury, via inhibition of MPTP opening.

Chapter Six

THE MITOCHONDRIAL PERMEABILITY TRANSITION PORE:

A POTENTIAL TARGET FOR PROTECTING HUMAN MYOCARDIUM FROM LETHAL REPERFUSION INJURY BY IMPROVING ***CELLULAR VIABILITY AND DECREASING NECROSIS***

(Cardiomyocyte model)

6.1 Introduction

Next we go on to show the effect, of inhibiting the mitochondrial permeability transition pore at reperfusion using the known inhibitors, Cyclosporin-A (CsA) and sanglifehrin-A (SfA), on the percentage viability, apoptosis and necrosis of the human atrial cardiomyocytes after subjecting them to a period of lethal hypoxia / ischemia. We also aimed to quantify the percentage of apoptotic and necrotic cells simultaneously in the same experiments.

Opening of the MPTP in the first few minutes of reperfusion, following an episode of lethal ischemia is a critical determinant of cell death in ischemia-reperfusion. Opening of the MPTP results in both apoptotic and necrotic cell death[12,166,170,209], and the inhibition of its (MPTP) opening during ischemia-reperfusion injury has been demonstrated to confer protection in various experimental models[68,130,139,202,214,223,229,274,275]. Although cyclosporin-A (CsA) is a potent inhibitor of MPTP opening, it also has other actions, which include inhibiting the activity of the protein phosphatase, calcineurin and therefore CsA may mediate cardio-protection via this anti-apoptotic mechanism. The novel immunosuppressant, sanglifehrin-A (SfA), has been shown, to also act as a potent inhibitor of MPTP opening[68,139,143,229,233,234] and has been demonstrated to be a more potent inhibitor of MPTP opening than CsA, and in addition SfA does not inhibit calcineurin. Studies have examined the effect of inhibiting MPTP opening using CsA or SfA at the crucial time of reperfusion alone, when the MPTP has been demonstrated to open[68,139,143,229,233,234]. All these studies were carried out in various animal and cellular models.

However, to our knowledge there has been no study so far have examined the effect of inhibiting MPTP opening using CsA and SfA at the time of reperfusion in human cardiomyocyte model. Therefore the aim of this study was to determine whether pharmacologically inhibiting MPTP opening *at the time of reperfusion,* protects the human heart against lethal reperfusion injury, using percentage cell viability, apoptosis and necrosis as the measured end-points in a setting of hypoxia- reoxygenation injury.

6.2 Hypothesis

Opening of the MPTP at the time of reperfusion is a critical determinant of cell death and inhibiting its opening is a target for cardioprotection in the human heart.

In this study we set out to examine the role of the MPTP in mediating the cell death induced by lethal reperfusion injury in a human model. We used cyclosporin-A and sanglifehrin-A (does not inhibit calcineurin) to inhibit the MPTP opening that has been demonstrated to occur in the first few minutes of reperfusion. We administered these agents ***only at the time*** of reperfusion in order to demonstrate that the opening of the MPTP is an important mediator of lethal reperfusion injury. We used samples from the right atrium of humans undergoing cardio-pulmonary bypass, in a setting of hypoxia-reoxygenation model to demonstrate the effect of known pharmacological inhibitors on MPTP opening at the time of reperfusion. We used the human cardiomyocyte model to demonstrate that inhibiting the opening of MPTP at reperfusion can be cardio-protective and increase the percentage of cellular viability and decrease the percentage of apoptotic and necrotic cells.

6.3 Aim

To determine whether pharmacologically inhibiting MPTP opening at the onset of myocardial reoxygenation improves the survival of human atrial cardiomyocytes (also decreasing the necrosis and apoptosis) subjected to a period of sustained simulated ischemia.

6.3.1 Materials

1) *Cyclosporin-A:* (Sigma Chemicals, Poole, Dorset) were dissolved in 50% ethanol and added to the enzyme solution such that the final ethanol concentration was less than 0.005%. All other reagents were of standard analytical grade.

2) *Sanglifehrin-A:* (Novartis Pharma AG, Basel) was dissolved in dimethyl sulphoxide (DMSO, Sigma Chemicals, Poole, Dorset) and added to the modified Tyrode's buffer such that the final DMSO concentration was less than 0.01%. All other reagents were of standard analytical grade.
3) *Medium / Low-calcium (MC) solution:* comprising (in mM): NaCl 120, KCl 5.4, $MgSO_4$ 5, pyruvate 5, glucose 20, taurine 20, HEPES 10 and Ca^{2+} 1-2 μM (pH 7.4).
4) *Enzyme Solution* (mM): pH 7.4, Sodium Chloride (NaCl) 120, Potassium Chloride (KCl) 5.4, Magnesium Sulphate ($MgSO_4$) 5, Pyruvate 5, Glucose 20, Taurine 20, HEPES 10, Calcium Chloride ($CaCl_2$) 35 μM.
5) *Modified Esumi Buffer* (mM): pH 6.5, Sodium Chloride (NaCl), 137, Potassium Chloride (KCl), 12, Magnesium Chloride ($MgCl_2$) 0.49, Calcium Chloride ($CaCl_2$) 0.9, HEPES 4, Lactate 20
6) *Sodium Dithionite – 0.5 mM*

6.3.2 Isolated Human right Atrial Cardiomyocyte model

The human right atrial samples were taken from patients undergoing bypass operations and the cardiomyocytes isolated from them were placed on 6 well plates and subjected to 20 minutes of simulated ischemia (hypoxia) followed by 30 minutes of reperfusion (reoxygenation) (section 3.4 and 3.5). At the end of the reperfusion period, the percentages of viable cells, apoptotic cells and necrotic cells were determined using fluorescent microscope (section 3.5).

6.3.2a Method of subjecting human atrial cardiomyocyte using hypoxic chamber for assessment of viability and percentage necrosis and apoptois under fluorescent microscope

After stabilising the human atrial cardiomyocytes in the enzyme solution for 50-60 minutes in the incubation chamber the cells were transferred to the six well plates for the experiments. The cell suspension in the enzyme solution was divided into 2 ml solutions into eppendorffs

[2ml each – (1) for the control, (2) for hypoxia and reperfusion alone, (3) for hypoxia and CsA + reperfusion and (4) for hypoxia and SfA + reperfusion]. These cells were centrifuged at 1000 rpm for 1-2 minutes and re-suspended in the appropriate solutions i.e.

1) The control group: cells was re-suspended back in the enzyme solution into a well of the six well plates and transferred back to the incubator to be kept at 37°C.
2) Hypoxic group: the cells subjected for hypoxic protocols were re-suspended in the modified Esumi Buffer and transferred into the wells of the six well plates. This group consists of both the hypoxic group and the drug groups (CsA and SfA) as all of them were subjected for hypoxic protocol.

The cardiomyocytes in the plates were subjected to respective protocol as above - i.e. the control was re-suspended back into the enzyme solution in a well of the plates and put back in the incubator, and the cells subjected for hypoxic protocols were re-suspended into the modified Esumi Buffer and transferred into the wells of the six well plates. The plates for hypoxic protocol were transferred to the hypoxic chamber along with 0.5 mM of sodium dithionite (separately). The hypoxic chamber was closed airtight and the temperature was maintained at 37°C. The air in the hypoxic chamber was sucked out using tubing connected to the cold-water tap (one way) for the first 5 minutes to produce vacuum. For the next fifteen (15 minutes) minutes the hypoxic chamber was flushed with argon gas (BOC) to replace any oxygenated air left in the chamber and to maintain hypoxia (combined 20 minutes of hypoxia). The outlet for the argon gas from the hypoxic chamber was connected to an underwater seal to prevent air entering into the hypoxic chamber.

The ischemia / hypoxia was simulated by combination of (1) Modified Esumi Buffer, (2) Sodium Dithionate and (3) Hypoxia in the chamber. During this hypoxic period the control cells were counted for their baseline viability count under light microscope and the result is taken from the average of five fields from the single well by an independent observer.

At the end of the twenty minutes of simulated ischemia the hypoxic chamber was opened and the drugs Cyclosporin-A (CsA) and sanglefehrin (SfA) were added to the cells respectively that are subjected for the drug protocol. Now the cells were pippetted out into separate eppendorffs including the control and are centrifuged for 1-2 minutes at 1000 rpm. The cells from all the groups were re-suspended into the enzyme solution (The cells in the

drug groups will get the drug CsA and SfA along with the enzyme solution) in separate wells of the 6 well plates, which were in turn incubated at 37°C.

During the reperfusion period cardiomyocytes were subjected to Annexin-V (A-V) and Propidium Iodide (PI) and were worked under darkness to prevent photosensitisation as follows[265,266]. At the end of 20 minutes of reperfusion, 200ul of Annexin-V (A-V) was added to the cardiomyocytes in the wells and reincubated. Similarly at the end of 30 minutes of reperfusion 1ml of Propidium Iodide (PI) was added to the myocytes just before the cardiomyocytes were subjected for counting under fluorescent microscope. The percentages of viable cells, necrotic cells and apoptotic cells were counted under the fluorescent microscope at the end of 30 minutes of the reperfusion period. The percentages of viable cells, necrotic cells and apoptotic cells were taken as the average count of three fields from each well by an independent person.

The cells were accounted as follow (Figure 6.1):

- Live cells = rod shaped cells and no yellow stained nucleus.
- Necrotic cells = cells with yellow stained nucleus.
- Apoptotic cells = round cells and no yellow stained nucleus.

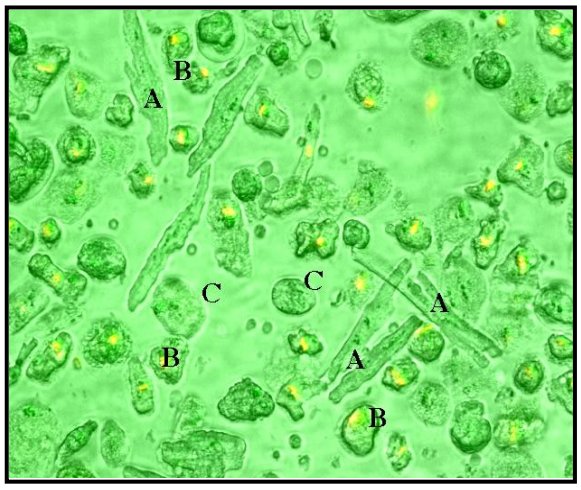

Figure 6.1 **Eample of picture showing Cardiomyocytes under fluorescent microscope after staining with Annexin-V and Propidium Iodide. A – Live cells (**rod shaped**); B – Necrotic cells (**round cells with yellow stained nucleus**) and C – Apoptotic cells (**round cells with no stained nucleus**).**

6.3.3 Experimental Protocols

The experiment protocols for the cellular viability of the atrial cardiomyocyte studies are presented in Figure 6.1. The cells were assigned to one of the following treatment groups:

(1) ***Control Group:*** *cardiomyocytes* (n=6) were stabilised in the enzyme solution *during* the entire experiment of 70 minutes (equivalent of 20 minutes of simulated hypoxia and thirty minutes of reperfusion) This group was used as a positive (positive) control for the further experiments.

(2) ***Hypoxic Group:*** *cardio*myocytes (n=6) were subjected to simulated ischemia for 20 minutes in the hypoxic chamber in the modified Esumi buffer along with sodium dithionate at 37°C followed by 30 minutes of reperfusion in the enzyme solution in the incubator.

(3) ***CsA-treatment***: *cardio*myocytes (n=6) were subjected to simulated ischemia for 20 minutes followed by reperfusion with cyclosporin-A (0.2 μmol/l) in the enzyme solution during the *entire* period of reperfusion (30 minutes).

(4) ***SfA-treatment***: *cardio*myocytes (n=6) were subjected to simulated ischemia for 20 minutes followed by reperfusion with sanglifehrin-A (1.0 μmol/l) in the enzyme solution during the *entire* period of reperfusion (30 minutes).

(5) ***DMSO Group:*** *cardiomyocytes* (n=3) were subjected to simulated ischemia for 20 minutes followed by reperfusion with DMSO in the enzyme solution *during* the entire experiment of reperfusion (30 minutes). This group was used as a positive control for the drug group SfA.

(6) ***50% Ethanol Group:*** *cardiomyocytes* (n=3) were subjected to simulated ischemia for 20 minutes followed by reperfusion with 50% ethanol in the enzyme solution *during* the entire period of reperfusion (30 minutes). This group was also used as a positive control for the drug group CsA.

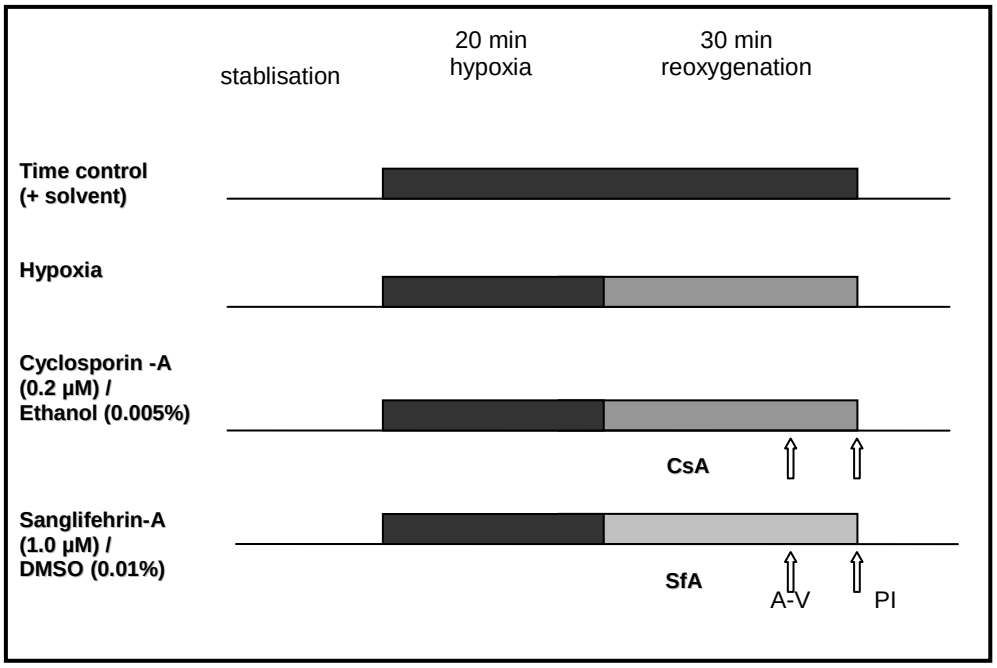

Figure 6.2: ***Experimental Protocols for Investigating the Effect of CsA and SfA Inhibiting MPTP Opening on Viability of Human Right Atrial Cardiomyocytes*.** CsA – Cyclosporin A; SfA – Sanglifehrin A; DMSO - dimethyl sulphoxide. (Time control includes solvent group).

6.4 Results

6.4.1 Exclusions

Samples were obtained from 36 patients with stable heart disease (27 men and 9 women; age range 51-78; mean age 67 years). When there were good yield of cells, it was able to allocate the cells for the above four-six groups of experiments. 14 samples were excluded because of poor / no cells in the digest or dead cells (no live cells) at the beginning of the experiment.

6.4.2 Experimental Data and results

The baseline percentage of viable cells from the time control ranged from 22% to 39% and the average was 29%. The percentage of viable cells in the groups: CsA (15.8±0.7) and SfA (15.0±1.1) were significantly higher (P<0.001) compared to the hypoxic control (10.8±0.9) as well as their vehicle controls i.e. DMSO (9.3±0.9) and 50% Ethanol (9.0±1.2), (Figure 5.2a). The percentage of viable cells in the time control (16.3±1.2) was also significantly higher than the hypoxic and vehicle control groups (Figure 5.2a).

The percentage of necrotic cells in the drug groups CsA (48.5±1.6) and SfA (47.6±1.2) were significantly lower (P<0.04) than the hypoxic group (54.8±1.0) and also significantly lower than their vehicle control groups i.e. DMSO (54.0±0.6) and 50% Ethanol (56.3±1.2), (Figure 5.2c).

Interestingly our experiment failed to show any statistical significant difference in the percentage of apoptotic cells among all the groups (Figure 5.2b).

Groups	Number	% Live cells	% Apoptotic cells	% Necrotic cells
1. Time Control	6	16.3±1.2#	37.3±3.4	46.5±4.0
2. *Hypoxic control*	6	10.8±0.9	34.2±1.0	54.8±1.0
3. CsA	6	15.8±0.7*	36.2±1.6	48.5±1.6*
4. SfA	6	15.0±1.1*	37.5±1.8	47.7±1.2*
5. DMSO	3	9.3±0.9	37.0±0.6	54.0±0.6
6. 50% Ethanol	3	9.0±1.2**	34.7±0.33	56.3±1.2**

Values are mean ± SEM.

Table 6.1: ***This table shows the percentage of Viable, Apoptotic and Necrotic cells at 30 minutes of reperfusion period.*** CsA – Cyclosporin A; SfA – Sanglifehrin A; DMSO - dimethyl sulphoxide.

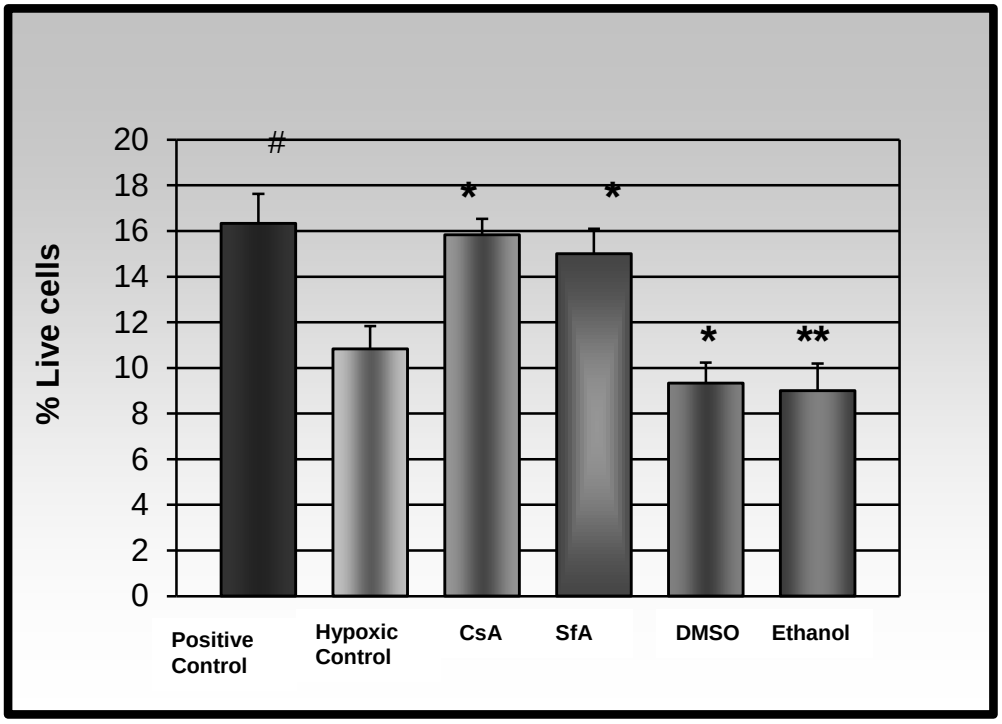

Figure 6.3.a: ***This graph shows the % of live cells (cardiomyocytes) at 30 minutes of reperfusion***. The % of live cells in the CsA and SfA groups were statistically higher than their hypoxic control counterpart, *P<0.001; as well as their vehicle groups, **P<0.001; whereas the % of live cells in the time control were statistically higher compared to the hypoxic and vehicle groups, #P<0.0002. CsA – Cyclosporin A; SfA - Sanglifehrin A; DMSO - dimethyl sulphoxide.

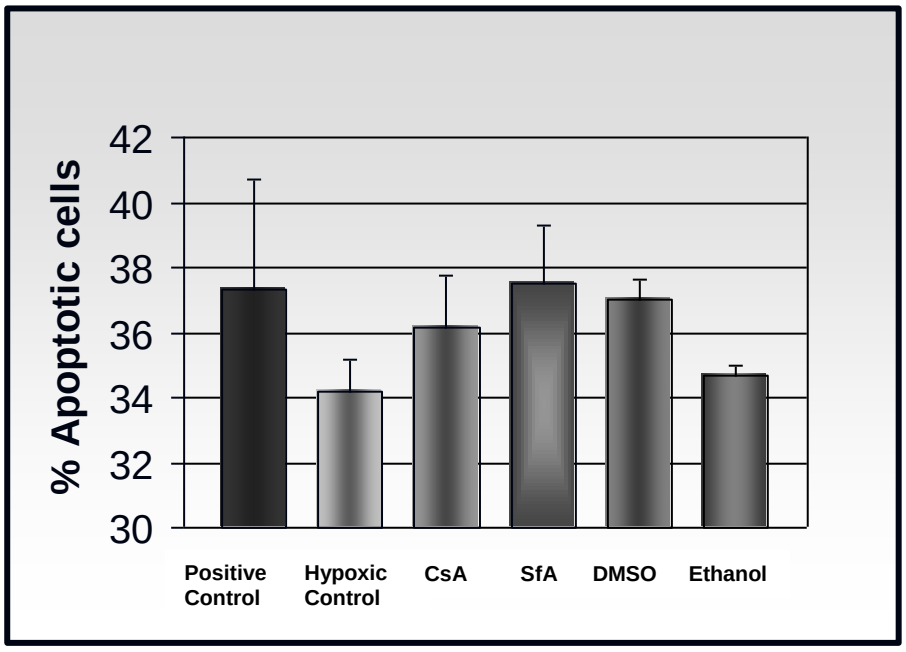

Figure 6.3.b: ***This graph shows the % of apoptotic cells at 30 minutes of reperfusion***. The % of apoptotic cells in all the groups were not statistically significant compared to each other, though there appears to be less apoptotic cells in the hypoxic and ethanol groups. CsA – Cyclosporin A; SfA - Sanglifehrin A; DMSO - dimethyl sulphoxide.

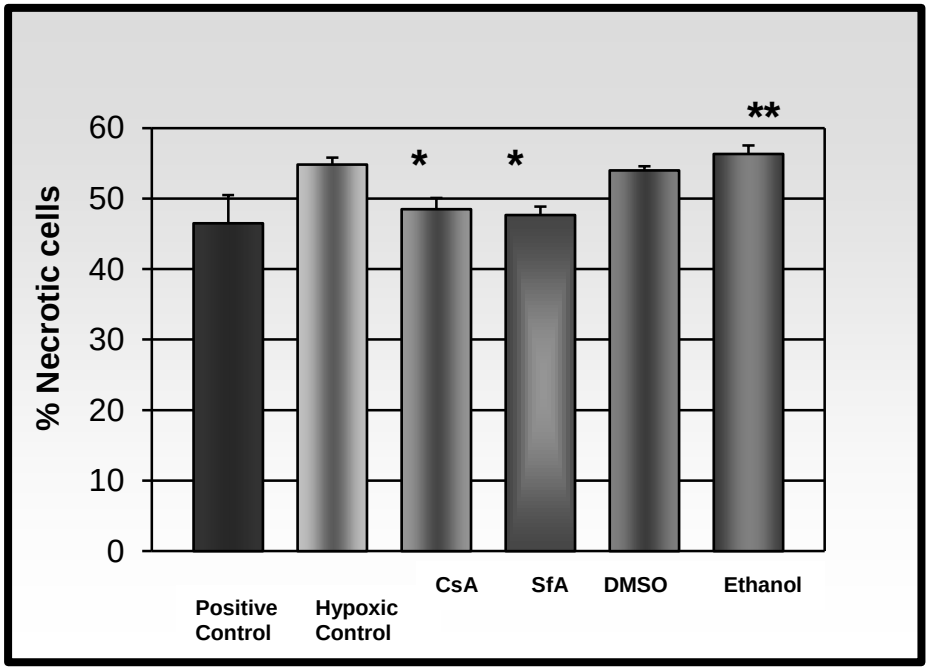

Figure 6.3.c: ***This graph shows the % of necrotic cells at 30 minutes of reperfusion***. The % of necrotic cells in the CsA and SfA groups were statistically lower than their hypoxic control counterpart as well as the ethanol vehicle group, *P<0.04; The % of necrotic cells in the 50% ethanol vehicle group were statistically higher than their time control counterpart, **P<0.01. CsA – Cyclosporin A; SfA - Sanglifehrin A; DMSO - dimethyl sulphoxide.

6.5 Discussion

In this part of the study we demonstrated that pharmacologically inhibiting MPTP opening for the first 30 minutes of reperfusion, following a lethal period of ischemia, using either cyclosporin-A (CsA) or sanglifehrin-A (SfA), protected the heart against lethal reperfusion injury, as evidenced by an increase in the recovery of the percentage live cells and a decrease in the percentage of necrotic cells. Importantly, the known MPTP inhibitors were only given at the time of reperfusion, to target the time period when MPTP opening has been demonstrated to occur[12,143,166,170]. Our experiments have shown that the opening of MPTP occurs during reperfusion and that by inhibiting its opening we could prevent the lethal effects of reperfusion injury and hence the MPTP is a target for cardioprotection in the human heart.

The improvement in the percentage of live cells and decrease in the percentage of necrotic cells, induced by CsA at reperfusion was not due to its effect on calcineurin but was most likely due to its suppression of MPTP opening. In support of this, we also demonstrated protection against lethal reperfusion injury using sanglifehrin-A, which does not inhibit calcineurin[139,143]. Studies have demonstrated that the pharmacological inhibition of the apoptotic signalling cascade during the reperfusion phase is able to attenuate both the apoptotic and necrotic components of cell death[53,219,277]. Our study was able to show attenuation of the necrotic component of the human cardiac cardiomyocytes.

The apoptotic component of cell death is believed to contribute to the extension of myocardial infarct size during reperfusion. Zhao and colleagues, demonstrated that pharmacologically inhibiting the reperfusion-induced apoptotic component of cell death resulted in improved contractile function of ischemic canine hearts[53]. Inhibiting MMP using MPTP inhibitors such as cyclosporine-A or bongkrekic acid have been demonstrated to inhibit apoptotic cell death[223,274,275], suggesting that MPTP opening is an obligatory mediator of apoptosis. However, recent data in CyP-D deficient mice suggests that the CypD-regulated MPTP may not be crucial for apoptotic cell death which may explain why we failed to see an effect on the apoptotic component of cell death[195,278]. Although in the chapter four we managed to show statistical significance in the recovery of percentage contractile force of the human atrial trabeculae and show statistically significant improvement in the percentage of live cells (in chapter 5 and 6), our study did not show any statistically significant difference in the percentage of apoptotic cells among all the groups. Yet there seems to be a slightly increased percentage of apoptotic cells in the drug group (Figure 5.2b).

Therefore, providing these agents were administered in a timely manner, we were able to inhibit MPTP opening despite the presence of the MPTP-inducing factors that prevail during reperfusion.

6.6 Conclusion

In conclusion, we have demonstrated that pharmacologically inhibiting MPTP opening during reperfusion protects the heart against lethal reperfusion injury as evident by increase in the percentage of viable cells and decrease in percentage of necrotic cells. From the results of this part of the study, it appears that the opening of the MPTP at the time of reperfusion mediates lethal reperfusion injury. The early period of reperfusion therefore represent a 'window of opportunity' for interventions directed to attenuating lethal reperfusion injury, via inhibition of MPTP opening.

Chapter Seven

THE MITOCHONDRIAL PERMEABILITY TRANSITION PORE:

A POTENTIAL TARGET FOR PROTECTING HUMAN MYOCARDIUM FROM LETHAL REPERFUSION INJURY BY ***DELAYING OPENING OF THE MPTP IN HUMAN ATRIAL CARDIOMYOCYTES***

7.1 Introduction

Next we go on to show that MPTP opening occurs in human cardiomyocytes and also the direct effect, of inhibiting the mitochondrial permeability transition pore using the known inhibitors, Cyclosporin-A (CsA) and sanglifehrin-A (SfA), against injury induced by oxidative stress. We aimed to quantify the time delay in opening of the MPTP induced by oxidative stress.

Opening of the MPTP in the first few minutes of reperfusion, following an episode of lethal ischemia is a critical determinant of cell death in ischemia-reperfusion. Opening of the MPTP results in both apoptotic and necrotic cell death, and the inhibition of its (MPTP) opening during ischemia-reperfusion injury has been demonstrated to confer protection in various experimental models[68,139,143,229,233,234]. Although cyclosporin-A (CsA) is a potent inhibitor of MPTP opening, it also has other actions, which include inhibiting the activity of the protein phosphatase, calcineurin. The novel immunosuppressant, sanglifehrin-A (SfA), has been shown, to also act as a potent inhibitor of MPTP opening. This drug has been demonstrated to be a more potent inhibitor of MPTP opening than CsA, and in addition SfA does not inhibit calcineurin[68,139,143,229,233,234]. All these studies were carried out in various animal and cellular models. Opening of the MPTP in adult rat myocytes was induced and detected using a well-characterised cellular model of oxidative stress[201,267-270].

However, to our knowledge there has been no study so far have examined the direct effect of inhibiting MPTP opening using known MPTP inhibitors CsA and SfA at the time of oxidative stress induced MPTP opening in human cardiomyocyte model. Therefore the aim of this study was to determine whether pharmacologically inhibiting MPTP opening can protect the human heart against lethal oxidative stress induced MPTP opening, using the percentage time delay of the opening of the MPTP as the measured end-point.

7.2 Hypothesis

Opening of the MPTP at the time of reoxygenation / reperfusion is a critical determinant of cell death and inhibiting its opening is target for cardioprotection in the human heart.

In this study we set out to demonstrate that MPTP opening does occur in human cardiomyocyte and also examine the role of the MPTP in mediating the cell death induced by lethal injury of oxidative stress in human cardiomyocyte model. We used cyclosporin-A and sanglifehrin-A to inhibit the MPTP opening that has been demonstrated to occur in the first few minutes of reoxygenation / reperfusion. We administered these agents ***at the time*** of opening of MPTP which is shown to occur at the time of reoxygenation / reperfusion in order to demonstrate that the opening of the MPTP is an important mediator of lethal reperfusion injury. We used samples from the right atrium of humans undergoing cardio-pulmonary bypass to demonstrate the effect of known pharmacological inhibitors on MPTP opening at the time of its opening. We used the human cardiomyocyte model to demonstrate the opening of MPTP and that inhibiting / delaying the opening of MPTP can be cardio-protective.

7.3 Aim

To demonstrate that MPTP opening occurs in human atrial cardiomyocytes subjected to oxidative stress, and that MPTP opening can be inhibited by known pharmacological MPTP inhibitors.

7.3.1 Materials

1. *Cyclosporin-A* (Sigma Chemicals, Poole, Dorset) were dissolved in 50% ethanol and added to the enzyme solution such that the final ethanol concentration was less than 0.005%.
2. *Sanglifehrin-A* (Novartis Pharma AG, Basel) was dissolved in dimethyl sulphoxide (DMSO, Sigma Chemicals, Poole, Dorset) and added to the enzyme solution such

that the final DMSO concentration was less than 0.01%. All other reagents were of standard analytical grade.

3. *Low calcium medium* (mM): pH 6.96, Sodium Chloride (NaCl) 120, Potassium Chloride (KCl) 5.4, Magnesium Sulphate (MgSO4) 5, Pyruvate 5, Glucose 20, Taurine 20, HEPES 10, NTA 5, calcium 1-2 µM.
4. *Enzyme Solution* (mM): pH 7.4, Sodium Chloride (NaCl) 120, Potassium Chloride (KCl) 5.4, Magnesium Sulphate ($MgSO_4$) 5, Pyruvate 5, Glucose 20, Taurine 20, HEPES 10, Calcium Chloride ($CaCl_2$) 35 µM.

7.3.2 Isolated Human right Atrial cardiomyocyte model of MPTP opening

Adult human atrial cardiomyocytes were isolated from according to the method in described in section 3.4. Isolated cardiomyocytes were seeded onto 25-mm round cover slips according to the method in described in section 3.6. Seeded cardiomyocytes, in enzyme solution, were incubated with the fluorescent dye, TMRM (3 µmol/l) for 15 minutes at 37°C, and visualised using confocal fluorescence microscopy, as described in section 3.6.

TMRM which is a lipophilic cation selectively accumulates into mitochondria according to the mitochondrial membrane potentia[271]. Opening of the MPTP was induced and detected using a cellular model of oxidative stress. Laser-illumination, of this TMRM that has already accumulated within the mitochondria, photosensitises the TMRM, generating reactive oxygen species (ROS) from within the mitochondria[272], which induce opening of the MPTP. Opening of the MPTP permeates the inner mitochondrial membrane resulting in uncoupling of oxidative phosphorylation. This leads to subsequent collapse of the mitochondrial membrane potential[267,279]. Oxidative stress generated on reperfusing ischemic myocardium also involves the excess production of ROS from within the mitochondria, and this model therefore simulates the events associated with lethal reperfusion injury. This model represents a widely reported and reliable way to reproducibly induce the loss of mitochondrial membrane potential, which has been unequivocally identified as MPTP opening[180,267,268,270,279].

The relatively high concentration of TMRM in the mitochondria causes auto-quenching of fluorescence and the fluorescence signal becomes a non-linear function of dye

concentration. The mitochondrial membrane depolarisation results in the loss of dye into the cytosol where the signal increases[272]. Laser-induced oxidative stress was applied until MPTP opening had been induced (indicated by collapse of the mitochondrial potential) and the duration of time taken to induce MPTP opening was measured (corresponding to the ROS burden required to induce MPTP opening).

7.3.3 Experimental Protocols

After loading with TMRM in the enzyme solution, cells were randomly assigned to one of the following treatment groups. Cells in the drug groups were incubated for 15 minutes with the drug(s) at 37˚C and were then subjected to the TMRM-oxidative stress protocol.

(1) ***Control Group:*** *cardiomyocytes* (n=11) were stabilised in the enzyme solution *during* the entire experiment.

(2) ***CsA-treatment***: cardiomyocytes (n=10) were perfused with cyclosporin-A (0.2 μmol/l) in the enzyme solution during the *entire* period of experiment along with TMRM.

(3) ***SfA-treatment***: cardiomyocytes (n=10) were perfused with sanglifehrin-A (1.0 μmol/l) in the enzyme solution during the *entire* period of experiment along with TMRM.

(4) ***DMSO Group:*** *cardiomyocytes* (n=6) were perfused with DMSO in the enzyme solution *during* the entire experiment. This group was used as a positive control for the drug group SfA.

(5) ***50% Ethanol Group:*** *cardiomyocytes* (n=6) were perfused with 50% ethanol in the enzyme solution *during* the entire experiment. This group was also used as a positive control for the drug group CsA.

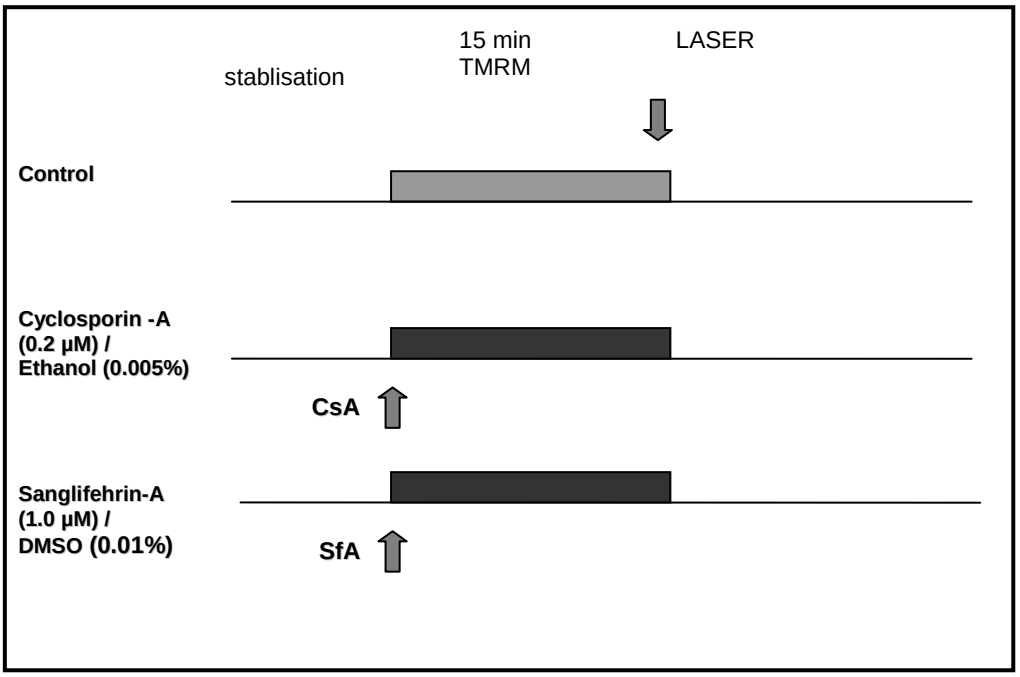

Figure 7.1: ***Experimental Protocols for Investigating the Effect of CsA and SfA on Direct Inhibition of MPTP Opening using oxidative stress model of TMRM.*** CsA – cyclosporin A; SfA – Sanglifehrin A; DMSO - dimethyl sulphoxide

7.4 Results

7.4.1 Exclusions

Samples were obtained from 35 patients with stable heart disease (28 men and 7 women; age range 49-74; mean age 62 years). When there were good yield of cells, it was able to allocate the cells for the above four-six groups of experiments. 4 samples were excluded because of poor / no cells in the digest or dead cells (no live cells) at the beginning of the experiment.

7.4.2 Experimental Data and results

The times taken to induce global mitochondrial membrane depolarisation were noted and in the control group, MPTP opening was routinely induced after (115.6±7.8) (see table 7.1) seconds of laser-induced oxidative stress (see figure 7.2). The times taken to induce global mitochondrial membrane depolarisation in the drug group CsA (189.1±10.2) and SfA (183.0±12.2) (see table 7.1) were significantly ($P<0.0001$) higher than the control group and their vehicle groups i.e. DMSO (109.8±8.4) and 50% Ethanol (99.8±5.2) (see table 7.1).

This observed global mitochondrial membrane depolarisation induced by laser induced oxidative stress actually represents opening of the MPTP and this was demonstrated by showing that it was sensitive to cyclosporin-A (CsA), the most reliable inhibitor of MPTP opening[96]. CsA was shown to extend the time required to induce opening of the MPTP from 115.6±7.8 seconds to 189.1±10.2 seconds ($P<0.0001$, figures 7.2). However, as well as inhibiting MPTP opening, CsA inhibits the phosphatase, calcineurin.

To exclude the effect of CsA-induced inhibition of calcineurin, we tested with sanglifehrin-A[139]. SfA also significantly prolonged the time taken to induce both MPTP opening to 183.0±12.2 ($P<0.0001$, figure 7.2). Furthermore, sanglifehrin-A was shown to be a similar potent inhibitor of MPTP opening as CsA in extending the time taken to induce MPTP opening.

Groups	Number	Time (seconds)
1. Control	11	115.6±7.8
2. CsA	10	189.1±10.2*
3. SfA	10	183.0±12.2*
4. DMSO	6	109.8±8.4
5. 50% Ethanol	6	99.8±5.2

Values are mean ± SEM.

Table 7.1: ***This table shows the time taken to induce MPTP opening (seconds) by laser induced oxidative stress.*** CsA – Cyclosporin A; SfA – Sanglifehrin A; DMSO - dimethyl sulphoxide.

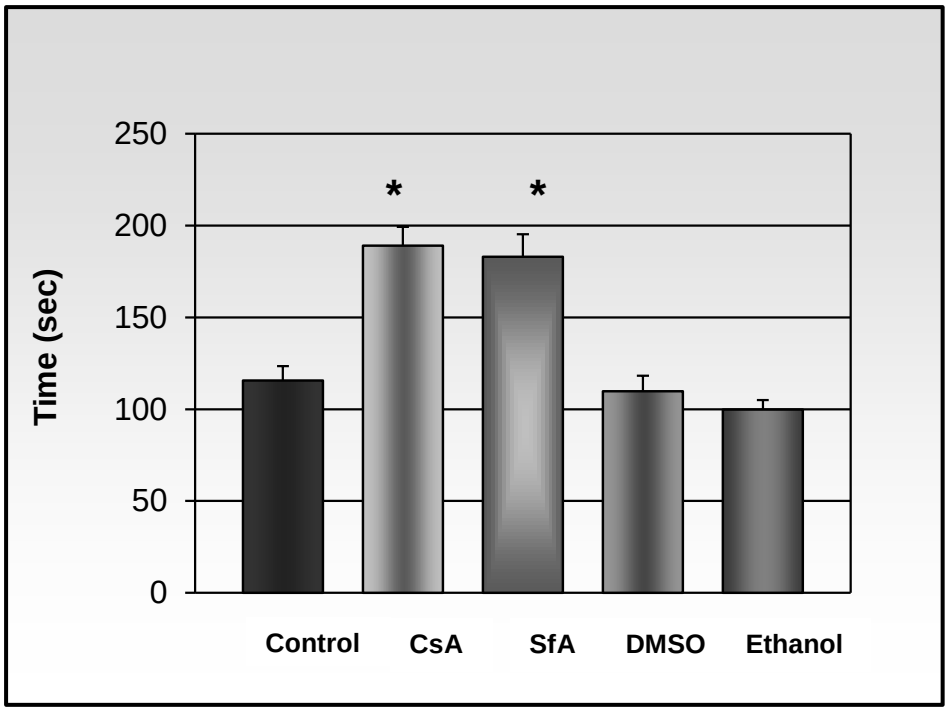

Figure 7.2: ***This graph shows the time taken for MPTP opening indicated by the membrane depolarisation due to oxidative stress induced by TMRM.*** The time taken for MPTP opening of the cells in the CsA and SfA groups were statistically higher than their control counterpart (including groups DMSO and 50% Ethanol), *P<0.0001. CsA – Cyclosporin A; SfA - Sanglifehrin A; DMSO - dimethyl sulphoxide.

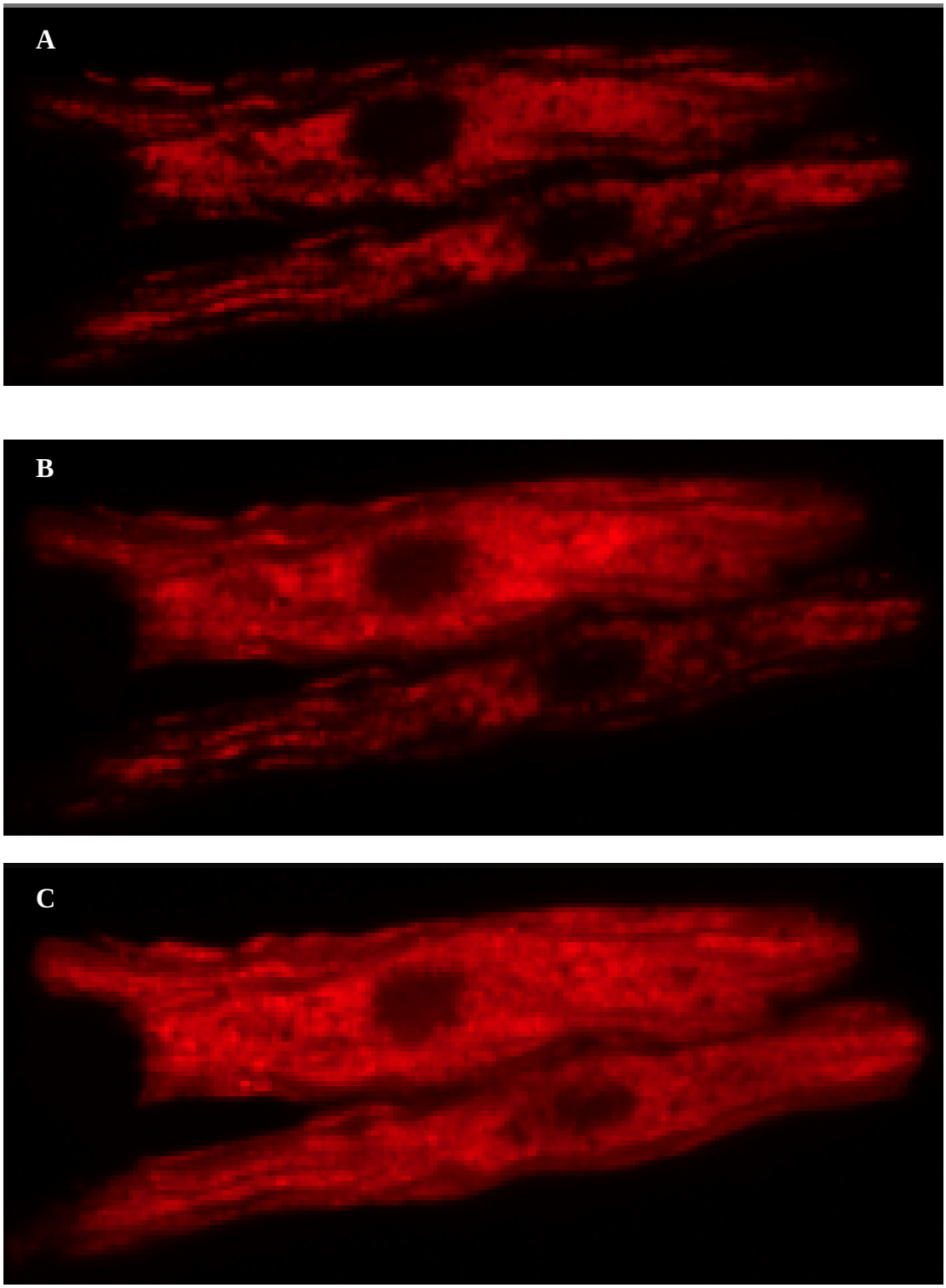

Figure 7.3: ***Confocal Images of TMRM-loaded Human Atrial Cardiomyocytes.*** Confocal fluorescent images are from time sequences in which two cardiomyocytes were subjected to laser-induced oxidative stress. They demonstrate the typical changes in mitochondrial membrane potential that occur in response to oxidative stress: (a) Beginning of oxidative stress. (b) Cardiomyocyte with partially depolarised membrane (localised areas devoid of TMRM fluorescence). (d) Cardiomyocyte with global mitochondrial depolarisation (as MPTP opening has taken place in most of the mitochondria).

7.5 Discussion

This technique has been used in a number of different laboratories and represents a reproducible technique for inducing and detecting MPTP opening in the intact cell[148,267-270,279,280]. We used an established model for both inducing and detecting MPTP opening in the intact human cardiomyocyte. Laser-illumination of TMRM generates oxidative stress from within mitochondria, inducing MPTP opening and is detected by global mitochondrial membrane depolarisation. Following the induction of MPTP opening continued oxidative stress results in ATP depletion.

The laser-induced collapse in mitochondrial membrane potential (detected by increased TMRM fluorescence) was demonstrated to be sensitive to the potent MPTP inhibitor, cyclosporine-A (CsA)[125]. We also demonstrated that the observed mitochondrial membrane depolarisation was sensitive to sanglifehrin-A[68,229,233,234].

In the current model to investigate MPTP opening, oxidative stress by laser illumination was continued until global mitochondrial membrane depolarisation has occurred which in turn reflects the opening of the MPTP. Both the control and drug-treated cells were exposed to equal burdens of oxidative stress. One would expect neither CsA nor SfA to completely suppress global mitochondrial membrane depolarisation (MPTP opening) under these extreme conditions of oxidative stress. From the current data it is clear that CsA and SfA increases the tolerance of the human myocardial cells for global mitochondrial membrane depolarisation to oxidative stress compared to control, suggesting that both CsA and SfA are able to delay the global mitochondrial membrane depolarisation and in turn suggesting that MPTP opening can be inhibited at the time of reperfusion.

7.6 Conclusion

In conclusion, we have demonstrated that MPTP opening does occur in human cardiomyocyte and pharmacologically inhibiting this MPTP opening protects the heart against lethal oxidative stress induced injury as evident by increase in the percentage of time duration for the opening of the MPTP. From the results of this part of the study, it appears that the opening of the MPTP mediates lethal injury. The early phase of reperfusion where MPTP opening is shown to occur, therefore represent a 'window of opportunity' for interventions directed to attenuating lethal reperfusion injury, via inhibition of MPTP opening.

Chapter Eight:

SUMMARY AND DISCUSSION

Coronary artery disease and myocardial infarction are a major cause for increasing mortality and morbidity in our population. To improve our current treatments novel therapeutic approaches are urgently required. Primary prevention strategies such as lifestyle changes, diet, exercise, anti-platelet agents, statins, ACE-inhibitors, HMG-CoA reductase inhibitors and secondary prevention strategies like anti-platelet agents, thrombolytic agents, primary PTCA, drug-eluting stents and surgery have made a significant impact.

The powerful phenomenon of ischemic preconditioning was studied and described by Murry et al in 1986[3]. This phenomenon of myocardial preconditioning has been well established and experiments have shown that it can deliver a powerful protection towards the heart against the lethal ischemia-reperfusion injury. In practice, preconditioning means intervening before the index ischemic episode of the acute coronary syndrome. This is difficult practically. Nevertheless preconditioning can be applied as an adjuvant to patients during acute coronary syndrome and to patients undergoing cardiac surgery.

Hence a practical cardio-protective strategy is to intervene after the index ischemic event. Currently adjuncts to reperfusion treatment strategies such as thrombolysis, primary PTCA and cardiac intervention offers a powerful protective approach.

To salvage the myocardium that is viable following ischemia, reperfusion process is essential and this reperfusion is paradoxically associated with cardiomyocyte death that was viable at the beginning of reperfusion[281]. This phenomenon is described as 'lethal myocardial reperfusion injury', and this clearly compromises the complete benefit of myocardial reperfusion[2,55]. Currently we do not have an effective therapeutic strategy to protect the myocardium against this lethal reperfusion injury. Therefore, novel strategies are urgently needed that are able to protect the myocardium against this lethal reperfusion injury and can be administered as an adjunct at the time of reperfusion to reduce the myocardial injury which

in turn will preserve and improve the myocardial function and the outcome of the of patients. The mitochondrial permeability transition pore (MPTP) has emerged as one of the important target for cardioprotection[68,177,282]. Opening of MPTP at the beginning of reperfusion of the myocardium following ischemia in the setting of acute ischemia–reperfusion / hypoxia-reoxygenation injury is an important and major determinant of cardiac dysfunction and cell death and hence opening of MPTP presents a potential target for human cardio-protection.

This thesis examined the importance of the MPTP as a potential target for human myocardial protection against the lethal hypoxia-reperfusion / ischemic-reperfusion injury. We found that opening of MPTP does occur in human cardiomyocytes. We have also identified that inhibiting this MPTP opening which takes place at reoxygenation / reperfusion is a potential target for cardio-protection when the protection was mediated by interventions that were applied solely at the time of reoxygenation / reperfusion.

The main findings of this thesis were: The MPTP is a critical determinant of myocardial death in the setting of hypoxia-reperfusion / ischemia-reperfusion injury, and pharmacologically inhibiting its opening, solely at the time of reoxygenation / reperfusion, protects the human heart against lethal reperfusion injury, as evidenced by;

1. Improving the functional recovery of the human right atrial trabeculae
2. Improving the human myocardial cellular viability
3. Decreasing the human myocardial cellular necrosis
4. Demonstrating that MPTP opening occurs in human cardiomyocyte and
5. Delaying the timing of the opening of the MPTP

The MPTP as a Mediator of Lethal Reperfusion Injury

In chapter 4, we demonstrated, using the isolated perfused human atrial trabeculae model, that the presence of the known pharmacological MPTP inhibitors, cyclosporin-A or sanglifehrin-A at the beginning of reoxygenation / reperfusion, protected the heart against lethal reperfusion injury, as evidenced by a significant increase in the recovery of the baseline

contracture following a lethal ischemic insult in the setting of hypoxia- reoxygenation. Importantly, we demonstrated that targeting reoxygenation / reperfusion was critical to the protective effect of these MPTP inhibitors, which supports the experimental findings that the MPTP opening occurs during post-ischemic reperfusion. The atrial trabeculae model has been well established and standardised and shown to work in our laboratory[260-262]. There are both advantages and disadvantages in using the right atrial muscle preparations as outlined in chapter 3. In vitro evidence from Ikonomidis et al was one of the early studies to show that ventricular cadomyocytes could be protected from SI by preconditioning[19]. Extensive studies at our department has shown that human atrial trabeculae can be preconditioned and this can be translated to better recovery of the contractile function following hypoxia and reoxygenation[18]. Though we are aware of the possible existence in the differences between atrial and ventricular cardiomyocytes, the human atrial model has been well established in our institution with reproducible results and hence this well established model was used in my experiments. Also our unpublished data from 1995 suggests that identical preconditioning effect can be induced in the ventricular tissue with a shorter hypoxic insult. The lack of tissue availability from ventricle is a limitation for conducting such experiments. Improvement in recovery of contractile function and reduction in infarct size secondary to preconditioning was shown by Jenkins et al and they showed that reduction in infarct size was proportional to improvement in the global left ventricular recovery[252]. In our experiment we assumed the same even though the experiment was performed in the human atrial tissue and there is no direct evidence. In addition the clinical studies support that the preconditioning does occur in humans[28,34,36,245-247].

A period of hypoxic superfusion and rapid pacing to stimulate ischemia has been shown to induce preconditioning in both animal models of regional and global hypoxia and in cell cultures[248-251]. The combination of hypoxia and rapid pacing in the human atrial trabeculae has been studied extensively and reproducibly in our institution and preconditioning has been shown to improve their functional recovery[260-262]. The hypoxic preconditioning protocol has also been previously demonstrated in this atrial trabeculae model to improve the recovery of myocardial contractile function[18].

Pharmacological Inhibition of the opening of MPTP by the immunosuppressive agent, CsA was discovered in the late 1980s[125]. In 1993, Griffiths and Halestrap first reported the CsA protection against the sustained myocardial ischemia reperfusion injury. They showed that the protection of CsA at a dose of 200 nM improved functional recovery in the isolated perfused rat heart model but not at 1 nM. CsA treatment also showed preservation of the myocardial ATP content in the above setting following acute ischemia reperfusion injury[130]. They also demonstrated that calcineurin was not responsible for the cardioprotection by adding tacrolimus, known calcineurin inhibitor, to their experiments and failed to show the myocardial protection against acute IRI in the presence of tacrolimus[130]. Following this in 1995 they investigated the timing of the opening of MPTP in relation to ischemia and reperfusion using a novel model of Hot-DOG technique[143]. They were able to show that the opening of the MPTP occurred in the first couple of minutes of myocardial reperfusion and that the MPTP remained closed during ischemia[143]. Opening of MPTP in the first few minutes of reperfusion was confirmed by a number of other studies subsequently[147,203]. Myocardial protection was shown by specifically inhibiting the opening of the MPTP at the time of reperfusion[203,229].

Our laboratory in 2002 were the first to demonstrate the protective effect of CsA against the myocardial infarct size in the isolated perfused rat hearts in that CsA was administered solely at the onset of myocardial reperfusion only to confirm that the opening of MPTP occurred primarily at the onset of myocardial reperfusion[229]. In addition, Sanglifehrin A was shown to be cardioprotective against ischemia reperfusion injury by administering them at the onset of myocardial reperfusion[68]. Sanglifehrin A inhibits mitochondrial CyP and it does not affect calcineurin activity and it was demonstrated that when SfA was administered after the first 15 minutes of myocardial reperfusion following ischemia cardioprotection was lost completely. This underpins the importance of the timing of opening of MPTP and their intervention during the first few minutes of myocardial reperfusion[68].

The understanding of the concept and timing of the opening of MPTP as an important mediator of myocardial reperfusion injury gives us an opportunity to intervene and inhibit the opening of MPTP and in turn protect the myocardium against ischemia reperfusion injury.

In 2008, Piot et al showed that a single bolus of CsA was cardioprotective in patients presenting with an acute myocardial infarction. In this study CsA was given at the time of percutaneous coronary intervention to the patients presenting with acute MI and they have a reduction in the myocardial infarct size by 30-40%[135]. 58 patients who were presenting with acute ST- elevation MI were randomly assigned to two groups. They either received normal saline or CsA bolus intravenously just before percutaneous intervention and they showed that the group receiving CsA bolus had smaller infarct size and a significant reduction in the release of Creatine Kinase (P=0.04)[135]. Interestingly further studies in porcine model have shown mixed results[136,284] and the reason for this mixed finding is not clearly evident.

Here we used this well-known MPTP inhibitor CsA, solely at the time of reperfusion / reoxygenation. As CsA can inhibit the protein phosphatase calcineurin in addition to inhibiting the opening of MPTP, we used SfA to show that MPTP inhibition is possible without calcineurin inhibiton[139] and this has been shown in langerdorff perfused heart model[139-142]. The specified concentrations of CsA and SfA have been demonstrated to inhibit the opening of MPTP in the isolated perfused rat heart model[68,130,139,229]. We used the same concentration as it was used in our laboratory for the inhibition of MPTP at the time of reoxygenation / reperfusion in the animal models as it was shown to be effective in preventing the opening of MPTP. The following clinical studies are supportive of our finding of CsA inhibition of MPTP at the time of reoxygenation. CsA protection against ischemia reperfusion injury has been concurred in other settings like neonatal cardioplegic arrest[137,285] and resuscitated cardiac arrest[138]. Leung and co-workers[137] demonstrated that CsA sensitive MPTP opening occurs at the time of reperfusion in a neonatal rabbit cardioplegic arrest model and that inhibition of this opening of MPTP using CsA or other MPTP inhibitors would be useful in reducing reperfusion injury during peri-operative period. Cour and colleagues[137] administered CsA in rabbits subjected to primary asphyxial cardiac arrest. Intravenous administration of CsA at the time of reperfusion has shown to improve survival and reduce myocardial necrosis. They also demonstrated inhibition of MPTP opening in isolated cardiac mitochondria by CsA.

The findings from this part of the study suggested that the MPTP opening during reoxygenation / reperfusion can determine cell death, and that inhibiting its opening provide a potential target of cardio-protection and improve its functional recovery.

In chapter 5 and 6, we demonstrated, using the human atrial cardiomyocyte model, that the presence of the known MPTP inhibitors, cyclosporin-A or sanglifehrin-A during the onset of reoxygenation / reperfusion, protected the heart against reperfusion injury, as evidenced by a significant increase in the recovery of the viable cells and a reduction in the necrotic cells following a lethal ischemic insult in the setting of hypoxia-reoxygenation. Isolated adult human right atrial cardiomyocytes provide cells, which are free from the neuro-hormonal influence in an environment, which can be controlled. MPTP opening results in both apoptotic and necrotic cell death[12,166,202,209,214] and the inhibition of its opening during reperfusion has been demonstrated to confer cardioprotection in various experimental models. In myocytes subjected to lethal hypoxia-reoxygenation, the presence of CsA improved cell viability[127]. Studies have demonstrated that both apoptotic and necrotic components of cell death can be reduced by inhibiting the apoptotic signalling cascade during reperfusion using pharmacological inhibitors[53,219,277]. Inhibiting MMP using MPTP inhibitors such as cyclosporine-A or bongkrekic acid have been demonstrated to inhibit apoptotic cell death[222,223,274,275] suggesting that MPTP opening is an obligatory mediator of apoptosis. Our study was able to show attenuation of the necrotic component of the human cardiac cardiomyocytes but failed to show any attenuation of the apoptotic component. Apoptosis can contribute to the extension of cell death and infarct size during reperfusion. Zhao and colleagues, demonstrated an improvement in the contractile function of canine hearts subjected to ischemia by inhibiting the reperfusion induced apoptotic component of cell death[53]. Although we could not demonstrate any significant reduction in the apoptotic component in the human atrial cardiomyocyte model, on the contrary we were able to demonstrate an improved contractile function of the human atrial trabeculae in the functional recovery model. This raises the question of whether apoptosis contributes to the functional recovery in our experiments. In our experiment the failure to shown any reduction in the apoptotic component of the myocytes could be explained by the fact that the hypoxic insult

could be severe and significant enough to cause necrosis on the fragile human cardiac myocytes and one suggestion would be do reduce the timing of the hypoxic insult.

Human right atrial cardiomyocytes were isolated from the right atrial appendages harvested from the patients undergoing elective cardiac surgery, just before going on to cardio-pulmonary bypass[263] by enzymatic dissociation using protease and collagenase digestion. We used the myocyte extraction method as used by Harding et al[263]. Though they used the protocol to extract myocytes from the ventricle, we utilised the same method to extract the right atrial cardiac myocytes and we were successful in isolating and carrying experiments on the right atrial cardiac myocytes.

Here again we used the same concentration of CsA and SfA in our mycocyte experiments, which is shown to potentially inhibit the MPTP opening at the time of reperfusion[68,229]. In chapter 5 first we attempted to characterise the model of ischemia / reperfusion in the human cardiomyocytes using hypoxic chamber to induce simulated ischemia / hypoxia simultaneously. Three time points (10, 30 and 50 minutes) were chosen to assess the cellular viability during the reoxygenation period following hypoxia. The 30-minute reoxygenation period was chosen as the time point for assessing cellular viability for further experiments of hypoxia and reoxygenation in chapter 6. Though we are aware that opening of MPTP occurs during the first few minutes of reoxygenation / reperfusion, we wanted to assess the cardiac cellular viability at three different time points as mentioned before (10, 30 and 50 minutes) using the well known MPTP inhibitors CsA and SfA.

In chapter 7, we demonstrated, using the human atrial cardiomyocyte model, that the opening of MPTP (global mitochondrial membrane depolarisation) does occur and that the presence of the known pharmacological MPTP inhibitors, cyclosporin-A or sanglifehrin-A protected the heart against lethal reperfusion injury, as evidenced by a significant increase in the time delay in opening MPTP (global mitochondrial membrane depolarisation), following laser induction (oxidative stress). MPTP opening in adult rat myocytes was detected using a cellular model of oxidative stress[68,139,143,229,233,234]. This model represents a reliable way to reproducibly inducing the loss of mitochondrial membrane potential and this has been unequivocally identified as MPTP opening[180,267,268,270,279]. The findings from this part of the

study suggested that the opening of the MPTP is a critical determinant of cell death and that inhibiting its opening provide a potential target of cardio-protection.

The MPTP and Cardio-Protection in Myocardial Preconditioning

We were also able to demonstrate that ischemic preconditioning of the human atrial trabeculae could protect the human myocardium from lethal reperfusion injury as evidenced by the functional recovery of the atrial trabeculae in the setting of ischemia reperfusion (chapter 4). Also in chapter 5 we were able to demonstrate a significant increase in the percentage recovery of viable human atrial cardiomyocytes subjected to hypoxic preconditioning followed by lethal ischemic insult in a setting of hypoxia-reoxygenation.

Several studies have been published, confirming that inhibiting MPTP opening is critical to the protection associated with myocardial preconditioning[140,280]. Preconditioning may inhibit MPTP opening at the time of reperfusion by several possible mechanisms including an indirect effect, in which known inducing factors of MPTP opening, such as mitochondrial Ca^{2+} load,[147,286-290] ATP depletion,[291,292] and oxidative stress[293-295] are counteracted by the effects of preconditioning, such that MPTP opening does not occur during the first few minutes of reperfusion. We did not investigate the association between IPC and MPTP in our study.

Chapter Nine:

CONCLUSION

9.1 Summary of Findings

This thesis has examined the role of the mitochondrial permeability transition pore (MPTP) in human myocardial protection and for the first time we have demonstrated the role of the MPTP as a viable target for myocardial protection with human cardiac muscle. We have demonstrated that the MPTP opening during reperfusion is a critical determinant of cell death in the setting of ischemia-reperfusion / hypoxia-reoxygenation injury, and therefore inhibiting its opening by administration of agents solely at the time of reperfusion, is an effective cardio-protective strategy. We also demonstrated that MPTP opening does occur in human myocardium and can be inhibited by the MPTP inhibitors CsA and SfA. We demonstrated that the interventions applied solely at the time of reoxygenation / reperfusion protected the human myocardium by targeting MPTP and the effect was similar to myocardial preconditioning.

Simulated hypoxia-regeneration injury was used in two different models where human atrial tissues were subjected to hypoxia-reoxygenation and we have found cardioprotection by inhibiting the opening of MPTP at the time of reoxygenation. Also for the first time we have demonstrated the cardioprotective effects of the pharmacological inhibitors using a human cardiomyocyte model for inducing and detecting MPTP opening. We used CsA and SfA to inhibit MPTP opening in our models.

We have used the human atrial trabecula model that has been well established in our laboratory[18,260-262,296] and others[21,297]. This is a robust model and is reproducible for hypoxia-reoxygenation injury. It has been shown before that the percentage recovery of the baseline contractile function is a measure of cardioprotection and this is very much reproducible. We were able to demonstrate the improvement in the recovery of the contractile function of the human atrial trabeculae function by inhibiting the opening of the MPTP using the known MPTP inhibitors for the first 30 minutes of the reoxygenation period. The functional recovery of the atrial trabeculae were similar to those subjected to hypoxic preconditioning and this

implies that MPTP opening is a critical determinant of reoxygenation injury and cell death in human myocardium.

We used a cardiomyocyte model for our next part of the study, isolated from the human right atrium, and subjected them to hypoxia-reoxygenation injury and we looked at the cellular viability of those human cardiomyocyte in relation to MPTP opening and their inhibition. We found an improvement in the cellular viability by inhibiting the opening of MPTP at the time of reoxygenation. In our experiments it was interesting to see attenuation in the necrotic component of the cell death but not in the apoptotic component. Protection against both necrotic and apoptotic component of cell death has been shown before by inhibiting MPTP opening[298]. We were only able to demonstrate a reduction in necrosis using hypoxia-reoxygenation injury in our human atrial cardiomyocyte model and we can attribute this to a limited reoxygenation time that was used in our experiment. We could speculate that there could have been an observed difference in the apoptotic component of cell death if we had extended the reoxygenation time period. Other explanation could be that MPTP opening is only involved in mediating necrosis but not apoptosis and this was supported by a recent study where over expression of Cyclophilin D promoted necrosis but appeared to inhibit apoptosis component of cell death[228].

Though many different models of ischemia and reperfusion injuries have demonstrated the effect of MPTP opening as critical determinant of cell death, to our knowledge this is the first time we have demonstrated the role of MPTP in the human heart as a determinant of functional recovery and cellular viability. Schneider et al[299] demonstrated the protective effect of CsA in the slices of human atrial tissues in regard to reoxygenation injury but they subjected the human atrium to CsA even before the hypoxic period and also MPTP was not investigated. In our study we aimed and administered the known MPTP inhibitors at the onset of reoxygenation period where the opening of MPTP occurred so that we could prevent their lethal effects of reoxygenation injury. We also demonstrated the direct protective effect of these drugs on MPTP using a human cardiomyocytes model.

In this study, subjecting the human atrial tissues to hypoxia-reoxygenation injury we have demonstrated the protective effects of known MPTP inhibitors CsA and SfA. We used both of these inhibitors because CsA can be cardioprotective not only by inhibiting the opening of MPTP but also by inhibiting calcineurin and more importantly SfA is a more specific inhibitor of MPTP opening as it does not inhibit calcineurin[139,141]. We administered both these known MPTP inhibitors particularly at the onset of reoxygenation following a lethal period of ischemia so that we could target the opening of MPTP that has been observed and demonstrated to occur at the onset of reperfusion/reoxygenation[143,147,202,203]. Translating this to the clinical settings it would be easier to apply cardioprotective strategies at the time of reperfusion.

In conclusion, for the first time we have show that the human myocardium can be protected from lethal hypoxia-reoxygenation injury by inhibiting the MPTP opening at the time of reoxygenation. Inhibition of the opening of MPTP may therefore provide a novel target for cardioprotection in the clinical settings of reperfusion.

9.2 Clinical Implications

In this thesis, we have provided evidence that pharmacological intervention at the start of reperfusion / reoxygenation protects the human heart by inhibiting the MPTP opening which occurs during the first few minutes of reperfusion / reoxygenation. Therefore, the first few minutes of reperfusion / reoxygenation offer a potential target for cardio-protection which would be under the control of the operator, such as after an acute myocardial infarction or at the time of cardiac surgery (clinical settings of ischemia-reperfusion injury), and from the findings of this thesis, targeting the MPTP opening, can offer protection that is comparable to myocardial preconditioning.

Importantly, we demonstrated that the opening of the MPTP during reperfusion was a major determinant of lethal reperfusion injury and was responsible for a significant loss in the percentage of myocardial functional recovery and cellular viability and a significant increase in the percentage of human myocardial necrosis following an episode of lethal ischemia /

hypoxia. We demonstrated that inhibiting MPTP opening during reperfusion by administering known pharmacological MPTP inhibitors, the human myocardium could be protected.

Reliable pharmacological agents need to be developed that will specifically inhibit the MPTP opening, safe to use, rapid action and no adverse effects. They can be used at the time of reperfusion, in the clinical settings of ischemia-reperfusion injury, such as at the time of reperfusion (by either thrombolysis or primary PTCA) following an acute coronary syndrome, acute myocardial infarction and at the time of cardiac surgery. However, the drug would have to be administered as an adjunct to the reperfusion treatment strategy to be effective, as we have demonstrated in this thesis that targeting the post-ischemic reperfusion is critical to protection.

In 2008, Piot et al showed that a single bolus of CsA was cardioprotective in patients presenting with an acute myocardial infarction. In this study CsA was given at the time of percutaneous coronary intervention to the patients presenting with acute MI and they have a reduction in the myocardial infarct size by 30-40%. 58 patients who were presenting with acute ST- elevation MI were randomly assigned to two groups. They either received normal saline or CsA bolus intravenously just before percutaneous intervention and they showed that the group receiving CsA bolus had smaller infarct size and a significant reduction in the release of Creatine Kinase (P=0.04). The reduction in the infarct size by cyclosporin suggests that reperfusion injury occurs in humans[9,53]. The possibility of new pharmacological target for treating patients with ongoing coronary syndrome to reduce lethal reperfusion injury is evident[53,61]. Staat et al described a similar reduction in infarct size using ischemic post conditioning[61]. Post conditioning also reduces the extent of reperfusion injury by inhibiting the permeability-transition pore opening[229,244]. These data require confirmation in a larger clinical trial. However these studies are supportive of our study that MPTP opening does occur in the human myocardium and it is a potential target for preventing lethal ischemia-reperfusion injury by the application of known MPTP inhibitors largely at or just after the time of reoxygenation / reperfusion.

Therefore, the local intra-coronary delivery of a small concentration of CsA or SfA, at the time of primary angioplasty for an AMI or following cardiac surgery, may provide a

potential approach for harnessing the protection gained from inhibiting MPTP opening at the time of reperfusion[300].

9.3 Future Directions

Further experimental studies are required to investigate newer classes of drugs which inhibit MPTP opening and which can be demonstrated to offer protection against lethal reperfusion injury by inhibiting MPTP opening. Known inhibitors like CsA and SfA need to study further for their safety and efficacy to be used in human subjects. This will become an effective adjunct to the reperfusion strategy and hence salvage of human myocardium in an acute myocardial infarction setting. Also it will be interesting to see whether other interventions which have been demonstrated to protect the heart against lethal reperfusion injury, when applied solely at reperfusion, such as the phenomenon of 'ischemic post-conditioning' or the opening of the mitochondrial K_{ATP} channel, do so by inhibiting MPTP opening and could be used in combination with pharmacological MPTP inhibitors at the time of reperfusion.

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