

**Testing metabolic and pharmacological agents for  
recovery following *de novo* acute heart failure in an  
isolated rat heart model**

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“If we knew what it was we were doing, it would not be called  
research, would it?”

**Albert Einstein**

# ***DECLARATION***

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**Date**

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# ***Abbreviations***

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AHF- Acute Heart Failure

AHA- American Heart Association

ADCHF- Acute Decompensation of Chronic Heart failure

ATP- Adenosine triphosphate

ACC- Acetyl CoA carboxylase

ACS- Acute Coronary Syndromes

Adr- Adrenaline

BCl2- B-cell lymphoma 2

BSA- Bovine serum albumin

BPM- Beats per minute

CVD- Cardiovascular Disease

CO- Cardiac Output

CAD- Coronary Artery Disease

cAMP- Cyclic Adenosine monophosphate

Co A- Co enzyme A

CPT1- Carnitinepalmitoyltransferase 1

CPT2- Carnitinepalmitoyltransferase 2

CHF- Chronic Heart Failure

CFTR- Cystic fibrosis transmembrane conductance regulation

CytC- Cytochrome C

ESC- European Society of Cardiology

eNOS- Endothelial nitric oxide synthase

FAT- Fatty acid translocase

FABP<sub>pm</sub>- Fatty acid binding protein in the plasma membrane

FACS- Fatty acyl-CoA synthase

FADH<sub>2</sub>- Flavin Adenine Dinucleotide

FFA- Free fatty acids

FOXO1- Forkhead box protein O1  
GTP- Guanosine triphosphate  
GLUT1- Glucose transporter 1  
GLUT4- Glucose transporter 4  
GAPDH- Glyceraldehyde 3-phosphate dehydrogenase  
GPCR- G protein coupled receptors  
GIK- Glucose- Insulin- Potassium  
HDL- High Density Lipoprotein  
HF- Heart Failure  
HG- High Glucose  
HR- Heart Rate  
HIV/AIDS- Human immunodeficiency virus infection / acquired immunodeficiency syndrome  
IPC- Ischaemic preconditioning  
Jak- Janus kinase  
LV- Left Ventricular  
LVDP- Left ventricular developed pressure  
LVEDP- Left ventricular end diastolic pressure  
LVESP- Left ventricular end systolic pressure  
LG- Low Glucose  
mPTP- Mitochondrial permeability transition pore  
NADH- Nicotinamide adenine dinucleotide  
OPTIMIZE HF- Organized Program to Initiate Lifesaving Treatment in Hospitalized Patients with Heart Failure  
PDH- Pyruvate Dehydrogenase  
RAAS- Renin Angiotensin Aldosterone System  
ROS- Reactive Oxygen Species  
RISK- Reperfusion Injury Salvage Kinase  
RPP- Rate Pressure Product  
Ran- Ranolazine

S1P- Sphingosine-1-phosphate

S1PR1-5- Sphingosine-1-phosphate receptors 1-5

SphK1/2- Sphingosine kinase 1 or 2

SAFE- Survivor Activating Factor Enhancement

STAT3- Signal transducer and activator of transcription 3

THESUS- HF- The Sub-Saharan Africa Survey of Heart Failure

TNF $\alpha$ - Tumor necrosis factor  $\alpha$

UN- United Nations

VEGF- Vascular endothelial growth factor

WHO- World Health Organization

## ABSTRACT

### Testing metabolic and pharmacological agents following *de novo* acute heart failure in an isolated rat heart model

*Gaurang Deshpande*

**Introduction:** Acute heart failure is a potentially lethal clinical emergency without any effective therapy. Using an isolated rat heart model, we established and validated a model of *de novo* acute heart failure to study novel putative cardioprotective therapies against acute heart failure, including glucose, insulin and the molecular agent sphingosine-1-phosphate (component of high density lipoprotein) for recovery.

**Methods:** In isolated rat hearts, the protocol consisted of three phases: stabilization at normotensive perfusion pressure, hypotensive acute heart failure and recovery by restoring normotension. Low glucose (2.5mM) and high albumin-bound free fatty acids (1.3mM) ( $\pm$  adrenaline  $10^{-8}$ M) were added in the perfusate. Molecular and metabolic agents were administered either alone or in combination in the acute heart failure or recovery phases. Effects of glucose, insulin and sphingosine-1-phosphate were observed on heart function, cell death and mitochondrial respiration.

**Results:** In the absence of adrenaline, combination of glucose and sphingosine-1-phosphate during acute heart failure improved functional recovery by increasing the heart rate. In the presence of adrenaline, glucose and sphingosine-1-phosphate administered separately were cardioprotective in the recovery phase by improving heart rate. The combination of glucose+insulin and glucose+sphingosine-1-phosphate in the recovery phase also increased heart rate. Glucose+sphingosine-1-phosphate+insulin increased heart rate and left ventricular developed pressure. Sphingosine-1-phosphate reduced expression of cytochrome C, but metabolic agents had no effect. AG490 (inhibitor of signal transducer and activator of transcription 3) attenuated the cardioprotective effect of sphingosine-1-phosphate with increased expression of the phosphorylated inactive form of this transcription factor protein.

**Conclusion:** We have established and validated an *ex-vivo* model of *de novo* acute heart failure. The combination of metabolic and molecular treatments improved heart function by increasing sinus node activity. Sphingosine-1-phosphate not only improved heart rate, but also reduced cell death, an effect mediated via activation of signal transducer and activator of transcription 3. Our data provide novel principles and approaches for the treatment of acute heart failure.

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# *Chapter 1*

## **INTRODUCTION**

### **Section 1: Burden of cardiovascular diseases**

#### **History of heart disease**

The industrial revolution originated two centuries ago and brought with it a comfortable lifestyle and associated diseases that are now epidemics. Despite comfortable lifestyles and access to excellent healthcare in many developed countries, mortality due to heart disease and cancer is escalating.<sup>1,2</sup> Cardiovascular disease is the number one cause of morbidity and mortality in the world,<sup>3</sup> predominantly in the developed countries and has also spread to the developing countries like India and South Africa. The previous dominance of infectious diseases and malnutrition as major contributors to mortality in developed countries has slowly changed to heart disease and cancer. Heart disease manifests itself in various formats such as ischemic heart disease, heart failure, valvular disease, congenital heart disease, hypertension, cardiomyopathies, rheumatic heart disease etc.

History suggests that heart disease is a complication of lifestyle changes and was believed to affect the richer and upper class people like the ancient Egyptians due to their sedentary lifestyle and very rich fatty diet.<sup>4</sup> Heart disease, however, predates the ancient Egyptians. In a recent publication from Thompson *et al*, the authors performed computed tomography (CT) scans on 137 mummies excavated from ancient Egypt, Peru, ancestral Puebloans (modern Southwest America) and Unangans of Aleutian Islands (modern Alaska) to evaluate atherosclerosis in ancient populations.<sup>5</sup> The scanning identified calcification of the arterial wall which was considered as indicative of atherosclerosis, and calcification along the expected course was considered probable atherosclerosis. The authors conclude that

atherosclerosis was prevalent in mummies from all cultures, even the hunter-gatherer cultures of Peruvians and Puebloans who predated agriculture. The use of fire for cooking and warmth generated smoke, inhalation of which may have been a key player in the progression of heart disease. Another hypothesis suggested by Opie *et al*<sup>6</sup> is that hunter gatherers often did strenuous physical work and were exposed to hunting hazards which led to profuse bleeding and often caused hypotension which, coupled with elevated catecholamines, led to acute heart failure and often death. Heart disease is therefore not just a modern disease, but a primitive disease that is now the major cause of mortality in the world.

### **Prevalence of heart disease**

According to World Health Organization (WHO), cardiovascular diseases account for around 30% of all deaths happening globally. It is estimated that by 2030, 23.3 million people will die annually due to heart disease ([http://www.who.int/cardiovascular\\_diseases/en/](http://www.who.int/cardiovascular_diseases/en/)). In Europe, cardiovascular disease is on the rise in younger adults.<sup>7</sup> The American Heart Association (AHA) guidelines mention that more than 2200 Americans die of cardiovascular disease everyday, 1 death every 39 seconds<sup>8</sup>; one death out of every nine deaths is caused by heart failure.

### **Heart disease in South Africa**

In South Africa, cardiovascular disease (CVD) is a more prevalent cause of mortality than Human immunodeficiency virus infection / acquired immunodeficiency syndrome (HIV/AIDS) in the younger population (<35 years).<sup>9</sup> Additionally, there is an alarming increase in diabetes, high triglycerides, low high density lipoprotein (HDL) cholesterol levels, obesity and hypertension.<sup>9</sup> Kengne *et al* predict that by 2030, heart disease and stroke will cause more deaths than any other cause including HIV.<sup>10</sup> CVD claims the lives of almost 200 people per day in South Africa.<sup>9</sup>

Risk factors for cardiovascular disease in South Africa are predominantly high blood pressure, obesity, use of tobacco, high cholesterol, lack of physical activity and unhealthy diets.<sup>11</sup> The INTERHEART study aimed to evaluate the prevalence of myocardial infarction and found that hypertension and diabetes are the leading risk

factors of CVD in the black African population as compared to other ethnic groups.<sup>11,12</sup> The data from the government sources show a growing trend in obesity in adolescents which is a main contributor to many other complications. These risk factors put an individual at risk of not only cardiovascular disease but also stroke. The pie charts below give a brief description of the types of heart diseases in developing countries from 2001.

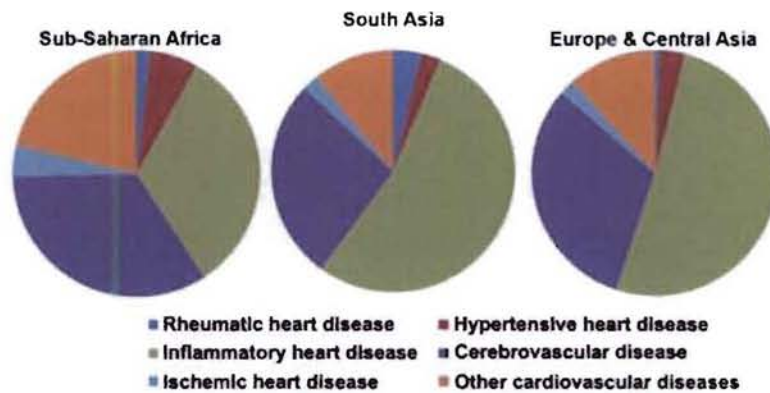
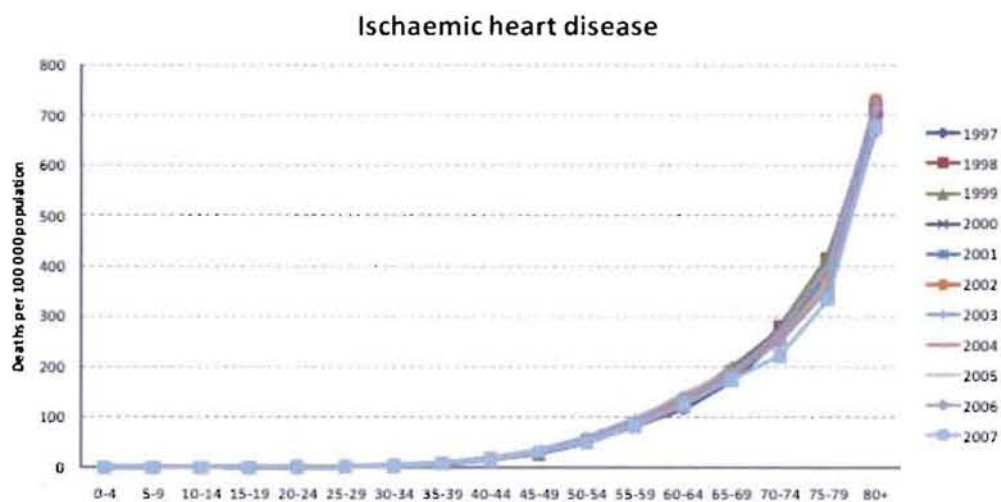
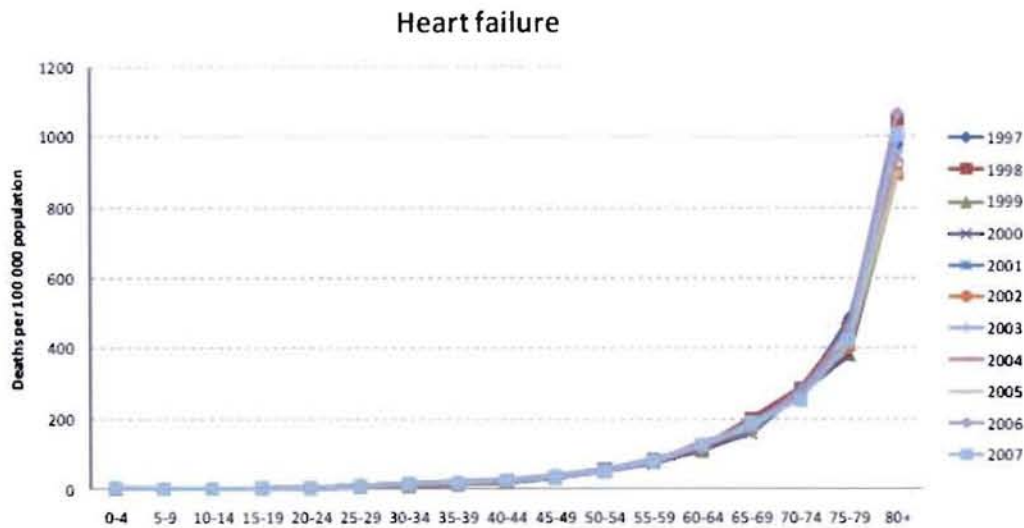


Figure 1-1-1: Causes of cardiovascular death in 2001<sup>11</sup>





**Figure 1-1-2:** Cause of death statistics for South Africa: Challenges and possibilities for improvement<sup>13</sup>

Hypertension is a major risk factor in the development and progression of cardiovascular disease, mostly with the end stage being heart failure (HF). The major endpoints of hypertension are cerebral hemorrhage, uremia or congestive heart failure.<sup>10,14</sup> This, coupled with lifestyle risk factors and obesity (which is rampant), makes hypertension a rapidly growing perilous factor for heart failure.

### Heart failure

The definition of heart failure has evolved with our understanding of the condition. The early definition of heart failure by Thomas Lewis in 1933 was: “heart failure is a condition in which the heart fails to discharge its contents adequately”. Eugene Braunwald in 1980 defined heart failure as “a pathophysiological state in which an abnormality of cardiac function is responsible for the failure of the heart to pump blood at a rate commensurate with the requirements of the metabolizing tissues.”<sup>15</sup> Heart failure has also been defined by J Cohn as “the inability of the left ventricle to fill or empty properly”.<sup>16</sup> However, heart failure is not limited to an impairment of filling and emptying. A more elaborate and detailed definition is provided by Katz: He defines heart failure as “a clinical syndrome in which heart disease reduces cardiac output, increases venous pressures and is accompanied by molecular abnormalities

that cause progressive deterioration of the failing heart and premature myocardial cell death.”<sup>17,18</sup> The European Society of Cardiology (ESC) defines heart failure clinically as “a syndrome in which patients have typical symptoms (e.g. breathlessness, ankle swelling and fatigue) and signs (e.g. elevated jugular venous pressure, pulmonary crackles and displaced apex beat) resulting from an abnormality of cardiac structure or function.”<sup>19</sup>

### **Prevalence of heart failure**

Heart failure is a rapidly progressing disease with high morbidity and mortality. In the United States alone, almost 5.7 million people present with heart failure every year and the disease accounts for 55,000 deaths every year. Heart failure costs over \$34.4 billion to the nation<sup>8,20</sup> In Europe, there are over 15 million patients with heart failure and the prevalence is high amongst the elderly. The ESC estimates that 50% of patients with heart failure will die within 4 years.<sup>19</sup>

### **Heart failure in Africa**

Heart failure in Sub-Saharan Africa is a major contributor to morbidity and mortality in Africa. Causes of heart failure, however, are not the same as in the developed world. The western world has a high incidence of ischemic heart failure whereas hypertensive heart failure is widely prevalent in Africa.<sup>21</sup> Other factors involved in the etiology of heart failure are rheumatic heart disease and cardiomyopathies which constitute a major aetiology of heart failure cases in the African continent. The other factor is the lack of proper healthcare amenities in the economically challenged countries. High prevalence of HIV and other infectious diseases also contributes to the pathophysiology of heart failure.<sup>21</sup> In the “Heart of Soweto” study conducted by Sliwa<sup>22</sup> and colleagues, 43% out of the 1960 patients that presented with heart failure, were *de novo*. The majority of patients were females (57%) and the most common ethnicity was black Africans (88%). The women patients presented with factors like obesity while men were predominantly smokers, with both sexes presenting with an elevated heart rate. The authors point out that the community in general was not very educated and the lack of basic amenities in the surroundings coupled with low income only makes the patients’ plight worse. The patients mostly

presented with cardiovascular risk factors like systolic and diastolic hypertension (60%), smoking (47%), obesity (34%) and dyslipidemia (19%). The types of heart failure that were principal were hypertensive heart failure, idiopathic cardiomyopathy, right heart failure, very few cases of ischemic cardiomyopathy and valvular disease.<sup>22</sup>

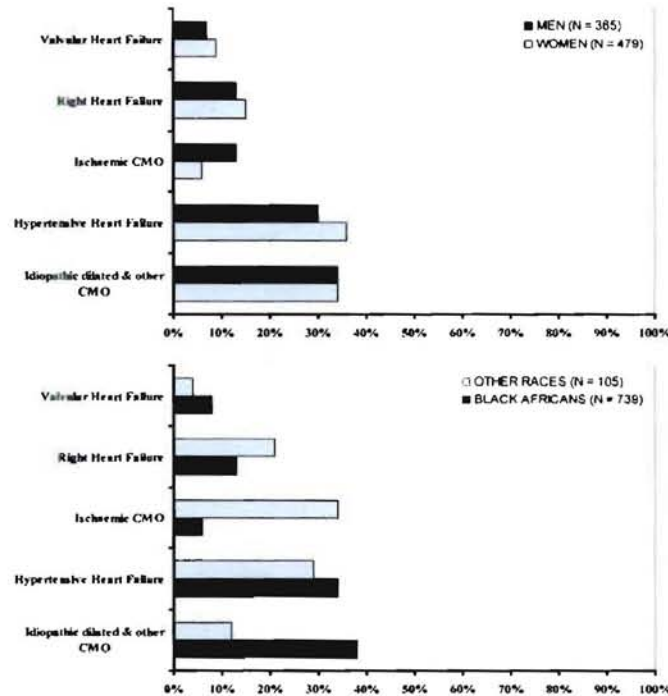


Figure 1-1-3: (Copied from) Causes of heart failure in the "Heart of Soweto" study<sup>22</sup>

### Types of heart failure

Heart failure can be broadly classified as chronic and acute. Chronic heart failure, as the name suggests, is a condition that develops over time while acute heart failure is a rapid deterioration of the heart's function. Acute heart failure can either be acute worsening of chronic heart failure called "decompensation" or can occur as new onset of heart failure called "*de novo*" acute heart failure.<sup>23</sup>

## **CHRONIC HEART FAILURE**

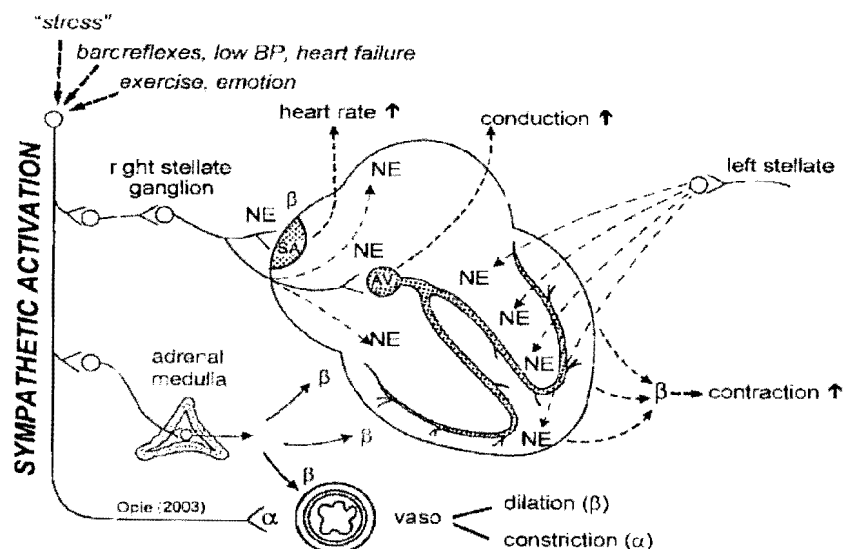
### **Chronic heart failure is a progressive disease**

Many cardiac events will lead to chronic heart failure over time. This episode may be an occlusion of an artery leading to a massive infarct, a slow progression of hypertrophy due to increasing pressure and volume stress on the heart or a cardiomyopathy. The common endpoint of these aetiologies is the decline in the inotropy and lusitropy of the heart muscle. An infarction leads to cell death via apoptosis or necrosis. The compensation that occurs in the heart is to aid the heart muscle to pump harder so as to maintain an optimal cardiac output and homeostasis of the cardiovascular system. This compensation mechanism activates the adrenergic nervous system and preserves water and salts to maintain cardiac output. Besides these activations, prostaglandins and nitric oxide are activated to control the vascular tone.<sup>24,25</sup>

### **Neurohormonal mechanisms in heart failure**

Researchers have found that the over-expression of certain biological compounds is responsible for the progression of heart failure. This model of heart failure is called the neurohormonal model of heart failure. The heart is under the control of the sympathetic nervous system and the increase of heart rate during "fight or flight" situations is mainly regulated by the sympathetic nervous system.<sup>26</sup> Thus, the prolonged over-expression of adrenaline has a deleterious effect on the heart as the heart has to work harder to keep up the extra demand of blood, thus leading to or worsening the haemodynamic imbalance. Oxidation of circulating free fatty acids is upregulated in the presence of catecholamines, thus inhibiting protective glucose uptake. Therapy using  $\beta$ -adrenergic blockers has been shown to improve outcome in patients with heart failure and is now standard therapy. The mechanism by which neurohormonal activity worsens the performance of the heart is by modulating the fuel consumption in the heart. The failing heart is forced to use the "expensive" free fatty acids as fuels to generate ATP which require more oxygen; it impairs the utilization of glucose as a fuel by down-regulating glycolysis, which is protective for a failing heart as glucose is "cheap" fuel.<sup>24,27</sup> Mann and Bristow<sup>24</sup> suggest that the

neurohormonal model of heart failure is not adequate to explain the progression of the disease; even though the conventional therapy is to antagonize the beta receptors, if this model was indeed adequate, this treatment should have curbed the disease by now. They suggest that the beta blockers aren't blocking all the receptors and that many systems in the body are affected in heart failure and release many other neurohormonal compounds into the blood stream. Hence, the rennin-angiotensin aldosterone system is not completely blocked, leading to disease progression.<sup>24,28</sup>



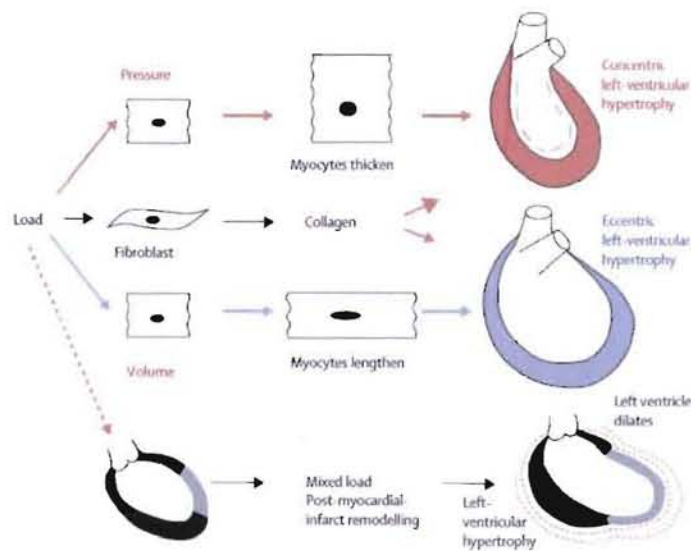
**Figure: 1-1-4 Mechanisms and outcomes of sympathetic stimulation<sup>29</sup>**

### **LV remodeling as a progressive model of heart failure**

Left ventricular hypertrophy in response to changing conditions in the heart is considered to be an adaptive mechanism in times of stress to maintain optimal pumping function in healthy individuals. Prolonged adaptation to a stress causes changes in the ventricular walls, known as remodeling.<sup>30</sup> The nature of this

remodeling is dependent on the type of stress imposed on the heart walls. This eventually contributes to the failure of the myocardium and can accelerate heart failure when combined with neurohormonal stimulation and/or prolonged haemodynamic alterations.<sup>24,31</sup> Opie *et al* describe the LV remodeling in heart failure in 3 ways: Remodeling caused by pressure overload, remodeling caused by volume overload and remodeling caused by a myocardial infarction. The pressure overload-induced remodeling involves the thickening of the cardiomyocytes (refer figure 1-1-5), making the LV smaller and causing it to attain concentric hypertrophy. This adaptation is needed to pump the blood out with more force. Volume-induced remodeling, as the name suggests, is an adaptive mechanism when the left ventricle needs to pump out a large amount of blood. This causes the cardiomyocytes to elongate (refer figure) and creates more space within the left ventricle to store and pump more blood.<sup>30</sup>

Remodeling after a myocardial infarction happens in order to compensate for the loss of viable myocytes. Due to a part of the myocardium being infarcted, the pressure on the viable cells in the ventricles is tremendous and there is extra volume that needs to be pumped out as well. This causes an initial beneficial adaptation for the ventricle, but more ischemic insults and prolonged hemodynamic stress on this ventricle can cause dilation and eventually failure.<sup>30,32</sup>



**Figure 1-1-5: Patterns of ventricular remodeling<sup>30</sup>**

## Section 2: Acute heart failure

The ESC defines acute heart failure as “the term used to describe the rapid onset of, or change in, symptoms and signs of heart failure.”<sup>19</sup> Acute heart failure is a complex condition which cannot be bound by an individual method of mechanism and/or treatment. It varies extensively with respect to the aetiology and pathophysiology and clinical presentations. Hence, the treatment of acute heart failure is as varied as the presentation and does not follow the one-size-fits-all ideology.

### **Acute heart failure morbidity and mortality**

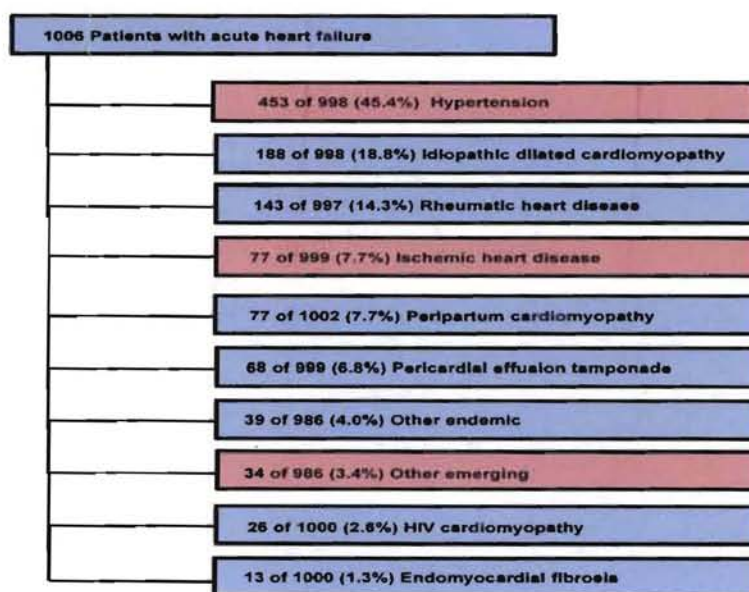
Acute heart failure holds the highest post-discharge cardiovascular diagnosis in elderly patients. Morbidity and mortality associated with AHF are high with a third of the patients being re-hospitalized or dead within 90 days of discharge.<sup>23</sup> Acute heart failure can be broadly classified into two subtypes: acute decompensation of chronic heart failure (ADCHF) and new onset or “*de novo*” acute heart failure. The first year mortality in patients with acute heart failure is high when a comparison is made between patients with acute decompensation of chronic heart failure and patients with new onset or “*de novo*” acute heart failure.<sup>2,23,33</sup> Some of the precipitating factors of acute heart failure are: ADCHF, acute pulmonary oedema, hypertensive heart failure, cardiogenic shock caused by hypotension leading to hypoperfusion of tissue, right heart failure and superimposed acute coronary syndromes. In the United States alone, the percentage of patients being hospitalized for acute heart failure rose by 174% from 1979 to 2003 and cost the country a massive amount of \$15.4 billion.<sup>34</sup> In Europe, data from various registries indicate that acute coronary syndromes are the major cause of AHF with in-hospital mortality being highest in patients with cardiogenic shock (40-60%). Almost 50% of the patients were re-hospitalized within 12 months.<sup>35</sup>

In an independent study performed in Finland, Lassus *et al.* examined the long term survival in patients after hospitalization for acute heart failure between ADCHF and *de novo* heart failure.<sup>2</sup> They observed that both groups had a high mortality in the first year post-hospitalization, with ADCHF patients faring worse. This mortality,

however, was reduced in the *de novo* AHF patients in the subsequent years (75.6% vs. 44.4%). The ADCHF group patients had a higher mortality over a 5 year period compared to the *de novo* patients.<sup>2</sup>

### Statistics of acute heart failure in Africa

Very few studies look at prevalence of acute heart failure in sub-Saharan Africa. Damasceno *et al.*<sup>36</sup> started the THESUS-HF trial where they examined 1006 patients from 9 countries. The participating countries were Senegal, Sudan, Ethiopia, Nigeria, Kenya, Uganda, Cameroon, Mozambique and South Africa. Their preliminary findings were that patients presenting with acute heart failure had a mean age of 52.3 yrs, were mostly black African women (50.8%) and almost 46% of the cases presented with hypertension as the cause of acute heart failure, while rheumatic and ischemic heart disease accounted for 14.3 and 7.7% respectively. One of the major reasons for this is the lack of proper medical attention in the subcontinent and the poor economic background of patients. The table below summarizes the causes of acute heart failure in sub-Saharan Africa.<sup>36</sup> Amongst rural Nigerians, prevalence of acute heart failure is very often accompanied by the presence of untreated hypertension.<sup>37</sup>



**Figure 1-2-1: Causes of acute heart failure in THESUS-HF<sup>36</sup>**

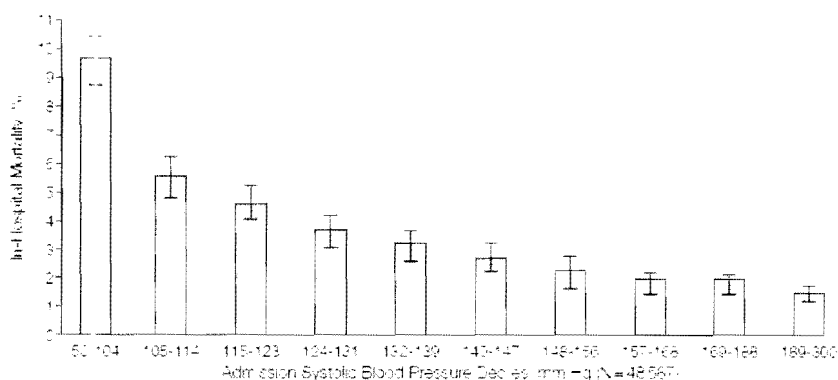
The study is the first of its kind to assess acute heart failure in the African continent. The results suggest that the disease affects members of both sexes equally even though the causes are different. The most important finding of this study is hypertension being the prime cause of acute heart failure and not ischemic heart disease as in the developed world.<sup>36</sup>

### **Aetiology of acute heart failure**

Causes of acute heart failure can broadly be classified into three categories: worsening of chronic heart failure (acute decompensation), ischemic acute heart failure and non ischemic acute heart failure.<sup>38</sup> Despite the cause, the major finding in all acute heart failure patients is the systemic and pulmonary congestion with or without decreased cardiac output.<sup>39</sup> Post-discharge mortality of acute heart failure patients remains high, the solution to which may lie in the proper classification of the patients based on aetiology. The majority of patients coming into the emergency room with acute heart failure have a worsening of chronic heart failure; however, there are other factors like coronary heart disease, valve disease and hypertension as well as non-ischemic conditions like renal failure, diabetes and anaemia that can cause acute heart failure.<sup>39</sup> Ischemia may cause acute heart failure; however, ischemia may be caused by acute heart failure as well. Acute decompensation may be caused by a factor completely independent of CAD and worse of all, all these complexities may be present in a patient presenting with acute heart failure. Such complex presentations of the disease warrant proper classification of patients and development of tailor-made therapies.<sup>40</sup> Acute heart failure can manifest itself independent of ischemic or non-ischemic causes, called "*de novo*" or new onset acute heart failure. One of the examples of this condition is Takotsubo cardiomyopathy, also known as "apical ballooning syndrome" or "broken heart syndrome". Patients with Takotsubo cardiomyopathy have elevated dilation of the left ventricular wall near the apex. This is thought to be caused by high circulating catecholamines and in some cases may cause necrosis by cyclic adenosine monophosphate (cAMP)-mediated calcium overload.<sup>41</sup> The numerous amounts of precipitating causes for the same disease and the lack of time in the emergency room contribute to high mortality associated with acute heart failure.

## Pathophysiology of heart failure

Acute heart failure involves severe and dire hemodynamic and neurohormonal changes that may or may not cause myocyte death. These abnormalities may be caused by cardiac (example: atrial fibrillation) or non-cardiac (example: renal failure) factors. A consistent finding in the majority AHF patients is elevated left ventricular diastolic pressure, which may or may not be accompanied by reduced cardiac output. This is caused by severe pulmonary congestion observed in patients with diastolic dysfunction.<sup>39</sup> In patients with worsening of chronic heart failure, neurohormonal factors play a pivotal role in the progression of the disease. The activation of the renin-angiotensin aldosterone system (RAAS) and the sympathetic nervous system in patients with left ventricular dysfunction causing reduced output increases peripheral vascular resistance and hence causes an increase in afterload.<sup>42</sup> This in turn leads to increased preload and a vicious cycle begins which worsens the condition of the left ventricle. Presence of catecholamines also leads to tachycardia which is deleterious by causing cell death and leading to more myocardial damage. Systolic pressure measurement is a promising marker to differentiate the patients with high risk for mortality vs. those with low risk. Lower systolic pressures were associated with high in-hospital mortality in the OPTIMIZE-HF registry and have been confirmed by the consistent data from the EuroHeart Failure Survey II where cardiogenic shock associated with hypotension and lower blood pressures were associated with increased mortality.<sup>43,44</sup>

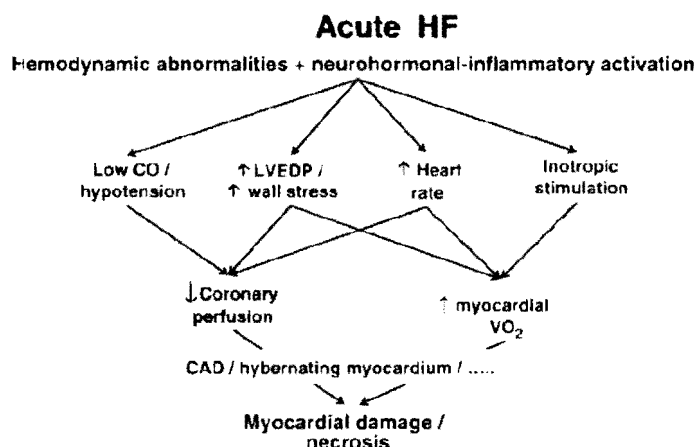


**Figure 1-2-2:** In-hospital mortality rates by admission systolic pressures<sup>43</sup>

## Myocardial damage in acute heart failure

Hemodynamic alterations leading to acute heart failure are associated with acute coronary syndromes and ischemic heart disease. The severity of an infarction and coronary artery disease may be the trigger for acute heart failure or vice versa. Especially in *de novo* acute heart failure patients, acute coronary syndromes play a pivotal role as a pathogenic mechanism leading to acute heart failure. A proposed pathway by Metra *et al.*<sup>23</sup> is described here (Fig1-2-3):

An episode of *de novo* acute heart failure coupled with hemodynamic abnormalities and the neurohormonal pathway may lead to hypotension, increased wall stress, and elevated heart rate owing to the elevated catecholamines which rapidly deteriorates the cardiac perfusion and may cause the myocardium to undergo necrosis. This mechanism supports the notion that reduced systolic blood pressure predicts higher in-hospital mortality.<sup>23,43</sup>



**Figure 1-2-3: Mechanisms of myocardial damage in patients with AHF<sup>23</sup>**

## Possible molecular and cellular mechanisms in acute heart failure

A number of aetiologies and precipitating factors for acute heart failure and the lack of basic research make it very tedious to pinpoint the exact mechanisms involved. However, there are a few proposed hypotheses for progression of Takotsubo cardiomyopathy. Takotsubo cardiomyopathy is an acute heart failure syndrome which is characterized by dilation of the left ventricular wall and reduction of

contractility of the apex and midventricular regions.<sup>45</sup> The hypothesis that this is caused by a supra-physiological level of circulating plasma catecholamines has stood the test of time. One explanation of the sympathetic overdrive and myocardial hypoperfusion observed in patients with Takotsubo cardiomyopathy is ischemia. Stress can cause vasoconstriction in patients without coronary disease. This is supported by observations by Kurisu *et al.*, who noticed coronary spasms in over 70% patients in stressful situations.<sup>46,47</sup> Another proposed mechanism is cell death due to elevated catecholamines. The mechanism suggested by Wittstein *et al*<sup>46</sup> involves catecholamine-mediated myocardial stunning by reducing myocyte viability, caused by increasing the calcium in the cells mediated by cyclic AMP. Catecholamines are sources of ROS and may cause cell death by attenuating antioxidants, by manipulating the sodium and calcium transporters and thus causing a calcium influx in the cell leading to death.

These mechanisms are proposed and need further research before they can be confirmed. The heterogeneity of the disease and the multiple causes and effects make it difficult to zero in on a mechanism that will improve our understanding of the disease.

This thesis is focused on the molecular and cellular mechanisms involved in the recovery after an episode of *de novo* acute heart failure on an isolated rat heart and will examine the effects of different molecular and metabolic agents on recovery. This will hopefully provide an insight into the treatment and the pathophysiology of *de novo* acute heart failure.

### **Our model of acute heart failure**

There are growing data regarding the clinical trials and registries in acute heart failure, but the mechanisms and metabolism involved remains elusive. Keeping this in mind, we established a model of *de novo* acute heart failure using an isolated rat heart on the Langerdorff retrograde perfusion apparatus.

Since reduced systolic pressure is associated with worse outcomes, our animal model of acute heart failure induces failure on the isolated rat heart by lowering the perfusion pressure and changing the substrate content from glucose to fatty acids in the presence of adrenaline to mimic the effects of neurohormonal stimulation. We

notice reduced coronary perfusion and left ventricular parameters and heart rate are the endpoints. We aim to look for protection against acute heart failure by molecular agents and delineate the pathways involved in recovery when we administer sphingolipids like sphingosine-1-phosphate which is a component of high density lipoprotein (HDL) cholesterol and a preconditioning agent <sup>48</sup> which is protective against ischemic-reperfusion injury.<sup>49</sup>

Metabolism plays an imperative role in the progression of heart failure. Previous work by Booyesen *et al*<sup>50</sup>, describing the effects of adrenergic induced advanced dilation in rats by chronic isoproterenol administration discusses the effects of catecholamines on the left ventricle. Even though these effects were completely reversed after 4 months, it is an imperative finding for our validation of the model where we shall use adrenaline as one of the ways to induce AHF on the isolated rat hearts. The high speed generation and utilization of high energy phosphates is important in the maintenance of the heart's contractility. In our model of de novo AHF, we aim to impair the contractility by reducing its oxygen availability. Another study by Saupe *et al*<sup>51</sup>, on hypoperfusion induced contractile failure where they gradually reduced the oxygen availability of the hearts to examine the effect of hypoperfusion on production of ATP in these hearts. Another study by Elliott *et al*<sup>52</sup> showed that cardiac failure is mainly caused due to the changes in Pi and H<sup>+</sup> and are secondary to hypoperfusion. In our model, we subject the hearts to a prolonged hypotensive phase to reduce the contractility. We are interested in examining the metabolism involved in acute heart failure and testing metabolic agents like glucose and insulin as therapy.

# Section 3: Normal heart metabolism and proposed metabolism in an acutely failing heart

## Myocardial substrate utilization in the normal heart

In a healthy adult heart, almost all ATP generation occurs by means of oxidative phosphorylation in the mitochondria.<sup>53</sup> Glycolysis contributes the remainder of energy. In order to propose the metabolism in an acutely failing heart, it is imperative to understand the metabolism in a normal heart. Taegtmeyer refers to the human heart as a “metabolic omnivore” due to the variety of substrates used by the heart to generate ATP energy.<sup>54</sup> The heart uses carbohydrates, fatty acids, amino acids and even ketones to generate ATP. Fatty acids, however, are the major contributor to ATP generation as they generate almost 70% of the ATP needed by the heart.<sup>55,56</sup> This ATP production by FFA utilizes more O<sub>2</sub> molecules (see table) and is efficient in non-ischemic conditions.

**Table 1-3-1:** ATP yield per molecule of different substrates. Glucose yields 38 ATP per molecule while fatty acids Palmitate and Oleate yield 129 and 144 respectively. However, glucose is more efficient in consumption of oxygen to produce ATP. Even though it is a small difference of 11%, it is important in a failing or ischemic heart.

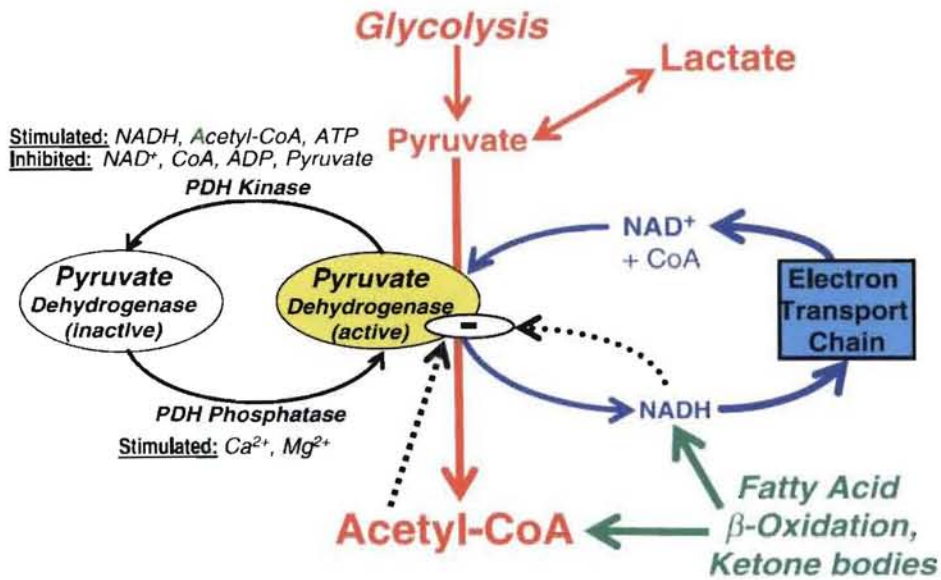
Molecule	ATP yield per molecule		ATP yield per carbon atom		ATP yield per oxygen atom taken up to (P/O ratio)	
	Old <sup>a</sup>	New	Old	New <sup>a</sup>	Old	New
Glucose	38 <sup>b</sup>	32 <sup>c</sup>	6.3	5.2	3.17	2.58
Lactate	18	14.75	6.0	4.9	3.00	2.46
Pyruvate	15	12.25	5.0	4.1	3.00	2.50
Palmitate <sup>d</sup>	130	105	8.1	6.7	2.83	2.33

(Copied from Opie LH, Heart Physiology: From Cell to Circulation)<sup>29</sup>

## CARBOHYDRATE METABOLISM

### Overview of Glycolysis

In a normal adult heart, almost 60-90% of acetyl-coA is generated by the  $\beta$ -oxidation of fatty acids while the remaining 10-40% comes from pyruvate oxidation which is derived from the glycolytic pathway and oxidation of lactate.<sup>53,57</sup> Glucose is taken into the cell by the activation of glucose transporters GLUT1 and GLUT4 (mostly GLUT4 in the heart).<sup>58</sup> The translocation of these transporters from the intra-cellular vesicles to the sarcolemma occurs in response to situations like ischemia, increased work or insulin stimulation. During exercise, the activity of AMP-activated protein kinase (AMPK) is also responsible for the translocation of GLUT4 transporters to the sarcolemma. Glycolysis occurs in the cytosol and converts glucose to either pyruvate or lactate based on the availability of oxygen. Even though pyruvate is formed in both aerobic and anaerobic conditions, it is oxidized under aerobic conditions. It is converted to lactate under anaerobic conditions.<sup>59</sup> The first step in glycolysis is the phosphorylation of glucose to glucose 6-phosphate by hexokinase followed by action of phosphoglucose isomerase to fructose 6-phosphate which by 6-phosphofructo-1-kinase forms fructose1,6-biphosphate where ATP is converted to ADP. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is the first enzyme that generates NADH from nicotinamide adenine dinucleotide ( $\text{NAD}^+$ ) by oxidation and phosphorylation of glyceraldehyde phosphate.<sup>60</sup> Downstream reactions in glycolysis produce 2 ATP and pyruvate from phosphoenol pyruvate in the aerobic conditions while in the absence of oxygen, NADH is reoxidized by lactate dehydrogenase, converting pyruvate to lactate. The pyruvate formed from glycolysis undergoes decarboxylation by the action of pyruvate dehydrogenase (PDH) to produce acetyl CoA which enters the Krebs cycle.<sup>53,60</sup>



**Figure 1-3-1:** Overview of Glycolysis. Acetyl-CoA is formed from pyruvate by the action of pyruvate dehydrogenase (PDH) which is the end product of Glycolysis. Two ATP and NADH are formed in the process. Acetyl-CoA subsequently enters the Krebs cycle. Copied from Stanley W et al.<sup>53</sup>

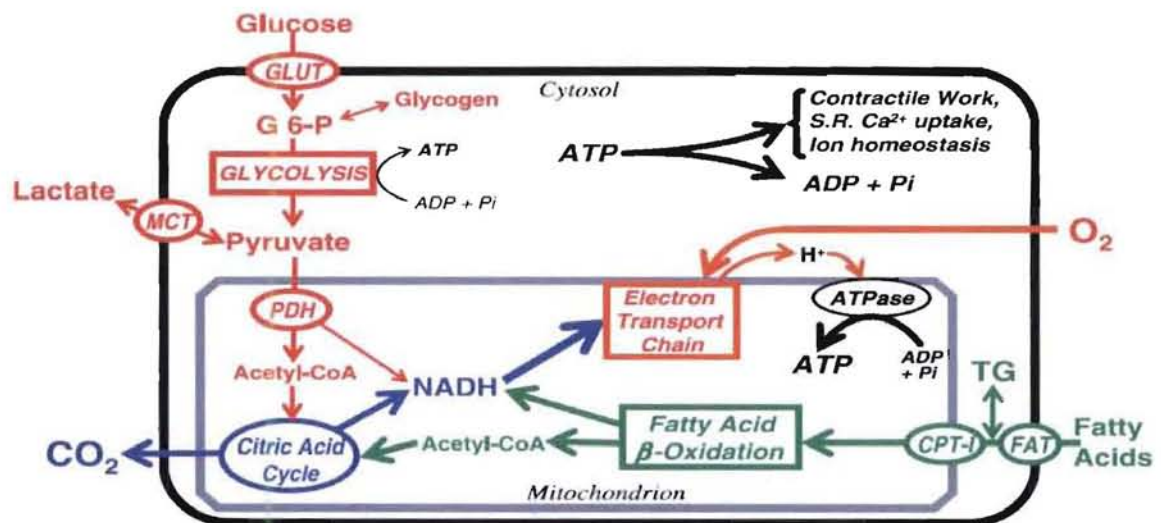
## FATTY ACID METABOLISM

### Overview of myocardial fatty acid metabolism

The rate of fatty acid uptake in the myocardium is defined by the concentration of non-esterified fatty acids in the plasma. These molecules are hydrophobic and are bound to proteins or covalently to co-enzyme A or carnitine. The transport of fatty acids through plasma is facilitated by being bound to albumin, very low density lipoproteins or triglycerides.<sup>53,59,61</sup>

Fatty acids enter the cell by passive diffusion or by means of a fatty acid translocase (FAT), which is a protein mediated transfer, or a fatty acid binding protein in the plasma membrane (FABP<sub>pm</sub>).<sup>62</sup> CD36 is a FAT which is predominantly found in the heart. Once the fatty acids have diffused across the sarcolemma, they bind to FABP and are then esterified to fatty acyl-CoA by the enzyme fatty acyl-CoA synthase (FACS). The products of FACS i.e. long chain fatty acyl-CoA are either converted to

long chain fatty acylcarnitine by carnitinepalmitoyltransferase I (CPT1) or are esterified to triglycerides by glycerophosphate acyl transferase. Diglyceride acyltransferase (DGAT) catalyses the reaction between diacylglycerol and acyl-CoA forming triglycerides.<sup>63</sup> CPT1 is responsible for the formation of long chain acyl carnitine from long chain acyl-CoA for transport between the inner and outer mitochondrial membranes. Once the transfer into the inner mitochondrial matrix is complete, carnitinepalmitoyltransferase II (CPT2) regenerates the long chain acyl-CoA in the mitochondrial matrix. This long chain acyl-CoA undergoes  $\beta$ -oxidation to generate acetyl-CoA, NADH and FADH<sub>2</sub> which enter the Krebs cycle and electron transport chain to generate ATP and oxygen.<sup>59,62,64</sup>



**Figure 1-3-2:** Overview of carbohydrate and fatty acid oxidation on the cell. FAT - Fatty acid translocase; CPT1 - carnitinepalmitoyltransferase 1; ATP - Adenosine triphosphate; GLUT - Glucose transporters; TG - triglycerides; PDH - Pyruvate dehydrogenase; G6P - Glucose-6-phosphate. (Copied from Stanley et al).<sup>53</sup>

### **Proposed metabolic patterns in acute heart failure**

To suggest a common metabolic path in acute heart failure is like describing the understanding of the mythical creature, the Hydra. Just as severing one head of the Hydra does not kill the beast, similarly, one metabolic process in AHF cannot possibly describe all the metabolic processes in an acutely failing heart. I shall attempt to describe the proposed metabolism in the *de novo* setting of acute heart failure in this thesis. To understand the metabolic patterns, it is first important to describe the precipitating factors for acute heart failure and what effect they have on the metabolism of the failing heart.

Acute heart failure manifests itself in multiple clinical presentations. Some of the common presenting factors are acute hypertension, volume overload, hypotension, atrial fibrillation, acute coronary syndromes, acute valvular heart disease, myocarditis, infections etc.<sup>65</sup> Dar and Cowie performed a comparison of precipitating factors for acute heart failure in various registries in Europe and the United States.<sup>66</sup> They found that acute coronary syndromes (ACS) were the most common precipitant of AHF in all registries where roughly 40% of cases were caused by ACS. Almost 40% of *de novo* AHF was caused by ACS while acute decompensation only contributed to around 20-25% of the cases, with ischemia being a common cause of cardiogenic shock which had the most in-hospital mortality rates. Other causes were atrial fibrillation and to a lesser extent infections (20%). In these registries, it is important to note that the cause of AHF was primarily ischemic and that the patients were aged 70-75 years, thus making AHF a "disease of the elderly".

Para acute hypertension, defined as systolic pressure >150mmHg on admission for the Helsinki-Zurich registry and >200mmHg for the New York registry was another precipitant of AHF in these patients but had very little in-hospital mortality.<sup>44,66</sup> In the OPTIMIZE-HF (Organized Program to Initiate Lifesaving Treatment in Hospitalized Patients with Heart Failure; n=48,567) study by Gheorghiade *et al*,<sup>43</sup> it was shown that patients with acute heart failure with reduced systolic pressure at admission showed higher in-hospital mortality. They also reported that the majority of patients in the study were aged 70-75 yrs which is consistent with the data from the various European studies.<sup>43</sup> To understand the metabolism of AHF in these patients we need a thorough understanding of the precipitating factors and the effects they have at the cellular level. The important points such as mitochondrial energetics, neurohormonal

activation, utilization of fuels, production of reactive oxygen species (ROS) and calcium metabolism in mitochondria need to be studied.

Studies from the African continent done by Damasceno *et al*<sup>36</sup> describe hypertension as the major cause of mortality in 1006 patients with acute heart failure. The study was performed in nine countries and the authors report that patients with acute heart failure had a mean age of 52 yrs and that there was no difference between the sexes. The authors report that the rate of readmission and death is similar to that seen in the European and American registries and is very high.

**Takotsubo cardiomyopathy** is a subtype of acute heart failure characterized by rapid dilation of the left ventricle due to elevated catecholamines caused by an acute up-regulation of the sympathetic nervous system. This type of cardiomyopathy is also called stress cardiomyopathy, with supra-physiological levels of circulating catecholamines (as high as 37 times the normal value) in these patients.<sup>46</sup> They measured plasma catecholamines everyday from day 1 until day 9, finding that the catecholamines were elevated to the maximum on day 1 (37 times) with reduced concentrations by day 9, were still 10 times normal values. Almost all patients presented with chest pain with some patients requiring intra-aortic balloon pump on admission.

Wittstein *et al*<sup>41,45,46</sup> suggest multiple hypothesis that could explain the relation between elevated catecholamines and myocyte injury in these patients. The first hypothesis suggests the calcium overload mediated by cyclic adenosine monophosphate (cAMP) causing a reduction in myocyte viability due to apoptosis. The second hypothesis considers Takotsubo cardiomyopathy due to epicardial spasms that lead to ischemia and in succession, myocardial stunning and microvascular dysfunction owing to abnormal perfusion. The third hypothesis deals with the idea of catecholamines causing endothelial stunning, leading to reduced coronary flow.

Uchida *et al*<sup>67</sup> hypothesised that microvessel apoptosis is a link between stress and Takotsubo cardiomyopathy, suggested that elevated catecholamines cause apoptosis in vascular endothelial cells via the catecholamine- $\beta$  receptor-caspase 3 and catecholamine-BCI2-caspase pathway. They conclude that stress-induced up-regulation of catecholamines causes apoptosis of endothelial cells and microvessel

spasm, and that this in turn causes myocardial stunning, which leads to thinning of the ventricle walls.<sup>67</sup> Mebazaa *et al*<sup>68</sup> showed the correlation between elevated blood glucose and 30 day mortality in patients with and without diabetes mellitus. They found that patients with high glucose had a lower survival rate ( $p < 0.0001$  vs. survivors at the end of 30 days) both in patients with and without diabetes mellitus. The proposed hypothesis for this condition is that severe systemic stress, elevated blood glucose and an upregulation of sympathetic nervous system increase the concentration of circulating free fatty acids which are deleterious to the failing myocardium. Hyperglycemia is also shown to induce apoptosis, alter calcium metabolism and cause remodeling in infarcted hearts.<sup>69</sup>

### **Lessons learnt from clinical trials and proposed model of de novo AHF**

Clinical trials of AHF and Takotsubo cardiomyopathy (ischemic insult or an acute hypertension or hypotension due to haemorrhage triggers AHF or worsening of CHF) have shown that acute heart failure is caused by a heterogeneous combination of conditions, affecting both young and old people. Based on these hypothesis proposed by various authors, I think that acute heart failure almost always comprises of elevated catecholamines and the myocardial stunning leading to cell death seems like an area one should focus in order to improve the functional recovery of the heart and in turn, survival of patients. Elevated catecholamines suppress glucose uptake in the myocardium and may cause hyperglycemia if the insulin secretion is reduced. I propose that in an acutely failing heart, similar to a chronically failing heart with elevated catecholamines and elevated fatty acids, insulin secretion is reduced which causes reduced glucose uptake even if glucose is available. This kind of acute heart failure may be triggered due to an acute ischemic insult or hemorrhage causing hypotension and acute hypoperfusion of the tissue. I propose that hypoperfusion caused by metabolic abnormalities triggers apoptotic pathways and leads to cell death due to increased mitochondrial calcium and sodium content, formation of reactive oxygen species (ROS) and oxygen wastage associated with fatty acid uptake.<sup>70</sup> Impaired mitochondrial efficiency causes decreased ATP production in conditions like ischemia and chronic HF. Since most AHF is caused by decompensation of CHF<sup>19</sup>, I propose that in AHF too, there is a reduction in ATP production which may be improved by substituting the substrate utilization from

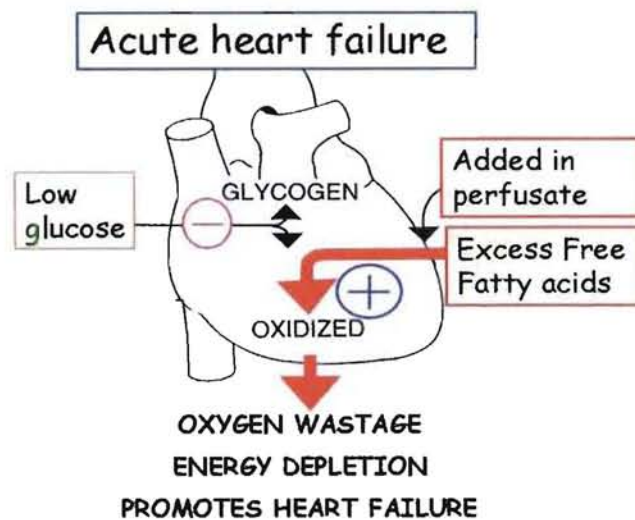
deleterious fatty acids to protective glycolysis and by administering insulin to facilitate glucose uptake.<sup>27,55,71</sup>

### **Proposed model of de novo acute heart failure**

Based on the clinical presentations, metabolic studies and mitochondrial function, my model of de novo AHF is based on the following:

- Reduced systolic pressure– which is associated with higher in-hospital mortality<sup>43</sup> and is a rationale to use a hypoperfusion in our model. Opie<sup>72</sup> in 1965 showed that left ventricular contraction could be calculated by multiplying heart rate and systolic pressure. I shall use this principle to reduce the left ventricular contractility by reducing the perfusion pressure. Studies by Bricknell and Opie<sup>73</sup> used a similar model of underperfusion to simulate ischemia to examine the effects of substrates on metabolic changes and lactate dehydrogenase release in ischemia.
- Elevated circulating catecholamines<sup>41,45,46</sup>–to inhibit glucose oxidation and promote fatty acid uptake.
- Increased fatty acids and reduced glucose in perfusate:<sup>27,74</sup>

This model uses a healthy isolated rat heart which is subjected to hypotension, reduced glucose, and elevated fatty acids in the perfusate and adrenaline to mimic the metabolic abnormalities in a failing heart. The hypotensive phase is achieved by reducing the perfusion pressure from 100 cm H<sub>2</sub>O to 20 cm H<sub>2</sub>O and fatty acids are introduced in the perfusate in this phase along with catecholamines while reducing glucose concentration in the perfusate.



**Figure 1-3-3:** Proposed substrate utilization in AHF (Copied from Opie LH, 2012)

We propose that in *de novo* acute heart failure with no prior history of heart disease, the use of glucose as a fuel for mitochondrial oxidation instead of fatty acids could serve as a therapy for acute heart failure, as has been argued for chronic heart failure.<sup>27</sup> In addition, glycolysis is protective because it is more energy-efficient and hence causes less oxygen wastage, thereby protecting the myocardium.<sup>73,75,76</sup>

### MITOCHONDRIAL FUNCTION AND CELL DEATH IN ACUTE HEART FAILURE

In a chronically failing heart as in an ischemic heart, mitochondrial dysfunction is closely related to worse outcomes in both animal models and patients.<sup>77,78</sup> We propose that in acute heart failure, similar stress situations like those in CHF and ischemia result in decreased ATP production and progressive worsening deterioration in mitochondrial integrity. Some of the stressors include impaired energetic, ROS production and excess calcium influx in the mitochondria. Rabuel C defines acute heart failure as a "reversible incapacity of the myocardium to provide a sufficient output for cellular metabolism".<sup>79</sup> This definition implies that there is an imbalance in energy utilization and energy production in an acutely failing heart which leads to reduced contractility and decline in mitochondrial function and ATP production.

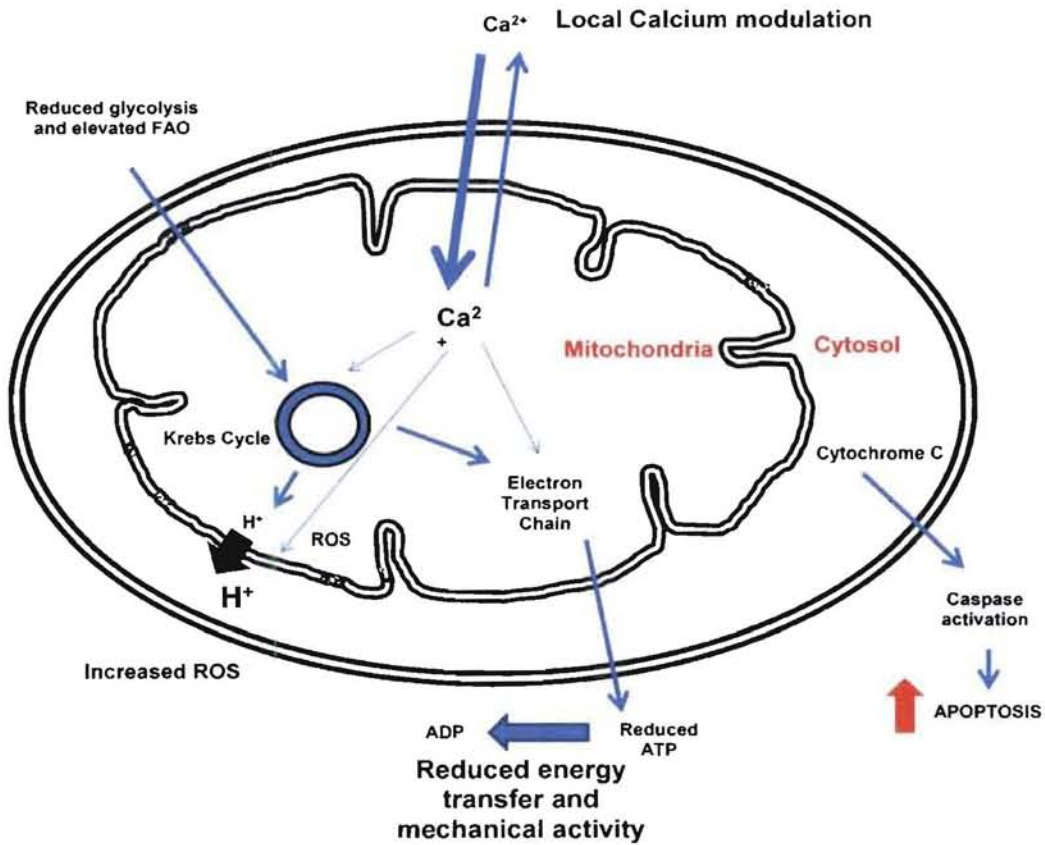
In my model of *de novo* acute heart failure, I shall examine the mitochondrial respiration on a Clarke type electrode after an episode of AHF to see if treatment with glucose and insulin in perfusate improves mitochondrial function as opposed to fatty acids.

### **Proposal: Mitochondria are involved in AHF**

**Myocardial energetics:** Acute heart failure is a rapid onset of, or change in, signs and symptoms of heart failure.<sup>19</sup> In CHF, mitochondrial energetic defects cause reduced creatine phosphate and ATP. The decrease in mitochondrial ATP production can be linked to altered substrate utilization, decrease of activity in the Krebs cycle enzymes or the enzymes of the electron transport chain. We propose that these defects in a chronically failing heart, when accelerated due to events like arrhythmias or an acute myocardial infarction, cause rapid deterioration of mitochondrial integrity and reduction in the production of myocardial high energy phosphates.

### **Reactive oxygen species (ROS) and calcium overload**

Mitochondria during acute coronary syndromes such as ischemia generate high amounts of reactive oxygen species<sup>80</sup> and cause oxidative stress, which impair the electron transport chain and generate even more ROS.<sup>29</sup> This causes damage to the mitochondrial membrane and may lead to cell death by opening of the mitochondrial permeability transition pore (mPTP).<sup>81</sup> Calcium modulation in a cell is also closely monitored by the mitochondria. They maintain homeostasis in the cell by maintaining the levels of calcium to prevent any changes in membrane potential. A rise in cytosolic calcium also causes a rise in the mitochondrial calcium content, leading to the opening of the mPTP, which leads to cell death.

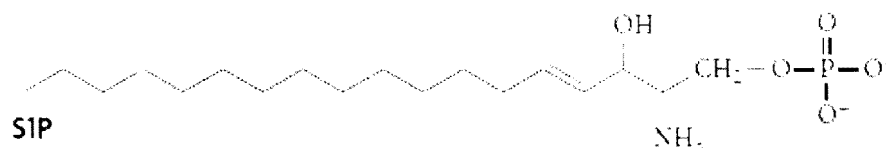


**Figure 1-3-4:** Proposed mitochondrial function in an acutely failing heart: ATP production is reduced due to utilization of free fatty acids (FFA) as substrates, hence reducing adenosine triphosphate (ATP) production, caspase activation leads to apoptosis and increase in free radicals causing oxidative stress. (Figure adapted from Rabuel C)<sup>79</sup>

# Section 4: Sphingosine-1-phosphate and acute heart failure

## Introduction

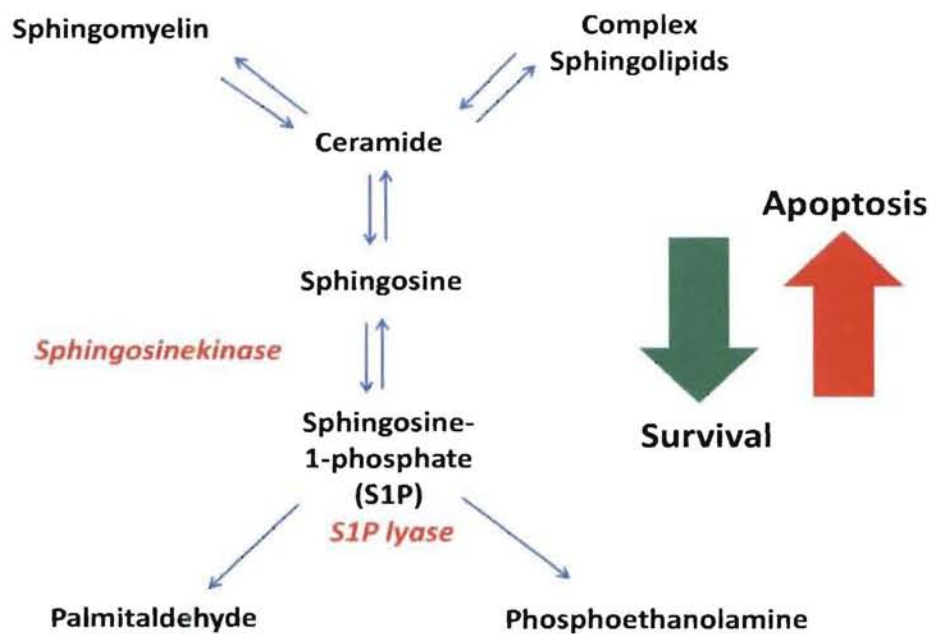
"It is a riddle wrapped in a mystery inside an enigma" is how Karliner describes the sphingolipid sphingosine-1-phosphate (S1P).<sup>82</sup>In 1884, "Father of Neurochemistry", JLW.Thudichum named the sphingolipids as such due to their mysterious chemical backbone and their inscrutable properties. The riddle of sphingolipids and their actions has now, however, been somewhat simplified, thanks to a lot of work in the world of sphingolipid signalling. S1P is now known to be involved in various cellular processes in the cardiovascular system, immune system and nervous system. S1P shows cardioprotective effects by decreasing infarct size after ischemic-reperfusion injury has been associated with cell proliferation, differentiation, permeability of vessels, cell motility and carcinogenesis and also has anti-apoptotic properties.<sup>17,83,84</sup>



**Figure 1-4-1:** Chemical structure of sphingosine-1-phosphate

Sphingomyelin produces many lipid components in the sphingomyelinase pathway, one of them being ceramide. Ceramide is then deacylated to sphingosine which is then phosphorylated by sphingosine kinase 1 or sphingosine kinase 2 to form sphingosine-1-phosphate. Ceramide and S1P have opposite effects in the cell.<sup>85</sup> While S1P is protective and non-apoptotic, ceramide is considered to be pro-apoptotic. The homeostasis between the two decides the fate of the cells. The main source of S1P in plasma is the erythrocytes, while minor sources include platelets

and endothelial cells. The concentration of S1P in human plasma is between 200-1000 nM.<sup>86</sup> Despite being present in such high concentrations in the plasma, a very low concentration of free S1P is available for activity, mostly because S1P is bound to high density lipoprotein (HDL) cholesterol molecules and albumin and, to some extent, by low density lipoprotein (LDL) cholesterol and very low density lipoprotein (VLDL) cholesterol molecules.<sup>87,88</sup>

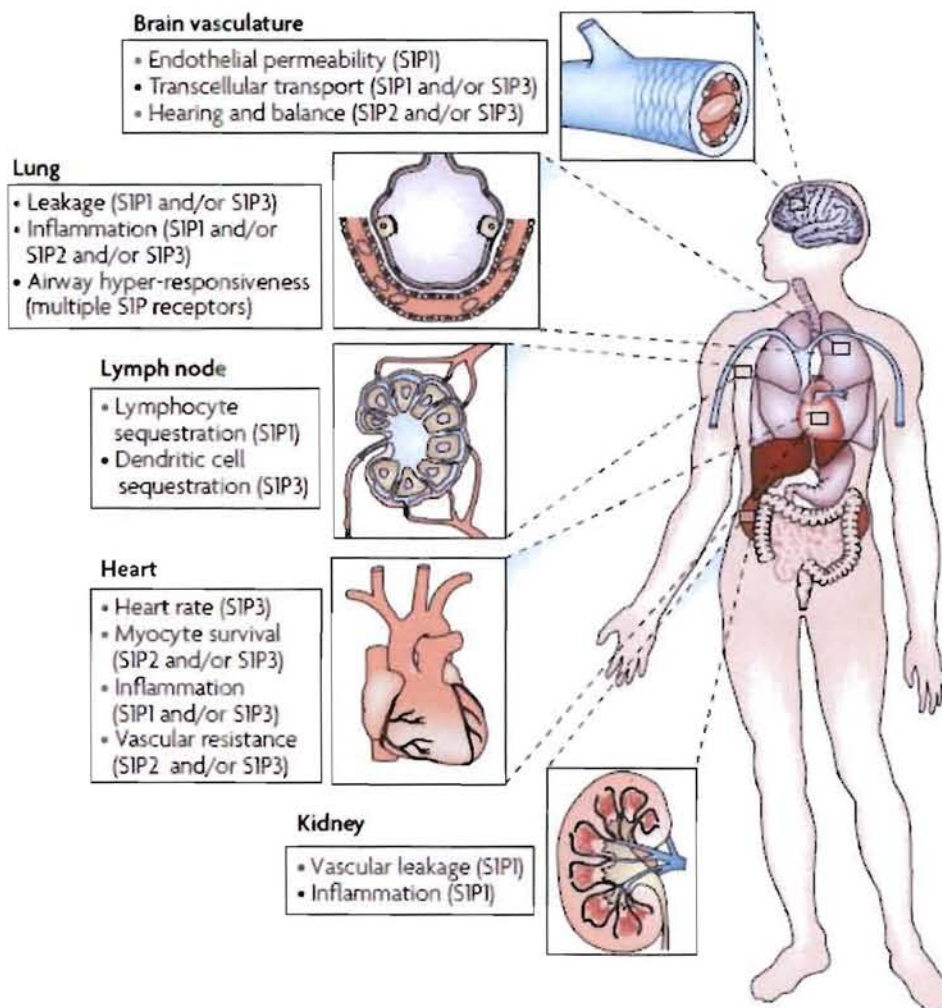


**Figure 1-4-2:** The synthesis of S1P from sphingomyelin. The reaction from ceramide to S1P is reversible in order to maintain cellular balance of the two opposing molecules. S1P lyase breaks down S1P into palmitaldehyde and phosphoethanolamine and is not a reversible step in the cascade. (Adapted from Kennedy et al)<sup>85</sup>

S1P has two modes of action: both by extracellular receptors and as an intracellular messenger. S1P binds to a group of cellular receptors called the G-protein coupled receptors or GPCR's which were discovered in the early 1990's and originally named as endothelial differentiation gene receptors.<sup>17,87</sup> S1P binds to its receptors and initiates a cascade of metabolic processes in various systems. Five receptors for S1P have been identified in the human body to date, namely S1PR<sub>1-5</sub>. These receptors are found in the cardiovascular system, nervous system and the immune system as well as the kidneys.

## S1P is an important intracellular messenger

S1P as an intracellular agent influences the regulation of calcium in the cell<sup>37,89</sup> and suppresses apoptosis.<sup>83</sup> Despite the fact that the mechanisms and intracellular targets of S1P remain unknown, Spiegel and Kolesnick state in their review that in cells like yeast which lack the GPCR's, phosphorylated sphingolipids modulate activities like cell growth and survival.<sup>83</sup> In plant cells, which also lack S1P receptors, intracellular S1P is responsible for calcium homeostasis and ion channels.<sup>83,90</sup> Above all, studies by van Koppen show that microinjection of S1P into mammalian cells induces calcium activity and cell proliferation.<sup>83,91</sup> S1P has a wide range of effects which are dependent on the signaling in which it is involved. However, for the interest of this thesis, I shall be focusing on the S1P receptors in the cardiovascular system only.



**Figure 1-4-3:** The different receptors of S1P in the body that regulate physiological processes. S1P1- S1P receptor 1; S1P2- S1P receptor 2; S1P3- S1P receptor 3.<sup>17</sup> S1PR1-3 have been identified in the heart so far and the physiological responses of S1P binding to each of the receptors is different. While S1P1 is mostly observed in endothelial cells, S1P2 is expressed in vascular smooth muscle cells which also express the S1P3 receptor.

### **S1P and cardiovascular diseases**

Sphingosine 1 phosphate is formed by the phosphorylation of sphingosine in the presence of sphingosine kinase (SphK) (Refer to figure in Introduction section above). SphK has two isoforms: Sphingosine kinase 1 (SphK1) and Sphingosinekinase 2 (SphK2). SphK1 levels are elevated in ischemic preconditioning (IPC), which is a process where short cycles of ischemia and reperfusion are administered before the actual ischemic insult.<sup>92</sup> IPC is cardioprotective and reduces infarct size. SphK1 is activated during IPC, promoting the formation of S1P, which in turn is cardioprotective and reduces infarct size. Lecour *et al* were the first to show protection by S1P in isolated rat hearts<sup>84</sup> and these results were confirmed in the isolated cardiomyocytes by Karliner *et al.*<sup>93</sup> In other studies using isolated cardiomyocytes, Zhang<sup>94</sup> and Vessey<sup>95</sup> showed that cardioprotection by S1P was equivalent to that of IPC (cycles of hypoxia and re-oxygenation, mimicking ischemia-reperfusion cycles) with a cell survival rate of around 93% compared to controls that were exposed to only hypoxia-reoxygenation and had a mortality rate of around 40%. S1P exerts its effects by binding to its GPCRs. There are 3 receptors in the heart and they modulate different functions, despite being expressed in the same cells. Endothelial cells mostly express the S1P1 receptor and to some extent S1P3 as well. On the other hand, S1P2 is found in vascular smooth muscle cells.<sup>17</sup> The vasoconstrictive effects of S1P were abolished by the addition of JTE013, an antagonist of S1P2. In the cerebral artery, S1P3 is responsible for vasoconstriction, demonstrating the distribution of S1P receptors and their heterogeneity. The effects of S1P are also dependent on the concentration in the plasma. In rat studies, a high concentration (>30mM) of S1P caused vasoconstriction while lower levels of plasma S1P (0.1-0.4mM) caused relaxation. The vasodilative effects of S1P are mediated through S1P1 and S1P3 and almost always require the activation of endothelial nitric oxide synthase (eNOS)

leading to the release of endothelial nitric oxide (NO).<sup>85</sup> In studies by Sugiyama *et al* on the canine sinus node, S1P increased the sinoatrial rate, while the ventricular blood flow and contraction were reduced.<sup>96</sup> Their study also revealed that S1P had no effect on the adenylate cyclase activity suggesting that S1P might be activating cyclic AMP via an indirect pathway.<sup>96</sup> The regulation of heart rate and sinus rhythm has been attributed to the S1P3 receptor as shown by Sanna *et al*.<sup>97</sup>

### **S1P and heart failure**

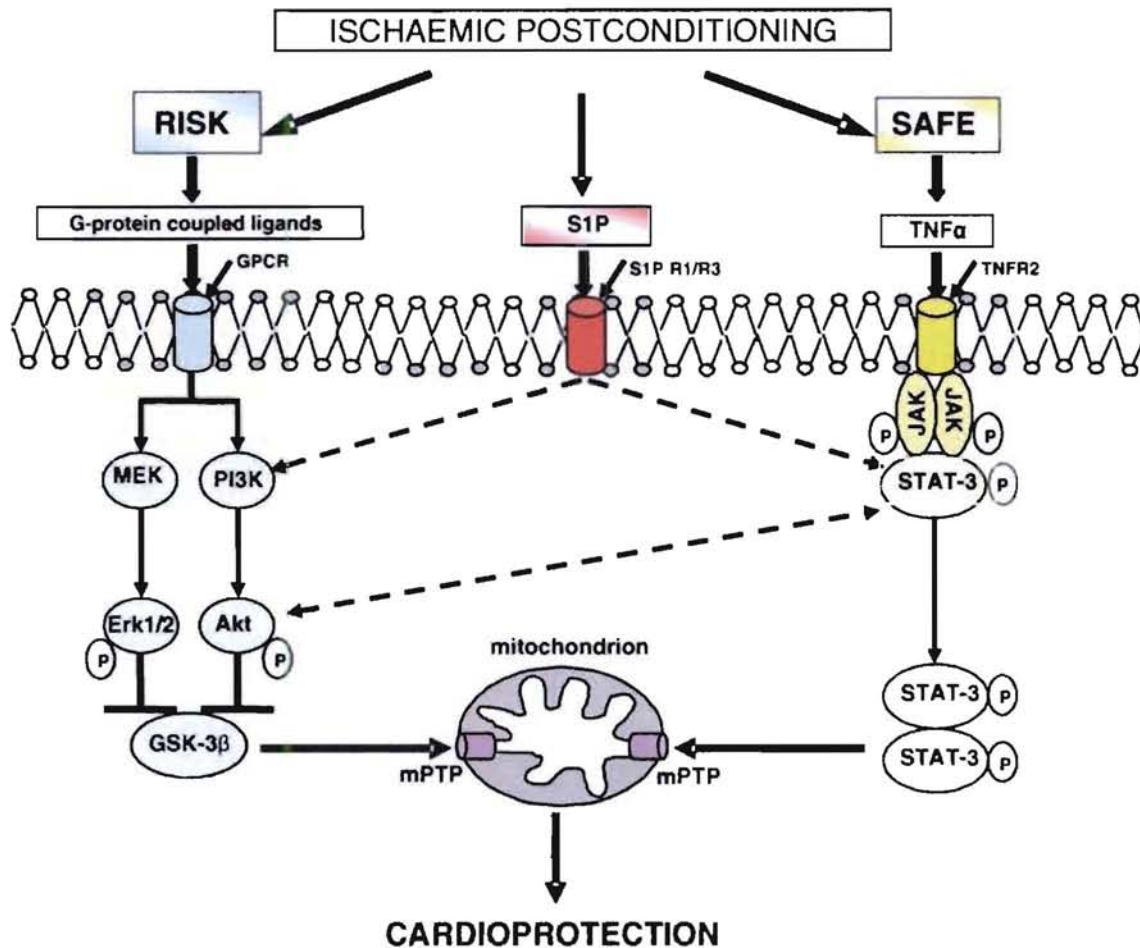
Very few studies have focused on the effects of S1P in the failing heart. In a study published by Knapp *et al*<sup>98</sup> in 2012, they attempted to measure the plasma concentrations of major sphingolipids in patients with chronic heart failure and healthy controls and found that there was no difference in plasma levels of S1P and ceramide between the two groups. However, they report that the levels of free sphingosine and sphinganine were reduced by 25% and 27% respectively in patients with systolic failure.<sup>98</sup>

Heart failure is associated with elevated fatty acid metabolism and reduction of protective glycolysis. In type 2 diabetes, ceramide concentrations in blood, muscle, adipose tissue and liver are increased, which is thought to be linked to progression of insulin resistance. Studies using pancreatic  $\beta$  cells showed a metabolic role of S1P. Exposure to high glucose stimulates sphingosine kinase (SphK) activity in the  $\beta$  cells, and leads to the secretion of insulin. This activation of SphK caused an increase in the plasma S1P concentration and this was abolished when SphK2 was knocked out. This suggests that the glucose-stimulated insulin secretion in the pancreas is mediated by SphK2.<sup>99</sup> This may be very important as a therapy in heart failure as the substrate utilization is altered with the heart utilizing fatty acids to generate ATP while wasting oxygen in the process. An up-regulation of insulin would cause the uptake of glucose, thus activating protective glycolysis and potentially salvaging the failing myocardium, suggesting a role of S1P in cardiac metabolism.

### **Mechanisms involved in protection with S1P in heart failure**

To delineate the pathways by which S1P exerts its cardioprotective effects is a challenge because S1P acts as an intracellular signaling molecule and also acts via

binding to its receptors to induce a variety of proliferative outcomes in the cells. Recently, S1P as an intracellular molecule has been shown to be transported by means of cystic fibrosis transmembrane conductance regulation (CFTR) in a mouse model of heart failure. The same study showed that CFTR expression is abolished in heart failure and that this is caused by elevated levels of TNF $\alpha$ .<sup>100,101</sup> Studies by Somers *et al*<sup>102</sup> show that S1P-induced cardioprotection after an ischemic-reperfusion injury is mediated by the activation of two powerful pro-survival pathways: namely Reperfusion Injury Salvage Kinase (RISK) which involves the activation of Akt/extracellular signal-regulated kinases (Erk)<sup>103</sup> and Survivor Activating Factor Enhancement (SAFE) pathway which requires the activation of tumor necrosis factor  $\alpha$  (TNF $\alpha$ )/signal transducer and activator of transcription 3 (STAT3) molecules. STAT3 requires translocation to either the nucleus or the mitochondria<sup>104</sup> post-phosphorylation. This transfer of phosphorylated STAT3 to the mitochondria is mediated by S1P in a mouse model of ischemic-reperfusion injury. However, the protection by S1P was abolished by the inhibitors of both RISK and SAFE pathways suggesting that the cardioprotective effects of S1P require the activation of both pathways and that S1P acts as a common ladder in the unification of these pro-survival pathways.<sup>102</sup> While the SAFE pathway is activated by a host of factors and molecules like insulin, opioids, HDL, melatonin, resveratrol and ischemic pre- and post-conditioning,<sup>104</sup> all these examine the SAFE pathway in the scenario of ischemic-reperfusion injury. There are no studies examining the effect of S1P in acute heart failure (AHF) or the involvement of pro-survival molecular pathways that might be active during AHF.<sup>104</sup> As Douglas Mann says, "One of the qualities of important research is that it frequently stimulates more questions than can be answered in a single study".<sup>105</sup> All these studies showing S1P promoting survival have sparked our interest in examining the effect of S1P in our model of AHF and we hope to delineate the molecular pathways involved if S1P is found to be protective. One of the hypotheses we propose is that since S1P increases the secretion of insulin, it could activate the SAFE pathway and lead to better recovery after an episode of AHF.



**Fig 1-4-4:** Cardioprotective RISK and SAFE pathways<sup>89</sup> (RISK- Reperfusion injury salvage kinase; GPCR- G protein coupled receptor; Erk- extra cellular signal regulated kinases; PI3K- phosphatidylinositol-3-OH kinase; GSK3β- glycogen synthase kinase 3 β; S1P- sphingosine-1-phosphate; S1PR1/R3- S1P receptors 1 and 3; SAFE- survivor activating factor enhancement; TNFα- tumor necrosis factor α; JAK- Janus kinase; STAT3- signal transducer and activator of transcription 3; mPTP- mitochondrial permeability transition pore)

## *Chapter 2*

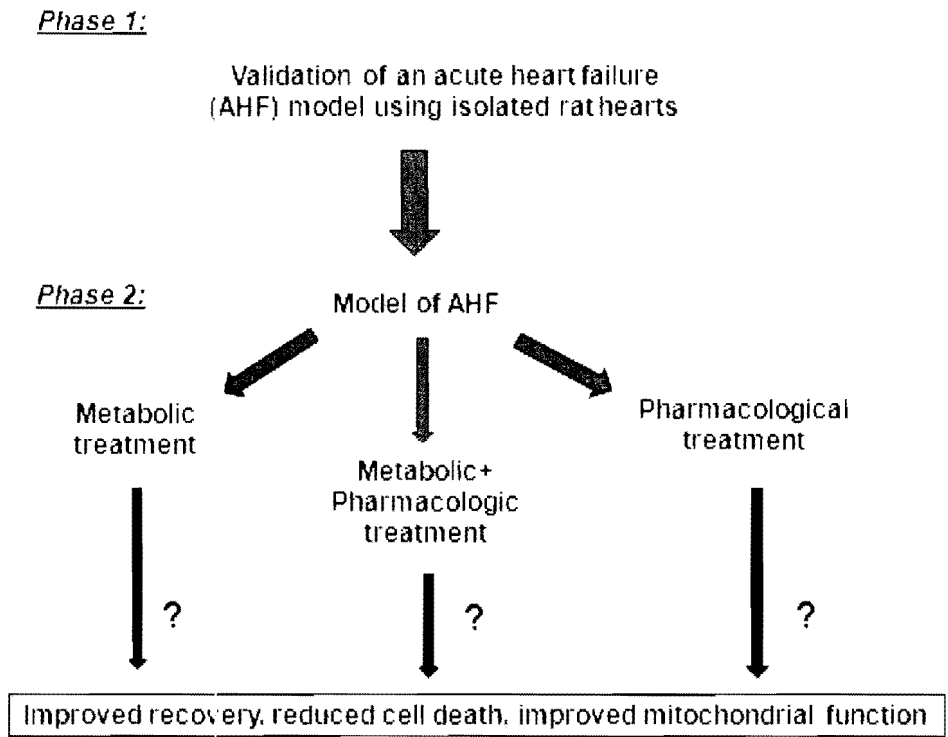
# **AIM and HYPOTHESIS**

### **AIM**

The lack of knowledge regarding the metabolism and effects of novel pharmacological agents in recovery against *de novo* acute heart failure is a possible major drawback in development of new treatments and therapies against AHF. Hence, the aim of this thesis is to establish a model of *de novo* acute heart failure using an isolated rat heart model on the Langendorff retrograde perfusion system and test pharmacological and metabolic agents for recovery and delineate the pathways involved in protection.

### **HYPOTHESIS**

- 1) Using our newly established model of *de novo* AHF, we hypothesize that the addition of molecular agent sphingosine-1-phosphate (S1P) improves the functional recovery after an episode of *de novo* acute heart failure and that the protection is mediated by the Survivor Activating Factor Enhancement (SAFE) pathway
- 2) We further hypothesize that glucose and insulin improve functional recovery after *de novo* acute heart failure and that they do so by reducing apoptosis in the heart.



**Figure 2-1:** Hypothesis that treatment with metabolic and pharmacological agents improves survival and recovery after AHF

# *Chapter 3*

## **METHODS**

To establish and validate a model of *de novo* acute heart failure and test metabolic and pharmacological agents for recovery, we used the following methods; which will be described in details here:

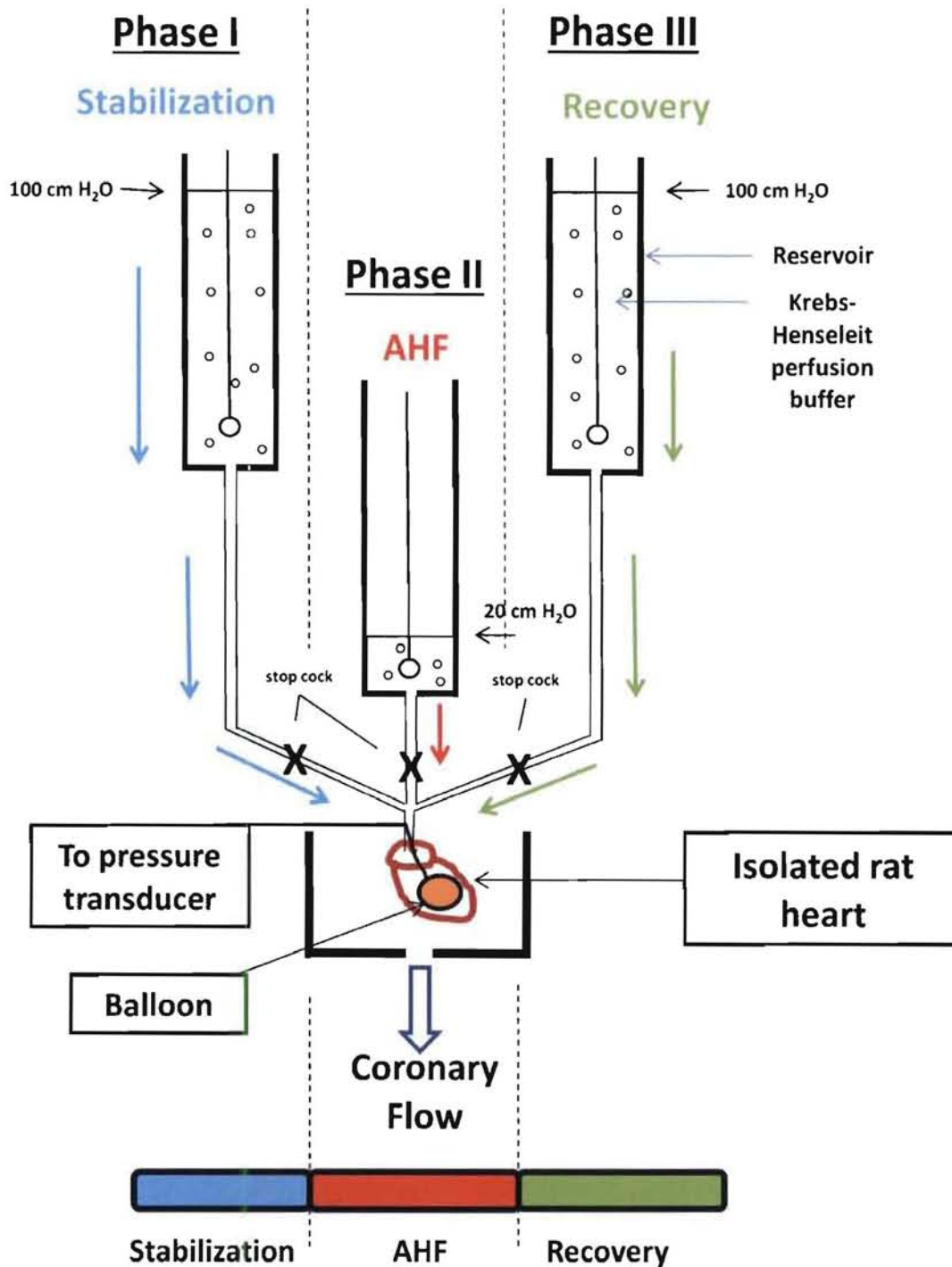
- Isolated rat heart Langendorff perfusion to determine the functional recovery of hearts
- Western blotting to determine the protein concentration for cell death and molecular mechanism determination
- Mitochondrial respiration measurement using a Clarke-type electrode

### **Isolated rat heart perfusions**

All experiments were conducted in male Long Evans rats weighing 250-320 g.

The rats were anaesthetised using sodium pentobarbitone (60mg/kg ip) in a syringe that was flushed with heparin to prevent blood clotting. The lack of pedal reflex tested the level of anaesthesia and once anaesthesia was established, the skin was incised at the xyphoid-sternum followed by the incision of the ribs to open the thoracic cavity. The heart was then removed rapidly and was transferred to a Petridish containing ice cold Krebs-Henseleit buffer.<sup>106</sup> The buffer consisted of sodium chloride (NaCl) (118.5mM, Sigma), sodium hydrogen carbonate (NaHCO<sub>3</sub>)(25mM, Merck), potassium chloride (KCl) (4.7mM, Sigma), magnesium sulphatehepta-hydrate (MgSO<sub>4</sub>. 7H<sub>2</sub>O) (1.2mM, Merck), potassium di-hydrogen phosphate (KH<sub>2</sub>PO<sub>4</sub>)(1.2mM, Sigma), glucose (11.1mM, Merck) and calcium chloride dihydrate (CaCl<sub>2</sub>) (1.3mM, Sigma). The aorta was cannulated and the heart was mounted on the Langendorff retrograde perfusion system. A 2-0 silk ligature was used to tie the heart on the cannula. A pressure transducer (AD Instruments) attached to a balloon was inserted into the left ventricle of the heart and the

pulmonary artery was snipped to release the Krebs-Henseleit buffer from the right ventricle. The balloon was inflated while inside the LV and the heart was to beat against the inflated balloon. The inflation of balloon was standardized when the LV developed pressure was maintained between 80-120mmHg in all hearts during the stabilization period. The mechanical beating of the heart was converted to an electrical signal which was subsequently read on the computer. Standard baseline parameters were set for every heart which were: HR between 240-420 BPM. The diastolic pressure was maintained below 12 mmHg. The acceptable baseline LV developed pressure was more than 80 mmHg while the coronary flow of the heart was between 8-12 ml per minute. The hearts were maintained at 37°C. The hearts were perfused at 100 cm H<sub>2</sub>O in this phase for 30 minutes.



**Figure 3-1:** Basic flow diagram of our Langendorff retrograde perfusion system to induce acute heart failure on an isolated rat heart.

The three phases i.e. Stabilization, Acute Heart Failure and Recovery. Each phase can be independently activated by switching the other reservoirs off at the stop-cock. The Stabilization phase and recovery phase were perfused at a 100 cm H<sub>2</sub>O while

the AHF phase had a perfusion pressure of 20 cm H<sub>2</sub>O. A balloon connected on one end to a pressure transducer was inserted into the left ventricle which converted the mechanical signal from the heart to an electrical signal.

The model consisted of three phases:

**Phase 1: Stabilization:** which took place over 30 min at 100 cm H<sub>2</sub>O perfusion pressure with glucose (11.1mmol/L) as the sole substrate in the perfusion buffer. The basal functional parameters were achieved in this phase. The accepted baseline parameters are described in the table below. Hearts were maintained throughout at 37°C. The purpose of this phase was to wash any metabolites and achieve the threshold parameters for heart rate, left ventricular developed pressure, diastolic pressure and coronary flow. Hearts that did not meet the parameters at the end of 30 min were excluded from the study.

#### **Exclusion criteria for isolated perfused hearts**

<b>Parameter</b>	<b>Values</b>
Heart rate (HR)	240-420 beats per minute (BPM)
Left ventricular end diastolic pressure (LVEDP)	0-12 mmHg
Left ventricular developed pressure (LVDP)	80-120 mmHg
Coronary flow rate (ml)	8-12 ml/min

**Phase 2: Acute heart failure phase:** This phase took place over 35 min where the perfusion pressure was dropped to 20 cm H<sub>2</sub>O and the glucose concentration was reduced to 2.5 mmol/L with addition of fatty acids (1.3 mmol/L) in the perfusate. Previously, Bricknell and Opie<sup>73</sup> established a model of low pressure global ischaemia with residual oxygen uptake to study the effects of substrates on tissue metabolism and measurement of lactate dehydrogenase and arrhythmias during reperfusion. They reduced the perfusion pressure from 100 cm H<sub>2</sub>O to 20 cm H<sub>2</sub>O to simulate a model of global ischaemia. We used the similar principle of reducing perfusion pressure to mimic the hypotension associated in patients with acute heart failure. The change in substrate concentration was made in order to reduce the uptake of glucose by the isolated heart. In their experiments, Hamilton and

Saggerson<sup>56</sup> found that in a dose response to measure the production of malonyl CoA concentration in myocytes, 2.5mmol/L of glucose significantly reduced the concentration of malonyl CoA when compared to physiological 5mM. Malonyl CoA<sup>56</sup> is formed by the carboxylation of acetyl CoA by acetyl CoA carboxylase (ACC). Malonyl CoA is responsible for the inhibition of the activity of carnitine palmitoyltransferase-1 (CPT-1) which regulates the uptake of fatty acids by transferring the long chain fatty acyl CoA through the membranes of mitochondria.<sup>62</sup> Hence, we decided to reduce the concentration of glucose in our model of acute heart failure to promote the upregulation of fatty acids. Fatty acids were introduced in this phase of the experiment. We used 1.3mmol/L of fatty acids bound to 1.4mol/l bovine serum albumin (BSA). Aasum and Larsen<sup>107</sup> in 1997 showed impaired ventricular function and calcium homeostasis when hearts were perfused with 1.2 mM Palmitate and glucose. We aimed to induce acute failure in the isolated rat hearts; hence, we used higher concentration of fatty acids.

**Phase 3: Recovery phase:** This phase lasted 30 min where perfusion pressure was restored back to 100 cm H<sub>2</sub>O but fatty acid and glucose concentration in the perfusate was unaltered from previous phase. We aimed to observe the effects of low glucose and high circulating fatty acids despite the improvement in perfusion pressure, thus mimicking the recovery phase in patients with hypotensive *de novo* acute heart failure or patients with hypotensive Takotsubo cardiomyopathy.

## **WESTERN BLOTTING**

### **Lowry protein determination**

5µl (in duplicates) of aliquots of each sample were added to 995µl distilled water which was followed by addition of 1ml Solution A (Na<sub>2</sub>CO<sub>2</sub> (20%) and CuSO<sub>4</sub>.5H<sub>2</sub>O (0.2 g) + dipotassium tartarate (0.4 g) in 100 ml were mixed 1:1 to make a final volume of 200 ml CTC reagent. To this, 200 ml of 10% SDS and 200 ml of 0.1 M NaOH were added. The total volume of Solution A was 600 ml. Samples were immediately vortexed and incubated for 10 min at room temperature. Following that, 500µl Folin's reagent (Merck); diluted 1:6 (Solution B) was added while vortexing because the color complex forms immediately. The samples were then incubated for

30 min and thereafter read at 750 nm on a Varian 120 dual spectrophotometer. BSA was used to construct a standard curve from 5-200 µg/ml and was read in the same way as the samples.

### **Bradford protein determination**

Protein determination was done using a Bradford assay.<sup>108</sup> The Bradford reagent was diluted to a 1x working solution with distilled water. In an Eppendorf, 5µl of protein sample and 95µl of distilled water were mixed, and 900µl of the Bradford reagent was added. After mixing, 200µl of each sample was loaded onto a microplate for the absorbance to be determined. . The components were protected from light as far as possible for the duration of the assay to prolong the stability of the working solution. BSA standards were added to separate wells and mixed, these served to construct a standard curve to determine the protein concentration. Protein extracts were added to separate wells (10µl per well) and mixed. Absorbance was measured at 595nm using a microplate reader (GloMax, Promega, Fitchburg, WI 53711) and protein concentration was determined from the standard curve. All samples were assayed in duplicate.

**Table 3-1:** Composition of buffers for protein extraction

	<b>Hypotonic Buffer</b>		<b>Hypertonic Buffer</b>
<b>1M HEPES pH 7.9</b>	1ml		500ul
<b>0.1M EDTA</b>	100ul		250ul
<b>1M DTT</b>	100ul		25ul
<b>0.1M EGTA</b>	100ul		250ul
<b>0.1M PMSF</b>	1ml		250ul
<b>1M KCl</b>	1ml	<b>5M NaCl</b>	2ml
<b>0.1M Na<sub>3</sub>VO<sub>4</sub></b>	1ml		-
<b>dH<sub>2</sub>O</b>	95.7ml		21.725ml
	100ml		25ml

### **Protein extraction: Cytosolic and Nuclear**

400ul of hypotonic buffer was added per tube. Crushed hearts were added in Eppendorff tubes and left on ice for lysis. 25 ul of 10% NP-40 (16x dilution of Nonident P40 / IGEPAL 100% with dH<sub>2</sub>O) was added to this and vortexed for 30 sec followed by incubation on ice for 5 min. The sample was vortexed again and then centrifuged for 3 min at 13200 RPM at 4°C. The resulting supernatant was collected which was the cytoplasmic fraction. The pellet was washed with 200µl hypotonic buffer and vortexed again followed by centrifugation at 4°C for 3 min at 13200RPM.

Extraction of the nuclear protein from the sample was carried out by, the discarding the supernatant and resuspending the pellet in 100ul of hypertonic buffer. This was then vortexed and left on ice for lysis for 20 min. After the lysis step, the sample was centrifuged for 7.5 min at 13200RPM at 4°C. The resultant supernatant contained the nuclear fraction.

Proteins were adjusted to the same concentration; diluted 1:1 with low ionizing buffer or Lamelli buffer before preparing gels.

**Table 3-2:** Components for gels

Lower Gel	2 gels	4 gels	Upper gel	2 gels	4 gels
<b>dH<sub>2</sub>O</b>	9.8ml	19.6ml		7.5ml	15ml
<b>1.5M Tris HCl pH 8.8</b>	5ml	10ml	<b>0.5M Tris HCl pH 6.8</b>	3ml	6ml
<b>20% SDS</b>	100ul	200ul		60ul	120ul
<b>Acrylamide (40% Bis)</b>	5ml	10ml		1.5ml	3ml
<b>10% APS</b>	100ul	200ul		80ul	160ul
			Bromophenol blue to colour		
<b>TEMED (ADD BEFORE POURING)</b>	40ul	80ul		40ul	80ul

To run the gels, the glass plates and combs were wiped clean with ethanol. The plates were set up and checked to avoid any leaks. The lower gel was poured into the space between plates and was allowed to set. This was followed by pouring the upper gel and the combs for wells were inserted. Approximately after 15 min when the gel was set, the assembly was removed and inserted into the running chamber

and the chamber was filled with running buffer. The combs were removed carefully and the wells were flushed to remove any air bubbles. The samples were denatured by suspending the Eppendorff tubes in boiling water for 5 min. The samples were then vortexed before loading.

3-10  $\mu$ l of protein ladder was added in the first lane and the samples in the subsequent lanes. Running buffer was added to the outer compartment of the gel tray to the same level as the inner compartment and the gels were run at 100V for ~2.5 hours or until the gel front reached the end of the gel. The transfer step in the protocol was prepared in this time.

To transfer the proteins from the gel to the membrane, PVDF membrane was cut to size of the gel and washed in methanol for 5 min followed by a wash with distilled H<sub>2</sub>O for 2 min. It was then left in cold transfer buffer. To set up the sandwich, ice cold transfer buffer was poured into dishes and the sandwich was prepared as illustrated.



**Figure 3-2:** Composition of layers for transfer of gels

Once the gel was run, the lower gel was cut and placed onto the membrane and extreme care was taken to avoid air bubbles between membrane and gel. The cassette was placed in the holder and transfer process took place at 0.02A overnight or 0.2A for 2 hours at 4°C. Following transfer, the membrane was removed and was fixed in methanol for 30s and then air dried completely.

The membrane was labeled, washed with Ponceau, rinsed with dist H<sub>2</sub>O and placed between cellulose acetate film and scanned. It was then washed with TBS-T once by hand followed by 3 X 5 min washes. This was then followed by blocking with milk powder in TBS-T for 2 hours at room temperature. This step was followed by 3 more

washes with TBS-T for 5 min. Membrane was exposed to primary antibody (1:3000-1:10,000 in TBS-T with 5% milk powder) overnight at 4°C.

The following day, membrane was washed 3x% with TBS-T and exposed to secondary antibody (1:3000 – 1:5000 in TBS-T) for 2 hours at room temperature. This was followed by more washing (3 X 5 min with TBS-T) and developed with ECL reagent (5 x 5min exposures with no light followed by 1 x 2sec white light exposure) for visualization.

## **MITOCHONDRIAL RESPIRATION MEASUREMENT**

Failing hearts are less energy efficient in utilizing the substrates for generation of ATP, the currency of energy for the cell. Five groups were studied: baseline hearts with no heart failure, control, S1P, Insulin and S1P + Insulin with the drugs having administered in the recovery phase. Also, the recovery phase was reduced to 7' in order to obtain as many viable mitochondria as possible. The substrates in the recovery phase were glucose (11.1mmol/L) and FFA (1.3mmol/L) for all hearts

The parameters measured were State2, State3, State4 respiration and respiratory control index (RCI). The mitochondrial protein was measured using a Qubit assay to normalize the values for all mitochondrial proteins.

### **Buffers for mitochondrial extraction and measurement of respiration on the oxygraph**

Isolation medium: The isolation medium to mince the heart and homogenize the heart consisted of 0.18M KCl, 0.001 M Ethylenediaminetetraacetic acid (EDTA) and the pH was set to 7.4 using Tris.

Incubation buffer: The incubation buffer was freshly made for every experiment. It consisted of 1.25 M Sucrose, 100mM Tris-HCl, 85mM K<sub>2</sub>HPO<sub>4</sub>

Glutamate (50mM) was used as substrate for mitochondrial respiration.

### **Mitochondrial Isolation Procedure**

The buffers were prepared as per instructions, the hearts were removed from the perfusion apparatus and immediately placed in Petri dishes containing ice cold isolation buffer. The heart was cut into very small pieces using small scissors. Once this was done, the hearts in the isolation buffer were transferred into a Teflon homogenizer. They were homogenized till the hearts were liquidized. This homogenate was then transferred into centrifuge tubes and spun for 10 min at 4°C at 2390 RPM. The resultant supernatant was transferred to another clean centrifuge tube and the pellet was discarded. This supernatant was then spun again at 4500 RPM for 10 min at 4°C. After centrifugation, the supernatant was discarded this time and the mitochondria containing pellet was resuspended in fresh incubation buffer. This mixture was then transferred into a fresh Eppendorff® tube.

### **Mitochondrial respiration measurement procedure**

The mitochondrial respiration studies took place on an Oxygraph (Oxytherm, Hansatech). The oxygraph contains a 1 ml chamber and has a S1 Clark type polarographic oxygen electrode disc. This forms the floor of the electrode chamber.

The chamber contained 316µl of incubation buffer. A baseline reading was taken for a minute to verify the stability of the calibration. Once that had been achieved, 20 µl of 50mM Glutamate was added as substrate in the chamber. 50 µl mitochondria were added to the chamber and respiration was measured in presence of glutamate. This represents State2 respiration in absence of ADP. Once this was stabilized, 14 µl of 10mM ADP was added to the chamber and the respiration was measured where ADP was rapidly taken up by the mitochondria to produce ATP. This was State3 respiration in the protocol. This phase was followed by State4 respiration where all ADP has been converted to ATP and the respiration is steady again. The RCI was calculated as a ratio of values of State3/State4. Respiratory control index (RCI) is a measure of viability of the mitochondrial population and a higher RCI indicated greater amounts of respiring mitochondria.

## *Chapter 4*

# **VALIDATION OF AN EX-VIVO** **MODEL OF DE NOVO ACUTE** **HEART FAILURE**

### **INTRODUCTION**

Acute heart failure (AHF) is associated high in-hospital mortality in patients presenting with low systolic blood pressures and adequate therapies are needed to improve the patients' health.<sup>43</sup> Most AHF results from acute decompensation of CHF where, a chronically failing heart deteriorates rapidly. The origins and pathophysiologies are multiple and our understanding of the disease mechanism remains very poor in part due to the paucity of basic research in the field.<sup>19,39,109</sup> The lack of an experimental animal model exploring the mechanisms of *de novo* AHF may explain the lack of knowledge and lack of efficient therapies. We therefore aimed to develop a simple and reproducible model to induce *de novo* AHF using an isolated rat heart model that we developed.<sup>49</sup> This model is to our knowledge, the first small animal model of AHF. Our model will hopefully contribute to better understand the pathophysiology of the disease and its underlying mechanisms and may serve as a tool to explore potential therapeutic targets for the disease.

Our model of *de novo* AHF mimics the clinical conditions of "cardiogenic shock" where the perfusion pressure that governs the external work in the perfused rat heart is abruptly reduced, as would happen when massive myocardial infarction in humans results in decreased myocardial contractility and hypotension (see table). Our model uses simulated hypotension with reductions in heart rate and substrates provided in the perfusate. The isolated heart model has both similarities to and dissimilarities from human clinical AHF with low blood pressure as inpatients with Takotsubo

'stress' cardiomyopathy<sup>46,110</sup> who may have AHF with lower systolic pressures (50-104 mmHg)<sup>43</sup> without any prior heart disease. Therefore, we tested the effects of high circulating catecholamines in our model of the acutely failing underperfused heart,<sup>73</sup> We also induced AHF by cardiac pacing,<sup>111</sup> or by the addition of adrenaline in increasing concentrations and we induced AHF by altering calcium and glucose concentrations in the perfusate.

**Table 4-1:** Comparison and differences between human AHF and our model

Conditions	Human AHF	Our model of AHF
Onset	Mostly acute-on-chronic (80%) very few <i>de novo</i> cases (20%) <sup>39</sup>	<i>De novo</i>
Hypertension	Almost 25% in Europe and USA; 45% in Africa <sup>36</sup>	Future work
Hypotension	10% of all patients in USA and Europe <sup>39,43</sup> ; Reduced systolic pressure associated with higher mortality <sup>43</sup> ; Known in patients with Takotsubo cardiomyopathy <sup>46</sup>	Reduction of perfusion pressure to induce hypotension
Catecholamines	No data available, however, in stress situations like AHF, catecholamines will be elevated; In Takotsubo cardiomyopathy, supra-physiological levels of circulating catecholamines <sup>41,46</sup>	High levels of adrenaline added in the AHF phase of the protocol
Substrates	No data available in AHF patients; elevated catecholamines will lead to up-regulation of FFA oxidation	Added high free fatty acids in perfusate; glucose concentration reduced

- **Hyperadrenergic stimulus to induce acute heart failure**

Patients with Takotsubo cardiomyopathy and acute heart failure, have supra-physiological levels of circulating catecholamines.<sup>46</sup> We therefore developed our model of acute HF in which we gave adrenaline in the acutely failing heart coupled with low glucose and high FFA in the perfusate. To validate an optimal model of *de novo* AHF, we used three concentrations of adrenaline for our study:  $10^{-6}$ ,  $10^{-8}$  and  $10^{-10}$  M to induce AHF and monitor recovery. Previous work from our laboratory by Horak and Opie<sup>70</sup> showed that adrenaline at  $10^{-8}$ M caused decreased efficiency of pressure work or “oxygen wastage” in catecholamine-induced myocardial injury. We tested three concentrations.

- **Glucose alteration in perfusate**

Glucose utilization for ATP production is reduced in a failing heart owing to the elevated adrenergic stimulus which promotes preferential fatty acid oxidation<sup>27</sup> 11.1mmol/L glucose is the standard concentration in isolated heart protocols while physiological glucose concentration is 5mmol/L. Previous studies on cardiac myocytes to investigate the changes in malonyl-CoA in response to glucose alterations have used the concentration of 2.5mmol/L.<sup>56</sup> However, this concentration of glucose has never been used in an isolated heart to induce *de novo* AHF. Hence we used two concentrations, 5 and 2.5mmol/L to examine the effects of each in the AHF and recovery phase. The fatty acids in the buffer were maintained at 1.3mmol/L in AHF and recovery phases.

- **Calcium modulation as a model of acute heart failure**

Calcium signaling plays an important role in the contraction of the heart. Contractility is compromised in a failing heart (Opie, LH, Heart Physiology; 4<sup>th</sup> edition). Calcium is shuttled in and out of the myocyte during every contraction-relaxation cycle. Altered calcium concentration in the heart is linked to risk of chronic heart failure, due to arrhythmias.<sup>112,113</sup> Hence we hypothesized that acute alteration of calcium concentration in the perfusate from 1.3 to 2.5mmol/L would promote contractile dysfunction, leading to *de novo* acute heart failure. The experiment was done in 3 phases: stabilization, AHF phase and recovery phase. The composition of the buffer for stabilization phase was same as described above. In the AHF and recovery phases, however, the calcium concentration was

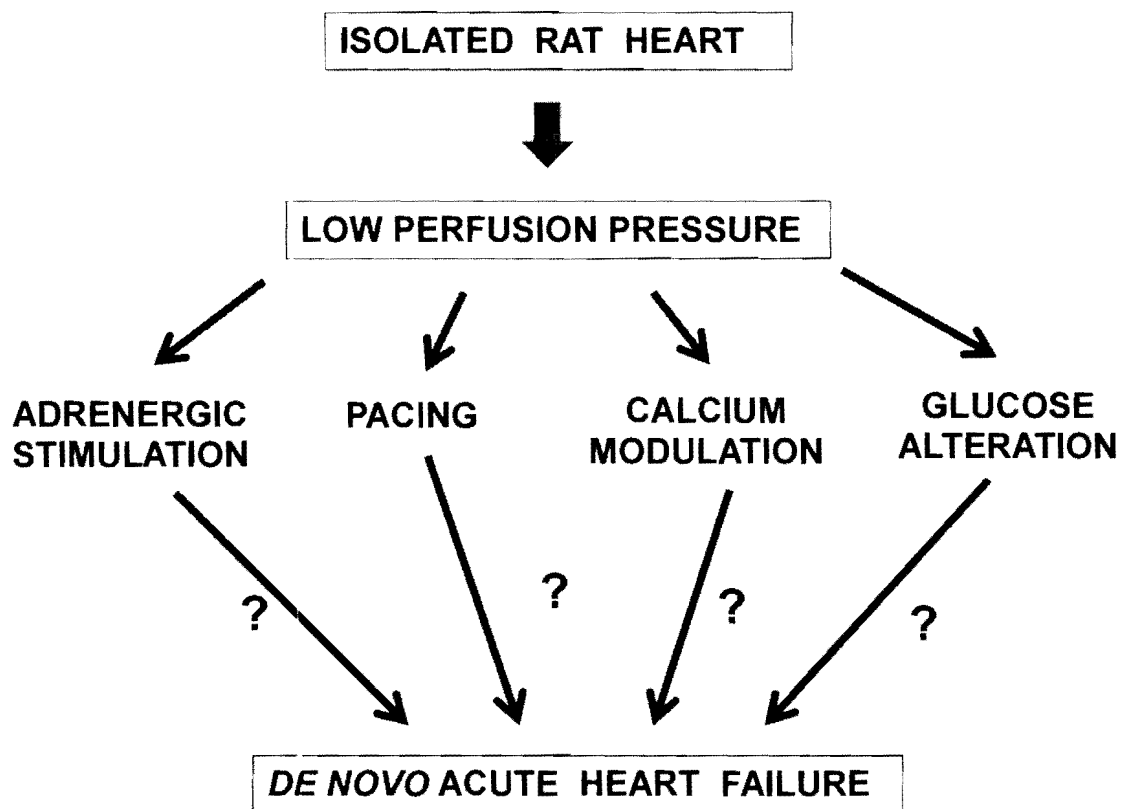
increased to 2.5mmol/L. The glucose concentration and fatty acid concentrations were 2.5 and 1.3mmol/L respectively. This is to force the heart to utilize more fatty acids over glucose and to promote heart failure. The perfusion pressure during the acute heart failure phase was 20cm H<sub>2</sub>O while the recovery phase was at 100 cm H<sub>2</sub>O. Temperature was maintained at 37°C throughout the protocol.

- **Pacing to induce acute heart failure**

Pacing has been used extensively as a model of chronic heart failure in canine studies by Stanley *et al.*<sup>111</sup> Pacing in dogs for 28 days caused LV hemodynamic alterations and dilation in their studies. We decided to pace rat hearts on our Langendorff retrograde perfusion system to examine whether acute pacing would cause acute heart failure. We examined 2 pacing speeds; 300 BPM and 150 BPM. The hearts were paced in the acute heart failure phase of the protocol for 35' in the presence of 1.3mmol/L FFA and 2.5mmol/L of glucose in the perfusate.

## **HYPOTHESIS**

We hypothesize that these different alterations used individually or in combination in the validation of the model will help us establish a reproducible model of *de novo* AHF.



*Figure 4-1: Hypothesis diagram to validate models to induce AHF*

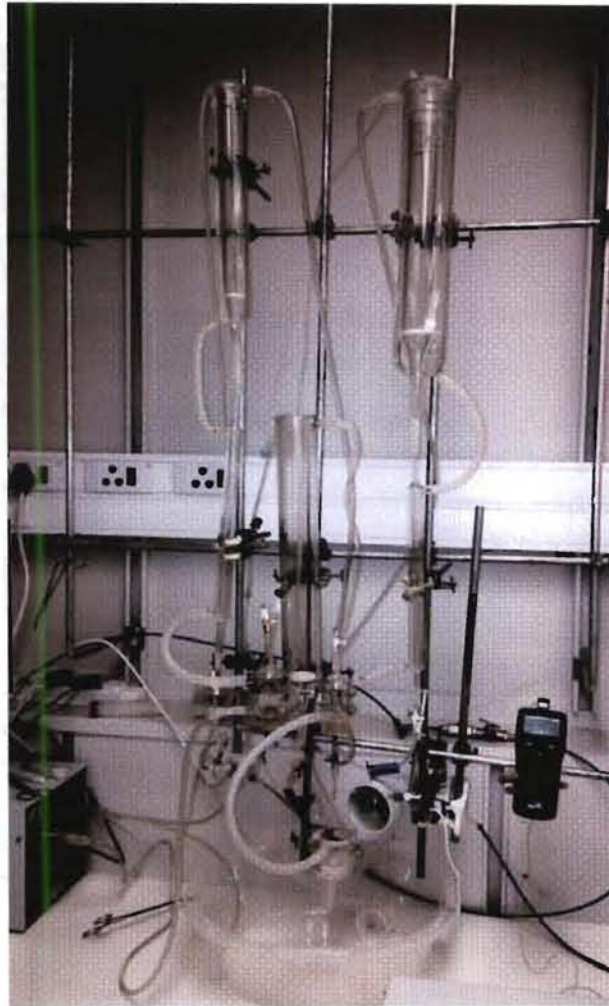
## **MATERIALS AND METHODS**

### **Animals**

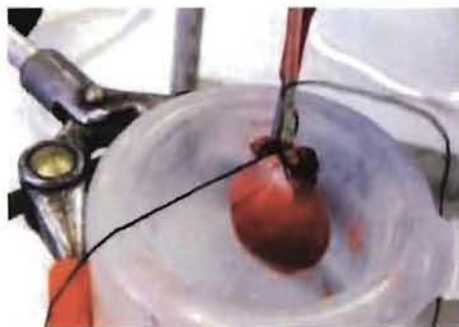
Adult Long Evans rats were used for the experiments in accordance with the Guide for the Care and Use of Laboratory Animals. The ethics committee of the University of Cape Town medical school approved all experiments (project no 011/038). The rats were housed in the animal unit at University of Cape Town, medical campus and had free access to food and water.

### **Isolated rat heart perfusions**

The heart perfusions were done as described in the methods sections in Chapter 2 of this thesis.



**Figure 4-2:** Langendorff retrograde perfusion system, modified to induce de novo acute heart failure (above); perfusion of the isolated rat heart via the cannulated aorta is shown (bottom)



**Protocols for isolated heart perfusions for functional recovery in this section**

**Table 4-2:** Protocol for different conditions to induce de novo acute heart failure



	Stabilization	AHF	Recovery
Glucose (mM)	11.1	5 Or 2.5	5 or 2.5
Adrenaline (M)	None	10 <sup>-6</sup> Or 10 <sup>-8</sup> Or 10 <sup>-10</sup>	None
Pacing (BPM)	None	150 Or 300	None
Calcium (mM)	1.3	1.3	1.3 Or 2.5

The experiments in each group were performed as follows:

- For experiments examining hyperadrenergic stimulus to induce acute heart failure:

The protocol consisted of 3 phases: stabilization, AHF phase and recovery. Glucose concentration for all hearts was maintained at 11.1mmol/L in stabilization phase. In the AHF phase, the glucose concentration was dropped to 2.5mmol/L and fatty acids (1.3mmol/L) were introduced in the perfusate. Adrenaline 10<sup>-6</sup> or 10<sup>-8</sup> or 10<sup>-10</sup> M was added to the hearts in this phase. The AHF phase was followed by recovery phase where the glucose and fatty acid concentration was same as the AHF phase and adrenaline infusion was stopped.

- **For experiments examining the effect of glucose modulation to induce acute heart failure:**

The protocol as described previously consisted of 3 phases: stabilization, AHF and recovery. Stabilization phase was characterized by perfusion by 11.1mmol/L glucose, in the AHF phase, fatty acids (1.3mmol/L) were added and the glucose concentration was either 2.5mmol/L or 5mmol/L in the perfusate. This phase was followed by recovery phase where the fatty acid and glucose concentration was maintained as the AHF phase.

- **For experiments examining the effect of calcium modulation to induce acute heart failure:**

The basic protocol in stabilization phase was similar to the experiments described above. The calcium concentrations in AHF and recovery phase were altered to 1.3mmol/L or 2.5mmol/L. No adrenaline was added to the hearts and glucose concentration was at 2.5mmol/L in AHF and recovery phase.

- **For experiments involving pacing to induce acute heart failure:**

The protocol for stabilization phase was same as in the other groups. In the AHF phase, fatty acids (1.3mmol/L) were introduced to the perfusate and glucose concentration was reduced to 2.5mmol/L. The hearts were paced at either 150 or 300 beats per minute (BPM) in the AHF phase. The pacing was stopped in the recovery phase and the substrate concentrations in the perfusate were same as those in the AHF phase.

### **End points**

The functional parameters measured were heart rate (HR), left ventricular developed pressure (LVDP), rate-pressure product (RPP), while functional recovery of the heart was calculated as the percentage of RPP against baseline stabilization values. We also monitored left ventricular end systolic pressure (LVESP) and left ventricular end diastolic pressure (LVEDP).

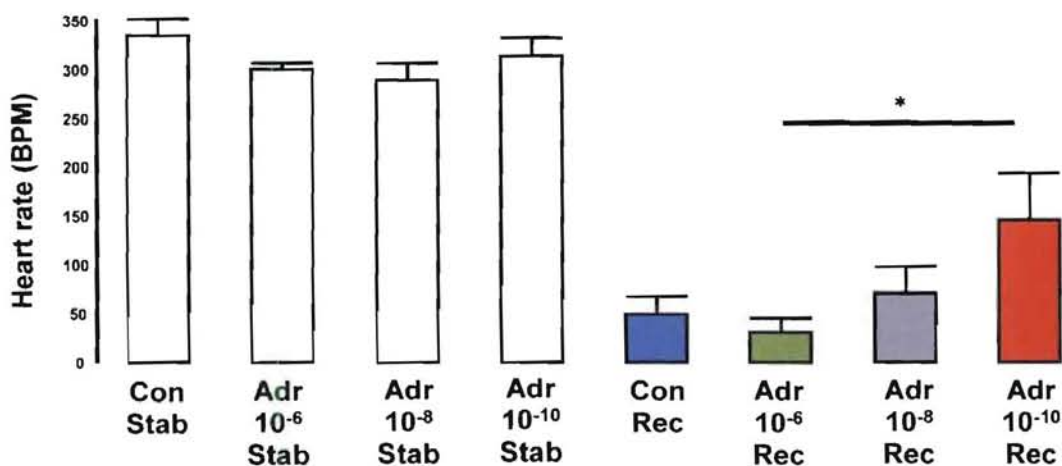
## Statistical analysis

All statistical analysis was done using InStat software. The data are expressed as mean  $\pm$  the standard error of mean (SEM). Tukeys test was performed between multiple groups with  $p < 0.05$  was considered significant. The sample size in these experiments was  $n=6-8$ .

## RESULTS

### Addition of adrenaline to induce acute heart failure

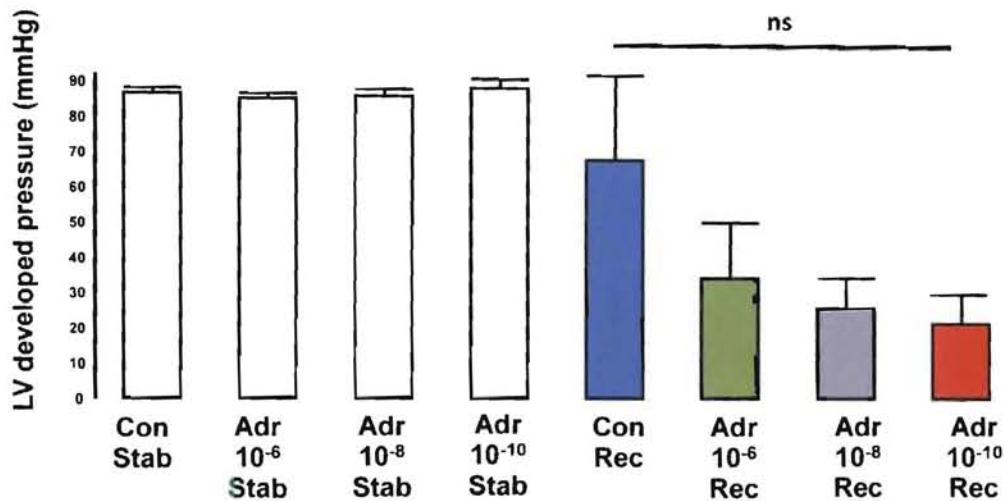
#### *Effect of adrenaline on recovery heart rate*



**Figure 4-3:** Effect of adrenaline on heart rate (HR)- Stabilization phase; Con- Control; Adr- Adrenaline; Rec- Recovery phase (\*-  $p < 0.05$  vs. Adr 10<sup>-6</sup> Rec; Tukey-Kramer Multiple Comparisons Test)

The stabilization phase heart rate for all hearts was 355.8 $\pm$ 16.4 BPM for controls, 300.8 $\pm$ 6.0 BPM for hearts treated with 10<sup>-6</sup> M adrenaline, 290.0 $\pm$ 16.9 for hearts treated with 10<sup>-8</sup> M adrenaline and 314.2 $\pm$ 18.9 BPM for hearts treated with 10<sup>-10</sup> M adrenaline. (Fig 4-3) The recovery hearts rates for the groups were: 50.2 $\pm$ 18.1, 31.0 $\pm$ 14.7, 71.0 $\pm$ 28.6 and 146.6 $\pm$ 48.9 BPM respectively. The stabilization values for all groups were significant over their respective recovery groups. Hearts treated with adrenaline 10<sup>-10</sup> M had a better recovery than hearts treated with 10<sup>-6</sup> M with  $p < 0.05$ .

### Effect of adrenaline on recovery left ventricular developed pressure

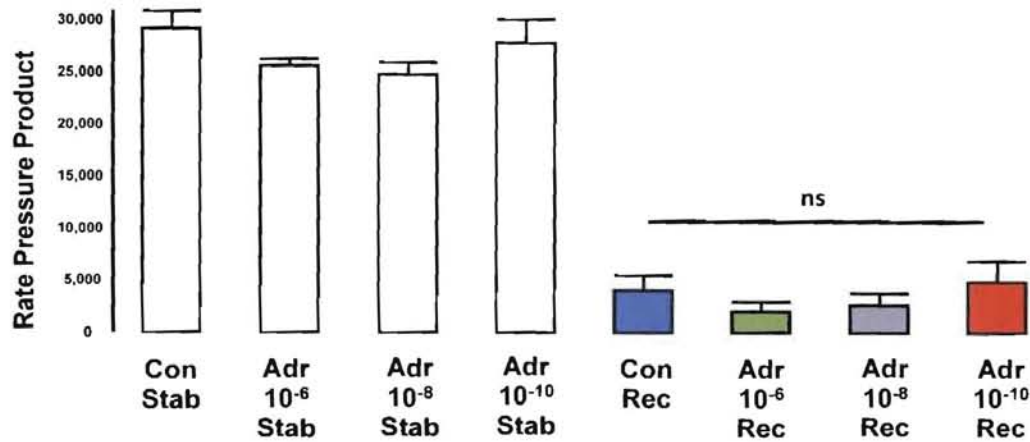


**Figure 4-4:** Effect of adrenaline on left ventricular developed pressure (LVDP)- Stabilization phase; Con- Control; Adr- Adrenaline; Rec- Recovery phase. (ns- not significant vs. adrenaline treated groups in rec; Tukey-Kramer Multiple Comparisons Test)

Stabilization LVDP for control, hearts treated with  $10^{-6}$  mol/L adrenaline,  $10^{-8}$  mol/L adrenaline and  $10^{-10}$  mol/L adrenaline were:  $86.8 \pm 1.5$ ,  $85.3 \pm 1.5$ ,  $86.2 \pm 1.9$  and  $88.5 \pm 2.5$  mmHg respectively. (Fig 4-4) The recovery LVDP for these groups was  $68.0 \pm 24.1$ ,  $34.6 \pm 15.8$ ,  $26.1 \pm 8.6$  and  $21.7 \pm 8.3$  mmHg respectively. There was no statistical significance between groups in the recovery phase.

### Effect of adrenaline on recovery rate-pressure product

The stabilization values were significantly higher than their respective recovery values but there was no significant difference between the various groups in the recovery phase. (Fig 4-5) Controls had a mean RPP of  $29196 \pm 1654$ , hearts treated with  $10^{-6}$ ,  $10^{-8}$ ,  $10^{-10}$  M adrenaline had RPP values of  $25677 \pm 607$ ,  $24896 \pm 1105$  and  $27956 \pm 2239$  in the stabilization phase respectively. At the end of recovery phase, the RPP of these four groups was:  $4087 \pm 1475$ ,  $2082 \pm 954$ ,  $2760 \pm 1108$  and  $4947 \pm 2061$  for controls and hearts treated with  $10^{-6}$ ,  $10^{-8}$ ,  $10^{-10}$  M adrenaline respectively.



**Figure 4-5:** Effect of adrenaline on rate pressure product (RPP). Stab- Stabilization phase; Con- Control; Adr- Adrenaline; Rec- Recovery phase; ns- not significant vs. adrenaline treated groups in rec; Tukey-Kramer Multiple Comparisons Test

**Table 4-3:** Effect of adrenaline administration on systolic and diastolic pressure and coronary flow

Systolic pressure (mmHg)				
	Control	Adrenaline 10 <sup>-6</sup> M	Adrenaline 10 <sup>-8</sup> M	Adrenaline 10 <sup>-10</sup> M
Stabilization	87.2±2.6	87.2±2.1	95.6±2.1	94.9±2.1
Recovery	113.7±19.3	68.7±15.2	71.1±16.2	22.5±7.8***
Diastolic Pressure (mmHg)				
	Control	Adrenaline 10 <sup>-6</sup> M	Adrenaline 10 <sup>-8</sup> M	Adrenaline 10 <sup>-10</sup> M
Stabilization	0.9±1.4	1.8±2.3	9.4±1.4	6.3±1.1
Recovery	45.6±11.4###	34.0±12.0#	45.0±8.7**/#	0.8±3.6
Coronary flow (ml)				
	Control	Adrenaline 10 <sup>-6</sup> M	Adrenaline 10 <sup>-8</sup> M	Adrenaline 10 <sup>-10</sup> M
Stabilization	8.5±0.3	9.4±0.3	9.3±0.2	8.9±0.4
Recovery	10.5±0.3	10.8±0.4	10.5±0.3	10.1±0.4

**Table 4-3:** \*\*\*-  $p < 0.001$  vs. Adrenaline 10-10M Stabilization; ###-  $p < 0.001$  vs. Stabilization; #-  $p < 0.05$  vs. Stabilization

In recovery phase the diastolic pressure with adrenaline  $10^{-10}$ M was significantly lower ( $p<0.01$ ) than adrenaline concentration  $10^{-8}$  M. Recovery phases of adrenaline controls,  $10^{-6}$  M ( $p<0.001$ ) and  $10^{-8}$  M ( $p<0.05$ ) were significantly higher than their values in the stabilization phase. There was no significant difference between coronary flows amongst the groups. (Table 4-3)

### **Summary**

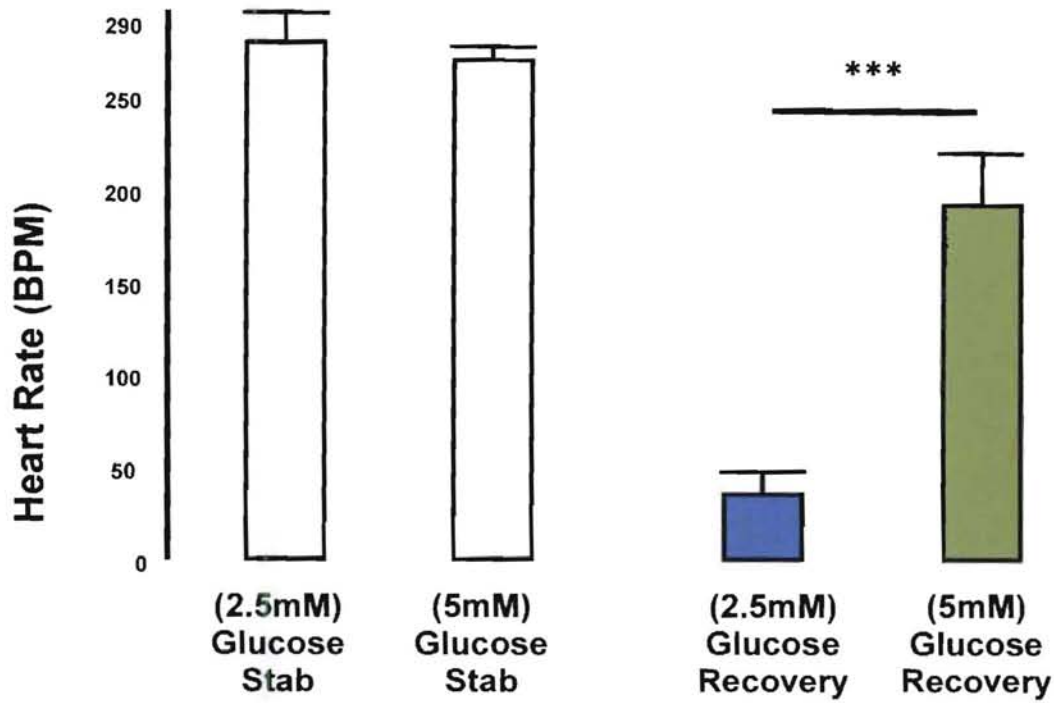
Provision of adrenaline reduced the recovery of the hearts when administered in the AHF phase. However, adrenaline at  $10^{-10}$ M concentration had a very high recovery in contractility while adrenaline at a concentration of  $10^{-6}$ M had a very poor contractility. Despite the statistical insignificant changes in rate pressure product, adrenaline at  $10^{-8}$ M concentration seemed to reduce both heart rate and LV developed pressure. Hence, adrenaline at  $10^{-8}$ M concentration will be considered for further experiments.

## **RESULTS**

### **Alteration of glucose in perfusate**

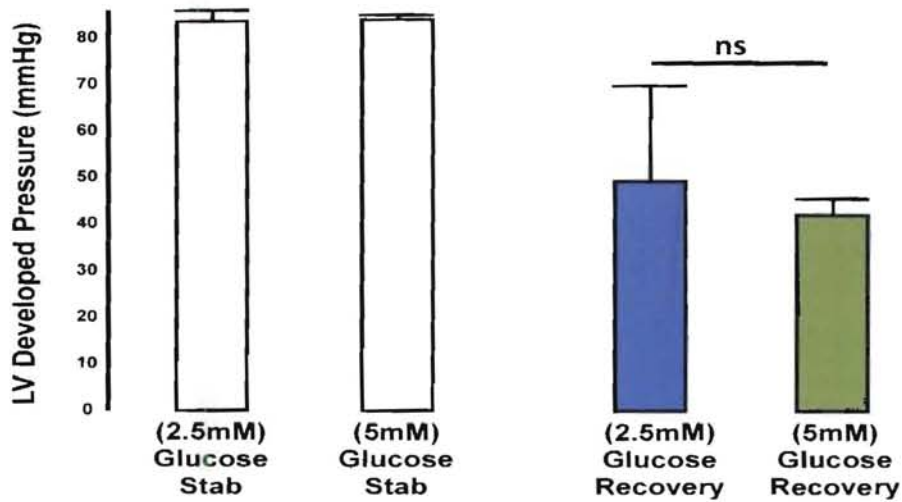
#### ***Effect of alteration of glucose concentration on heart rate***

The stabilization heart rates for hearts perfused with 5 and 2.5mmol/L glucose respectively were (Fig 4-6):  $270.4\pm 7.8$  and  $279.6\pm 16.3$  BPM. The hearts perfused with 5mmol/L glucose in the AHF and recovery phase had a significantly improved heart rate at the end of recovery phase ( $193.7\pm 28.0$  vs.  $35.7\pm 12.0$  BPM).



**Figure 4-6:** Effect of glucose alteration on heart rate (HR); \*\*\*-  $p < 0.001$  vs. 2.5mM glucose recovery; Tukey-Kramer Multiple Comparisons Test

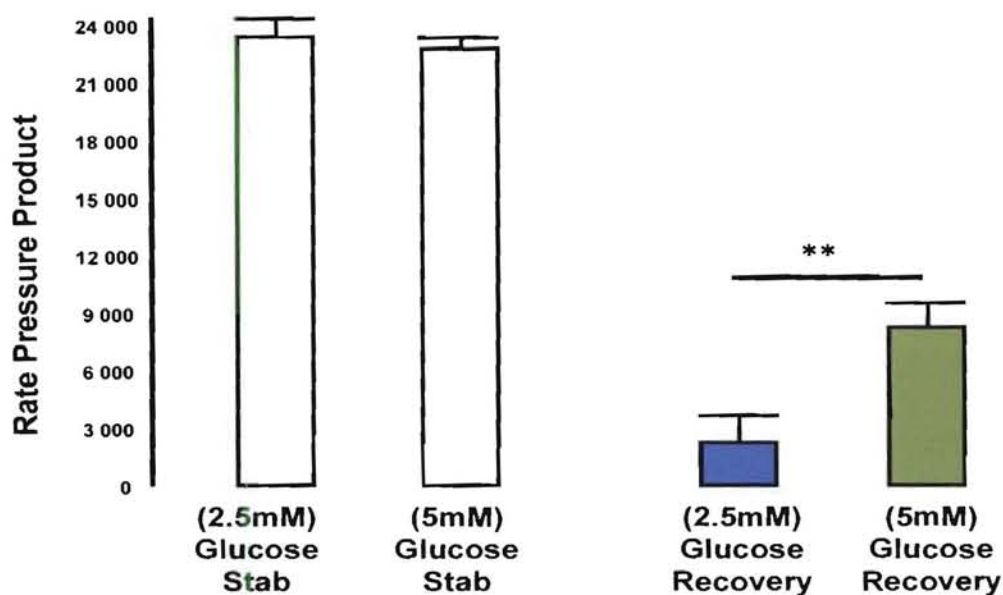
**Effect of glucose alteration on left ventricular developed pressure**



**Figure 4-7:** Effect of glucose alteration on LVDP; ns- not significant vs. 2.5mM glucose recovery; Tukey-Kramer Multiple Comparisons Test

The stabilization values of LV developed pressure for hearts perfused with 5mmol/L and 2.5mmol/L glucose were  $84.3\pm 0.6$  and  $83.8\pm 2.2$  mmHg respectively. (Fig 4-7) After an episode of AHF and subsequent recovery, the hearts perfused with 5mmol/L glucose had an LV developed pressure of  $42.1\pm 3.4$ mmHg and those perfused with 2.5mmol/L glucose had LVDP of  $49.4\pm 20.5$ mmHg. This change was not significant between groups in the recovery phase.

**Effect of glucose alteration on rate-pressure product**



**Figure 4-8:** Effect of glucose alteration on RPP; \*\*  $p < 0.01$  vs. 2.5mM glucose recovery; Tukey-Kramer Multiple Comparisons Test

Rate-pressure products for the stabilization period of the hearts perfused with 5mmol/L glucose and 2.5mmol/L glucose were  $22781\pm 515$  and  $23368\pm 1004$ . (Fig 4-8) At the end of the recovery phase, hearts perfused with 5mmol/L glucose had significantly improved rate pressure product values as compared to the hearts perfused with 2.5mmol/L glucose ( $8236\pm 1290$  vs.  $2254\pm 1300$ ). This increase was due to the improved heart rate between groups.

**Table 4-4:** Effect of glucose alteration on systolic, diastolic pressure and coronary flow:

<b>Systolic Pressure (mmHg)</b>		
	<b>2.5mmol/L Glucose</b>	<b>5mmol/L Glucose</b>
<b>Stabilization</b>	89.5±1.5	87.5±2.0 <sup>###</sup>
<b>Recovery</b>	68.8±12.3	41.5±3.4*
<b>Diastolic Pressure (mmHg)</b>		
	<b>2.5mmol/L Glucose</b>	<b>5mmol/L Glucose</b>
<b>Stabilization</b>	5.6±2.1	3.1±1.5
<b>Recovery</b>	19.6±16.6	0.0±0.6
<b>Coronary flow (ml)</b>		
	<b>2.5mmol/L Glucose</b>	<b>5mmol/L Glucose</b>
<b>Stabilization</b>	8.8±0.4	9.0±0.3
<b>Recovery</b>	10.3±0.5	11.3±0.2

**Table 4-4:** ###-  $p < 0.001$  vs. Stabilization; \*-  $p < 0.05$  vs. Recovery 2.5mmol/L glucose

### **Summary**

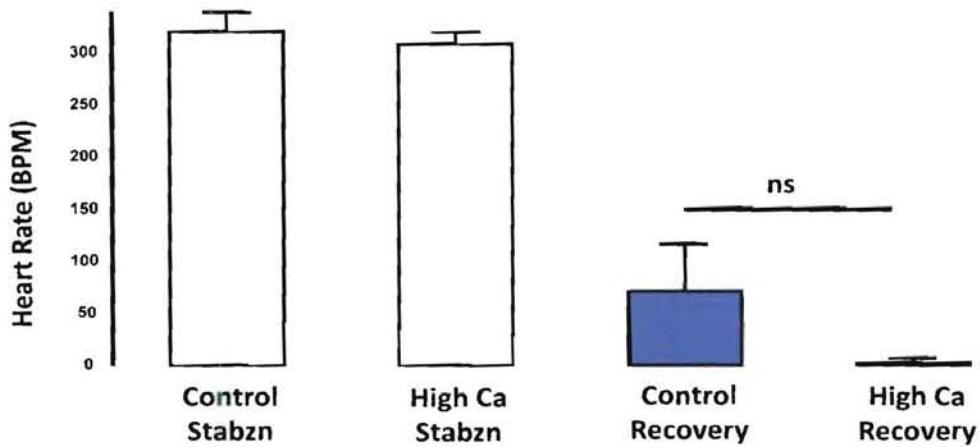
The reduction of glucose in the perfusate from hyperglycaemic 11.1mmol/L to physiologic 5mmol/L level had better recovery as compared to hearts perfused with 2.5mmol/L glucose. The reduced glucose availability in the 2.5mmol/L glucose perfused hearts in combination with the elevated fatty acids in the perfusate worsened the heart failure, thereby providing a low recovery value to act as a baseline concentration in the remaining experiments described in this thesis. (Table 4-4)

## **RESULTS**

### **Calcium modulation as a model of acute heart failure**

Calcium plays an important role in the contraction-relaxation of the cardiomyocytes. A overload of calcium may lead to arrhythmias, often seen in CHF.<sup>112</sup> Our hypothesis tests the acute calcium alteration in the perfusate to induce AHF. After 30' stabilization period, we studied the effects of normal (1.3mmol/L) calcium and high (2.5mmol/L) calcium perfusate concentrations on the heart rate.

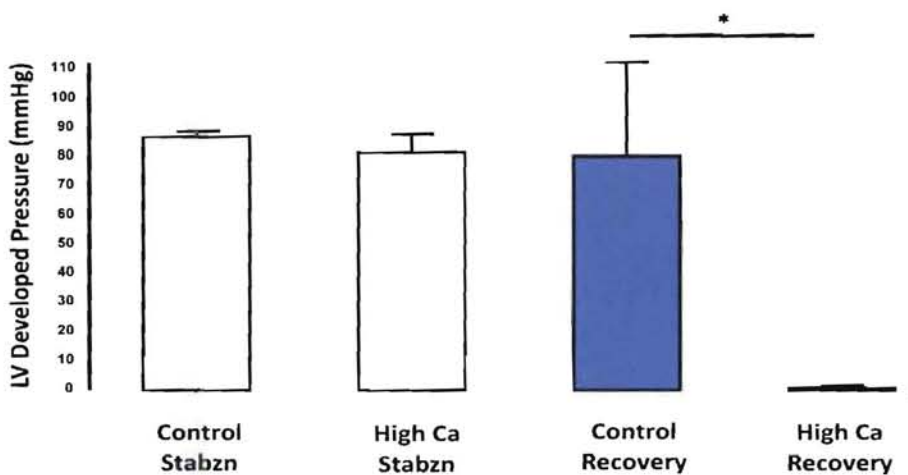
### Effect of calcium modulation on recovery heart rate



**Figure 4-9:** Effect of high calcium on heart rate; ns- Not significant vs. High Ca recovery; Tukey-Kramer Multiple Comparisons Test

After 30' stabilization period, heart rate normal (1.3mmol/L) calcium and high (2.5mmol/L) calcium were 309.6±10.7 vs. 321.6±18.0 BPM respectively. (Fig 4-9) After 35' of AHF phase and 30' of recovery phase, heart rates of normal calcium group were 71.8±45.0 BPM while the recovery heart rates in the high calcium group were 3.3±3.3 BPM. ( $p > 0.05$ ; not significant vs. high calcium)

### Effect of calcium modulation on recovery left ventricular developed pressure

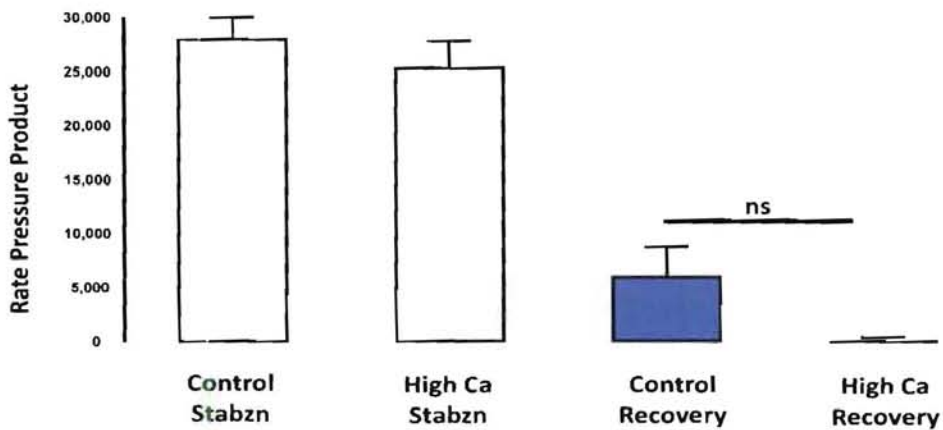


**Figure 4-10:** Effect of high calcium on LVDP; \*  $p < 0.05$ - vs. High Ca recovery; Tukey-Kramer Multiple Comparisons Test

At the end of stabilization, the LVDP in the normal calcium concentration group was  $87.0 \pm 1.3$  mmHg while that in the high calcium group was  $81.5 \pm 6.4$  mmHg. (Fig 4-10)

In the recovery phase, hearts with normal calcium (1.36mmol/L) exhibited LVDP of  $80.4 \pm 31.7$  while those with high calcium (2.5mmol/L) had a LVDP of  $0.9 \pm 0.4$  mmHg respectively ( $p < 0.05$ ) for recovery with normal calcium vs. recovery with high calcium)

**Effect of calcium modulation on recovery rate-pressure product**



**Figure 4-11:** Effect of high calcium on RPP; ns- Not significant vs. High Ca recovery; Tukey-Kramer Multiple Comparisons Test

Rate pressure product in the hearts perfused with 2.5mmol/L was very poor ( $2 \pm 2$ ) owing to the poor recovery heart rate and LV developed pressure when compared against 1.3mmol/L  $Ca^{2+}$  in perfusate ( $5915 \pm 2690$ ). These values were however, statistically insignificant. (Fig 4-11)

**Table 4-5:** Effect of calcium modulation on systolic, diastolic pressure and coronary flow

Systolic Pressure (mmHg)		
	Control	High calcium
Stabilization	$86.7 \pm 2.0$	$84.2 \pm 6.3$
Recovery	$129.6 \pm 10.0^{**}$	$54.6 \pm 21.5$
Diastolic Pressure (mmHg)		

	Control	High calcium
<b>Stabilization</b>	0.4±1.4	2.7±3.0
<b>Recovery</b>	49.2±22.3	53.7±21.3
<b>Coronary flow (ml)</b>		
	Control	High calcium
<b>Stabilization</b>	9.5±0.6	8.7±0.4
<b>Recovery</b>	11.5±0.6	10.5±0.2

**Table 4-5:** \*\*-  $p < 0.01$  vs. Recovery control

The increase in systolic pressure in controls was statistically significant vs. hearts perfused with high calcium ( $p < 0.01$ ). There was no significant change in the coronary flow between controls and high calcium treated hearts. (Table 4-5)

### **Summary**

The high concentration of calcium in the perfusate was detrimental for the hearts which showed almost no recovery. Hence, this model to induce acute heart failure was too severe and was discarded. We decided to maintain calcium concentration in our perfusate at 1.3mmol/L in all phases in all experiments described in the following sections.

## **RESULTS**

### **Pacing as a model to induce acute heart failure**

We next examined the effects of pacing on heart rate.

#### ***Effect of pacing on recovery heart rate***

The stabilization HR for controls, hearts paced at 150 BPM and 300 BPM were 351.1±18.1, 290.8±9.1 and 288.6±15.9 respectively. In the recovery phase, the heart rates for the same groups were: 54.4±28.3, 92.0±64.4 and 0.0±0.0 BPM respectively. The hearts paced at 300 BPM showed no recovery.

***Effect of pacing on recovery left ventricular developed pressure***

Stabilization values for LVDP in controls, in hearts paced at 150 and in hearts paced at 300 BPM were  $86.7 \pm 2.3$ ,  $84.7 \pm 1.6$  and  $85.6 \pm 2.3$  mmHg respectively. The recovery LVDP for the same groups was  $37.7 \pm 21.7$ ,  $19.1 \pm 14.8$  and  $1.1 \pm 0.5$  mmHg respectively. There was no significant difference between the groups in the recovery phase.

***Effect of pacing on recovery rate pressure product***

The rate-pressure product values were:  $30506 \pm 1978$  for controls,  $24612 \pm 621$  for hearts paced at 150 BPM and  $24717 \pm 1576$  for hearts paced at 300 BPM in the stabilization phase of the protocol.

The recovery values for control and hearts paced at 150 BPM were:  $3478 \pm 2248$ ,  $2136 \pm 1547$ ; hearts paced at 300 BPM had no recovery.

**Table 4-6:** Effect of pacing on systolic and diastolic pressures and coronary flow

<b>Systolic Pressure (mmHg)</b>			
	<b>Control</b>	<b>Pacing 150 BPM</b>	<b>Pacing 300 BPM</b>
<b>Stabilization</b>	88.0±4.1	87.0±3.8	90.5±5.2
<b>Recovery</b>	97.4±25.7	54.2±11.2	63.8±16.7
<b>Diastolic Pressure (mmHg)</b>			
	<b>Control</b>	<b>Pacing 150 BPM</b>	<b>Pacing 300 BPM</b>
<b>Stabilization</b>	1.3±1.8	2.3±2.7	4.8±4.0
<b>Recovery</b>	59.6±9.2	35.1±11.5	62.6±16.4
<b>Coronary flow (ml)</b>			
	<b>Control</b>	<b>Pacing 150 BPM</b>	<b>Pacing 300 BPM</b>
<b>Stabilization</b>	9.1±0.6	8.6±0.4	9.0±0.4
<b>Recovery</b>	10.3±0.2	9.7±0.1	10.3±0.3

### **Summary**

Pacing hearts in the AHF phase was a good model to induce *de novo* AHF, however pacing at 300 BPM was too harsh and the hearts showed no recovery. Pacing at 150 BPM seemed to induce failure and deserves merit but electrical stimulation of the heart was not nearly as good as the adrenergic stimulation leading to metabolic side effects. Hence, we used different concentrations of adrenaline to induce AHF.

### **DISCUSSION**

To our knowledge, our experiments demonstrate the first attempt to establish a model of *de novo* acute heart failure using isolated rat hearts. We tested three conditions to induce AHF: addition of adrenaline, pacing and increasing the calcium concentration in the acute heart failure phase and in the case of high calcium-induced AHF, we continued high calcium in the recovery phase.

Addition of adrenaline in our model mimics the hyperadrenergic stimulus associated with Takotsubo cardiomyopathy<sup>46</sup> and also an acute-on-chronic heart failure situation where the circulating catecholamines are elevated.<sup>27</sup> Our experiments with different

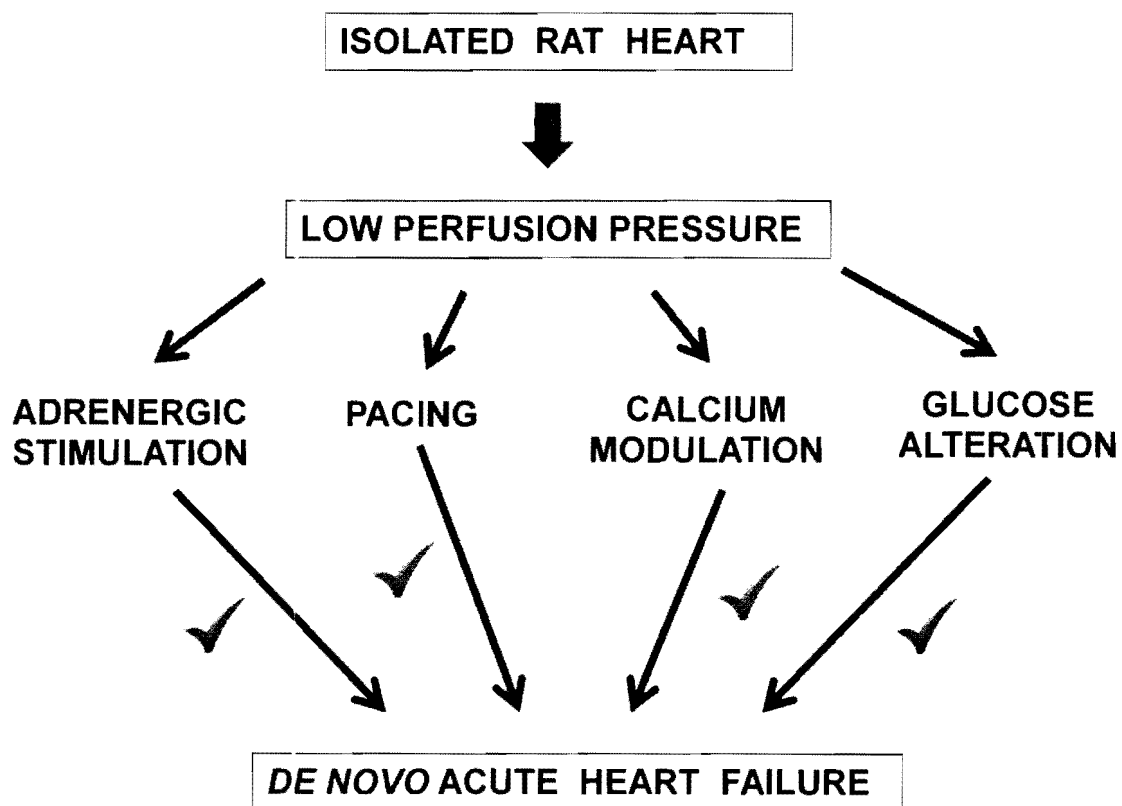
concentrations of adrenaline revealed that the concentration of adrenaline at  $10^{-10}$ M was too weak to induce heart failure due to the recovery of heart rate subjected to this concentration of adrenaline being the highest observed compared to the other groups. On the other hand, a concentration of  $10^{-6}$ M had the worst recovery of heart rate when compared to all the other groups. Adrenaline at  $10^{-8}$ M had a recovery similar to the control hearts and was considered a threshold recovery. Previous experiments in our lab in 1983 tested different concentrations of adrenaline on the release of lactate dehydrogenase (LDH) and found that at a concentration of  $10^{-8}$ M, there was oxygen wastage and decreased pressure work in the hearts.<sup>70</sup> The absence of adrenaline in control hearts was similar to those treated with  $10^{-8}$ M in our experiments, but in order to simulate the adrenergic stress situation, with expected metabolic changes, and not only focusing on the haemodynamic parameters; we chose to include adrenaline in our model of acute heart failure.

Pacing-induced heart failure has been used extensively to induce chronic heart failure in dogs by various authors.<sup>111,114-117</sup> These authors examined the effect of long-term pacing as a model of chronic heart failure in canines mostly focusing on end stage heart failure and examining mitochondrial function and metabolic changes in these hearts. To our knowledge, there is a lack of a model of AHF which uses pacing to induce acute failure in rodents. We compared non-paced hearts to hearts paced at 150 and 300 BPM respectively in the AHF phase of our protocol. Hearts paced at 300 BPM in the AHF phase had no recovery, proving that this pacing rate was too severe and was hence discontinued. Pacing at 150 BPM is a good model to induce AHF and is worthy of future work, being completely reversible thus permitting the researcher to monitor metabolic changes after recovery.<sup>118</sup> However, clinical or large animal studies using pacing as an acute stress to induce AHF are missing. We are interested in a metabolic model of acute heart failure with an adrenergic stimulus causing metabolic changes, and hence we decided to use adrenaline instead of pacing.

High calcium in the perfusate as a model to induce acute heart failure was tested in the AHF phase of our protocol. Heart failure is associated with mitochondrial changes.<sup>50</sup> Calcium plays an imperative role in the contraction of the heart. Abnormalities in the calcium concentration in the mitochondria may trigger lethal cell pathways like apoptosis.<sup>119</sup> We examined the effect of increased calcium in the

perfusate to induce AHF and observed the recovery in our model. When the concentration of calcium in the perfusate was raised from 1.3mmol/L to 2.5mmol/L, there was absolutely no recovery in both heart rate and contractility hence, it was concluded that the model was clearly too severe. The lack of recovery prompted us to discontinue this model for our further experiments; however, modulation of calcium in an acutely failing heart is an important model to study cell death associated with heart failure and needs further work to fully understand its merits.

We conclude from these experiments that addition of adrenaline, pacing, change of calcium concentration and reduction of glucose in perfusate all induce AHF most likely due to stimulation of different pathways. However, for this thesis, we have focused on adrenaline ( $10^{-8}$ mol/L) and reduction of glucose from physiological 5mmol/L to 2.5mmol/L in perfusate to induce AHF.



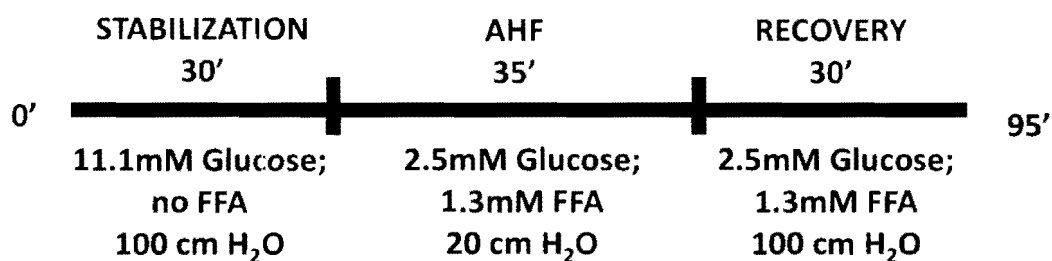
*Figure 4-12: Validation of models to induce AHF*

### **Models of acute heart failure**

Based on our different models of *de novo* AHF, we focus on two models of AHF, one without and one with adrenaline:

The model without adrenaline consisted of three phases: Stabilization phase, AHF phase and Recovery phase

- The stabilization phase is characterised by the perfusion pressure being kept at 100 cm H<sub>2</sub>O with glucose (11.1mmol/L) as the sole substrate in the perfusate
- The AHF phase is characterised by lowering the perfusion pressure from 100 cm H<sub>2</sub>O to 20 cm H<sub>2</sub>O. The glucose concentration in the perfusate is dropped to 2.5mmol/L and fatty acids (1.3mmol/L) are introduced into the perfusate
- In the recovery phase, the perfusion pressure is returned to 100 cm H<sub>2</sub>O while keeping the substrate concentration in the perfusate at the same level as in the AHF phase.



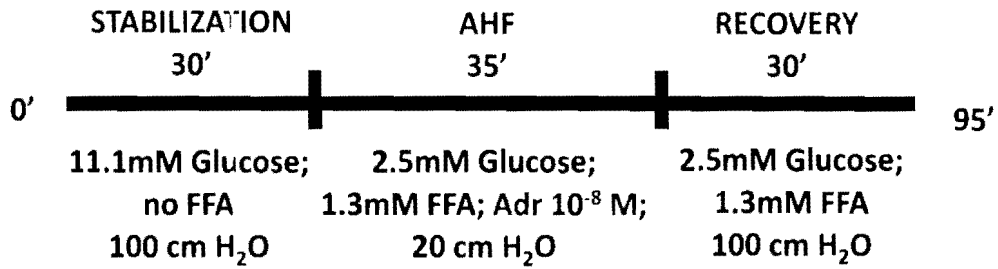
**Figure 4-13:** Protocol for model of AHF without adrenaline (model 1)

The severe model of AHF consists of three phases: stabilization phase, AHF phase and recovery phase.

- The stabilization phase is characterised by a perfusion pressure of 100 cm H<sub>2</sub>O with glucose (11.1mmol/L) as the sole substrate in the perfusate
- In the AHF phase, the perfusion pressure is dropped from 100 cm H<sub>2</sub>O to 20 cm H<sub>2</sub>O and the glucose concentration is reduced to 2.5mmol/L while fatty acids are introduced at 1.3mmol/L in the perfusate.

Adrenaline  $10^{-8}$ M is added in this phase to promote the uptake of fatty acids

- Recovery phase involves the perfusion pressure returning back to 100cm H<sub>2</sub>O while the substrates in the perfusate are maintained at the same level as in AHF phase.



*Figure 4-14: Protocol for model of AHF with adrenaline (model 2)*

## *Chapter 5*

# **TESTING METABOLIC AGENTS** **FOR RECOVERY FOLLOWING** **DE NOVO ACUTE HEART** **FAILURE**

### **INTRODUCTION**

A failing heart is metabolically associated with reduced production of ATP to sustain its function.<sup>120</sup> Carbohydrates and fatty acids are the essential fuels for the heart in the fed and fasted state respectively. After a carbohydrate rich meal, the amounts of glucose are elevated and insulin secretion is high, which reduces the releases of free fatty acids from the adipose tissue, thus promoting carbohydrates as major source (almost 75%) of energy. In the fasted state however, blood free fatty acid levels are raised and are oxidized preferentially over glucose. Fatty acids take up to 70% oxygen to generate energy (See table 5-1).<sup>29</sup>In an ischaemic heart, the enzyme pyruvate dehydrogenase is inhibited, thus reducing the amount of glucose uptake and fatty acids become the main source of energy. Malonyl CoA is inhibited by adenosine monophosphate- activated protein kinase (AMPK) and protective glycolysis is inhibited by inhibiting malonyl CoA. This leads to fatty acid oxidation which is “expensive” and deleterious to the heart.<sup>121</sup> Glucose via glycolysis generates ATP and is economical as compared to fatty acids.

**Table 5-1: Fuels of the heart: ratio of oxygen uptake<sup>29</sup>**

Conditions	Glucose (OER %)	Pyruvate (OER %)	Lactate (OER %)	Total CHO (OER %)	FFA + (TG) (OER %)
Insulin, low glucose <sup>a</sup>	6	44	51	101	3
Feeding	–	–	–	92	5
CHO meal <sup>b</sup>	68	4	28	100	–
Lipid meal	10	–	10	20	30 + (50)
Fasting, few hours <sup>c</sup>	31	2	28	61	34
Exercise	16	0	61	77	21
Exercise, recovery	21	2	36	59	36
Fasting overnight	27	1	11	38	62 + (14)

In 1962, Sodi-Pallares<sup>122</sup> infused glucose-insulin-potassium in patients with myocardial infarction with the hypothesis that it would lessen arrhythmias by improving the polarization in injured cells by potassium. There have been conflicting data in the literature about this concept. With the aim of increasing uptake of glucose and reducing that of FFA, provision of glucose-insulin-potassium (GIK) treatment has been shown to be cardioprotective against acute myocardial infarction in both experimental and clinical studies.<sup>123,124</sup> However, GIK has not been tested in acute heart failure. We tested the provision of glucose as a therapy in our model of *de novo* acute heart failure. We also tested the administration of insulin in the recovery phase to increase the uptake of glucose. We also test our hypothesis that metabolic therapy by glucose and insulin improves functional recovery after an episode of AHF and reduces cell death caused by apoptosis.

These experiments were done in two models of *de novo* AHF. The AHF phase in the mild model was characterized by reduced perfusion pressure, reduction of glucose concentration and introduction of supra-physiological levels of free fatty acids. The severe model had hyperadrenergic stimulus in form of adrenaline in the acute heart failure phase along with reduced perfusion pressure and change in substrate concentration.

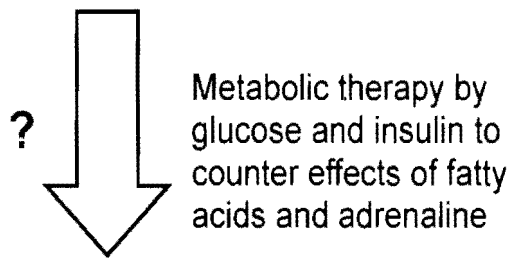
These experiments had three phases: stabilization, production of AHF and recovery. The buffer for stabilization was as previously described in chapter 2. The glucose

concentration in the AHF phase was 2.5mmol/L and the FFA concentration was 1.3mmol/L with the perfusion pressure at 20cm H<sub>2</sub>O. In the recovery phase, half the hearts were perfused with 11.1mmol/L glucose while the other half was perfused with 2.5mmol/L. The FFA concentration was 1.3mmol/L in all hearts. This phase had a perfusion pressure of 100cms H<sub>2</sub>O.

## HYPOTHESIS

We hypothesized that metabolic treatment by glucose and insulin would improve the functional recovery of the hearts after an episode of *de novo* acute heart failure.

### ACUTE HEART FAILURE



### IMPROVED FUNCTIONAL RECOVERY

**Figure 5-1:** Diagram hypothesizing improved recovery after treatment with glucose and insulin

## MATERIALS AND METHODS

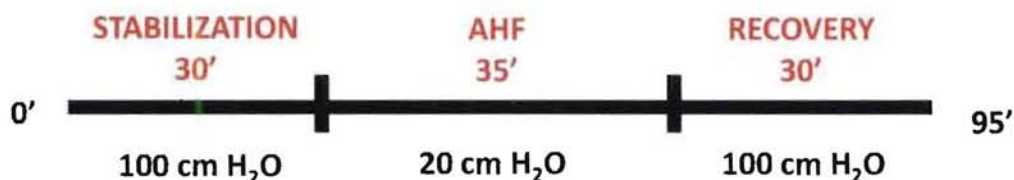
### Animals

As previously described in chapter 4.

### Isolated rat heart perfusions

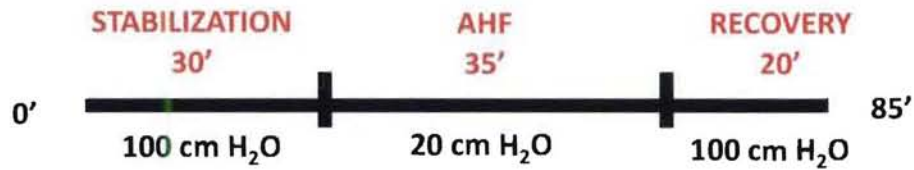
The heart perfusions were done as described in the methods sections in chapter 2 of this thesis.

*Protocols for isolated heart perfusions for functional recovery: and Western blotting in this section*



	Stabilization	AHF	Recovery
Model 1	11.1mmol/L Glucose; no FFA	2.5mmol/L Glucose; 1.3mmol/L FFA	2.5mmol/L Glucose; 1.3mmol/L FFA
Model 1	11.1mmol/L Glucose; no FFA	2.5mmol/L Glucose; 1.3mmol/L FFA	11.1mmol/L Glucose; 1.3mmol/L FFA
Model 2	11.1mmol/L Glucose; no FFA	2.5mmol/L Glucose; 1.3mmol/L FFA; 10 <sup>-8</sup> M Adrenaline	2.5mmol/L Glucose; 1.3mmol/L FFA
Model 2	11.1mmol/L Glucose; no FFA	2.5mmol/L Glucose; 1.3mmol/L FFA; 10 <sup>-8</sup> M Adrenaline	11.1mmol/L Glucose; 1.3mmol/L FFA
Model 2	11.1mmol/L Glucose; no FFA	2.5mmol/L Glucose; 1.3mmol/L FFA; 10 <sup>-8</sup> M Adrenaline	11.1mmol/L Glucose; 1.3mmol/L FFA; 0.3mU Insulin
Model 2	11.1mmol/L Glucose; no FFA	2.5mmol/L Glucose; 1.3mmol/L FFA; 10 <sup>-8</sup> M Adrenaline	11.1mmol/L Glucose; 1.3mmol/L FFA
Model 2	11.1mmol/L Glucose; no FFA	11.1mmol/L Glucose; 1.3mmol/L FFA; 10 <sup>-8</sup> M Adrenaline	11.1mmol/L Glucose; 1.3mmol/L FFA

**Protocol for isolated rat heart perfusion to evaluate the effect of metabolic therapy on cell death**



	Stabilization	AHF	Recovery
Model 2	11.1mmol/L Glucose; no FFA	11.1mmol/L Glucose; no FFA	11.1mmol/L Glucose; no FFA
Model 2	11.1mmol/L Glucose; no FFA	2.5mmol/L Glucose; 1.3mmol/L FFA; 10 <sup>-8</sup> M Adrenaline	2.5mmol/L Glucose; 1.3mmol/L FFA
Model 2	11.1mmol/L Glucose; no FFA	2.5mmol/L Glucose; 1.3mmol/L FFA; 10 <sup>-8</sup> M Adrenaline	2.5mmol/L Glucose; 1.3mmol/L FFA; 0.3mU Insulin
Model 2	11.1mmol/L Glucose; no FFA	2.5mmol/L Glucose; 1.3mmol/L FFA; 10 <sup>-8</sup> M Adrenaline	11.1mmol/L Glucose; 1.3mmol/L FFA
Model 2	11.1mmol/L Glucose; no FFA	2.5mmol/L Glucose; 1.3mmol/L FFA; 10 <sup>-8</sup> M Adrenaline	11.1mmol/L Glucose; 1.3mmol/L FFA; 0.3mU Insulin

**Western blots to examine the effect of metabolic therapy on cell death**

Lowry's method of protein quantification was used to assess the quantity of cytochromeC in the samples and the Western blots were run as described in the methods section in chapter 2.

**End points**

As previously described in chapter 4.

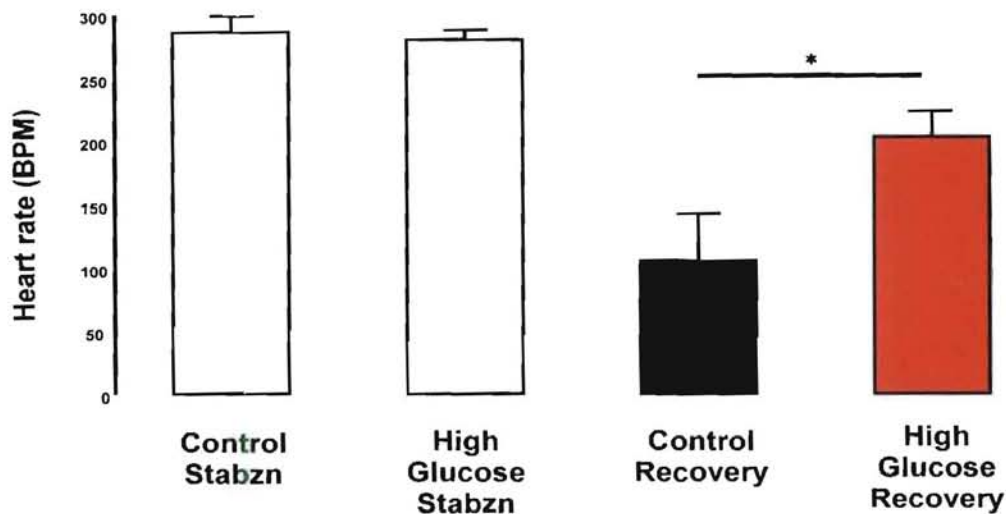
**Statistical analysis**

As previously described in chapter 4.

## RESULTS

### ***Effect of high glucose on heart rate when administered in recovery phase of model 1***

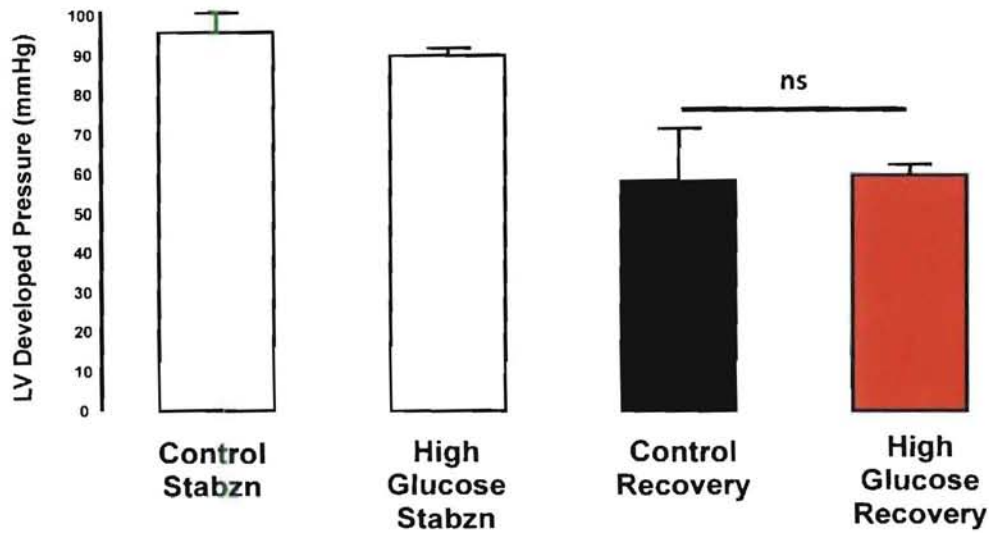
The mean heart rates in stabilization for controls and hearts treated with high glucose were:  $286.8 \pm 12.6$  BPM and  $281.0 \pm 7.3$  BPM respectively. (Fig 5-2) Recovery for controls was reduced to  $106.5 \pm 37.3$  BPM. Hearts treated with high glucose had a recovery HR of  $204.0 \pm 21.2$  BPM. Treatment with high glucose in recovery phase of the mild model improved the HR significantly ( $p < 0.05$ )



**Figure 5-2:** Effect of glucose on heart rate in recovery phase of model 1; \* -  $p < 0.05$  vs. control recovery; Tukey-Kramer Multiple Comparisons Test

### ***Effect of high glucose on left ventricular developed pressure (LVDP)***

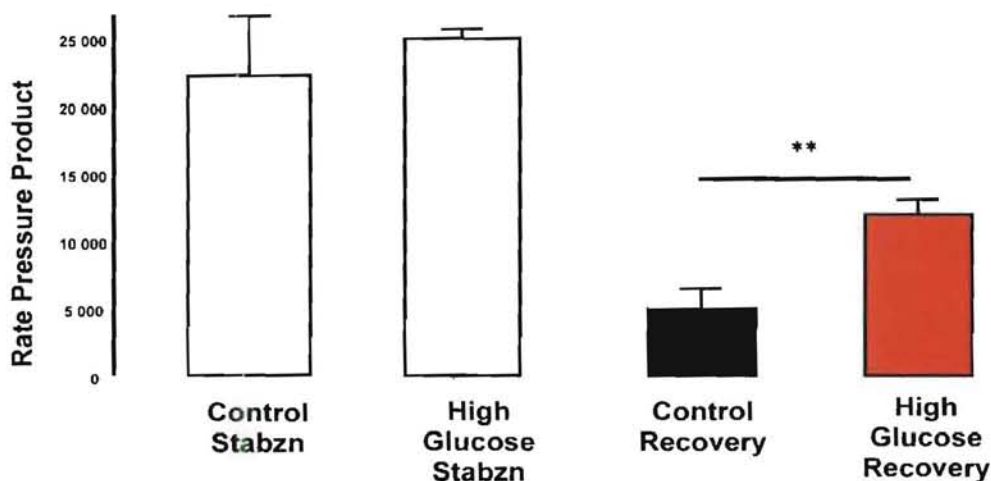
Stabilization values for controls and hearts treated with high glucose were  $96.0 \pm 5.0$  and  $90.0 \pm 2.1$  mmHg. Controls had a recovery LVDP of  $58.4 \pm 13.3$  mmHg and treatment with high glucose had LVDP of  $59.8 \pm 2.5$  mmHg. Treatment with high glucose in recovery phase did not affect the LVDP in these hearts. (Fig 5-3)



**Figure 5-3:** Effect of glucose on LVDP in model 1; ns- not significant; Tukey-Kramer Multiple Comparisons Test

**Effect of glucose on rate pressure product**

The mean stabilization RPP for control hearts was  $22259 \pm 4466$  and that for hearts treated with high glucose was  $25018 \pm 717$ ; After 35' of AHF and 30' of recovery phase, hearts treated with low glucose showed a recovery RPP of  $5018 \pm 1439$  while those treated with high glucose had a RPP of  $12024 \pm 1134$ . (Fig 5-4) Treatment with glucose in the recovery phase of the mild model significantly increased the RPP ( $p < 0.01$  vs. control recovery).



**Figure 5-4:** Effect of glucose on RPP in model 1; ###-  $p < 0.001$  vs. control recovery; ##-  $p < 0.01$  vs. high glucose recovery; \*\*-  $p < 0.01$  vs. control recovery; Tukey-Kramer Multiple Comparisons Test

**Table 5-2:** Effect of glucose on systolic pressure, diastolic pressure and coronary flow in model 1

<b>Systolic pressure (mmHg)</b>		
	<b>Control</b>	<b>High glucose</b>
<b>Stabilization</b>	97.1±4.7	93.8±1.2
<b>Recovery</b>	100.7±11.7	74.8±6.3
<b>Diastolic Pressure (mmHg)</b>		
	<b>Control</b>	<b>High glucose</b>
<b>Stabilization</b>	0.0±1.4	6.1±1.3
<b>Recovery</b>	42.2±7.2	14.9±6.5**
<b>Coronary flow (ml)</b>		
	<b>Control</b>	<b>High glucose</b>
<b>Stabilization</b>	9.0±0.3	9.6±0.5
<b>Recovery</b>	10.1±0.4	10.8±0.5

**Table 5-2:** \*\*-  $p < 0.01$  vs. Recovery control

The diastolic pressures in stabilization phase for both groups were  $2.4 \pm 1.3$  mmHg. However, following AHF and recovery phase, hearts treated with low glucose had a recovery diastolic pressure of  $42.2 \pm 7.2$  mmHg. (Table 5-2) Treatment with high glucose in the recovery phase significantly decreased this to  $14.9 \pm 6.5$  mmHg with the  $p < 0.01$  vs. recovery with low glucose.

### **Summary**

In our model 1 of *de novo* AHF where AHF was induced by reducing the perfusion pressure and altering substrate concentration in the perfusate, treatment with high (11.1 mmol/L) glucose improved the functional recovery by increasing the heart rate and also improved the relaxation of the ventricles by reducing the LVEDP.

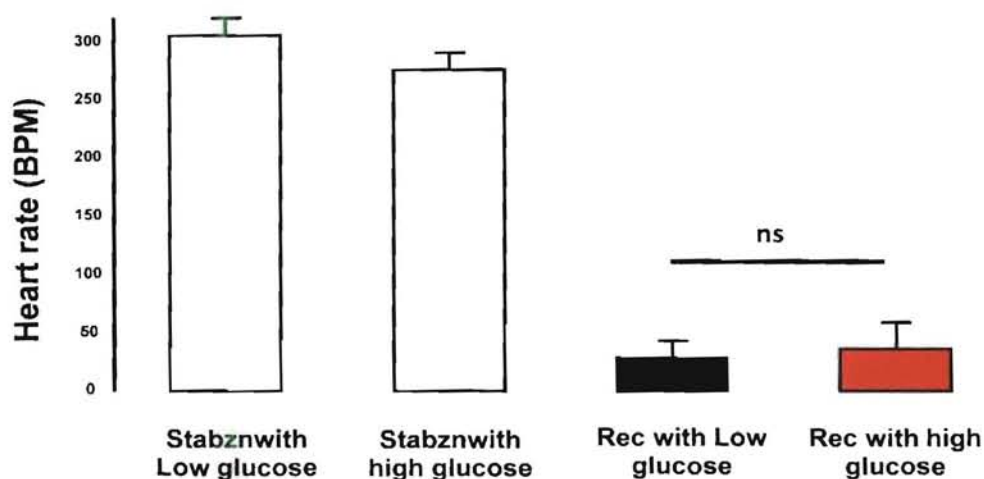
The following experiments shall be performed using model 2 which not only has reduction of perfusion pressure and substrate alteration in the perfusate, but a hyperadrenergic stimulus also.

## RESULTS

### *Effect of glucose on recovery in model 2 of AHF*

Glucose was cardioprotective when tested in our model 1. Hence, we decided to test glucose in model 2 of AHF. We tested glucose (11.1mmol/L) again in the recovery phase of the protocol; however, we added adrenaline ( $10^{-8}M$ ) to the existing hypotension and low (2.5mM) glucose and FFA (1.3mM) in the AHF phase.

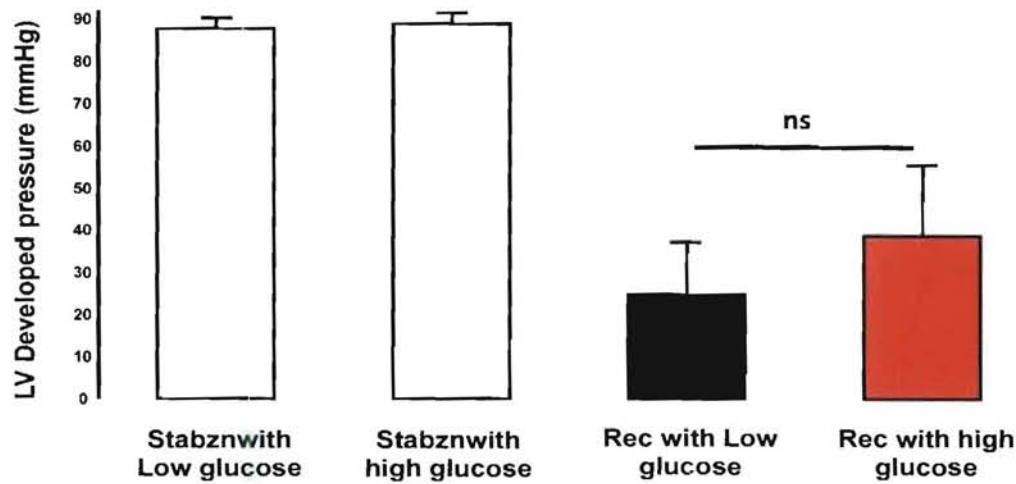
### *Effect of high glucose on heart rate in model 2*



**Figure 5-5:** Effect of glucose on HR in model 2; ns- not significant vs. Rec with low glucose; Tukey-Kramer Multiple Comparisons Test

Stabilization heart rates for low glucose group were  $306.5 \pm 15.0$  BPM and those for high glucose hearts were  $277.6 \pm 14.8$  BPM. (Fig 5-5) Recovery with low glucose in the severe model had a HR of  $28.5 \pm 15.0$  BPM while the recovery with high glucose was  $36.7 \pm 25.0$  BPM. The recovery between low glucose and high glucose in the recovery phase is statistically insignificant.

### **Effect of high glucose on left ventricular developed pressure**

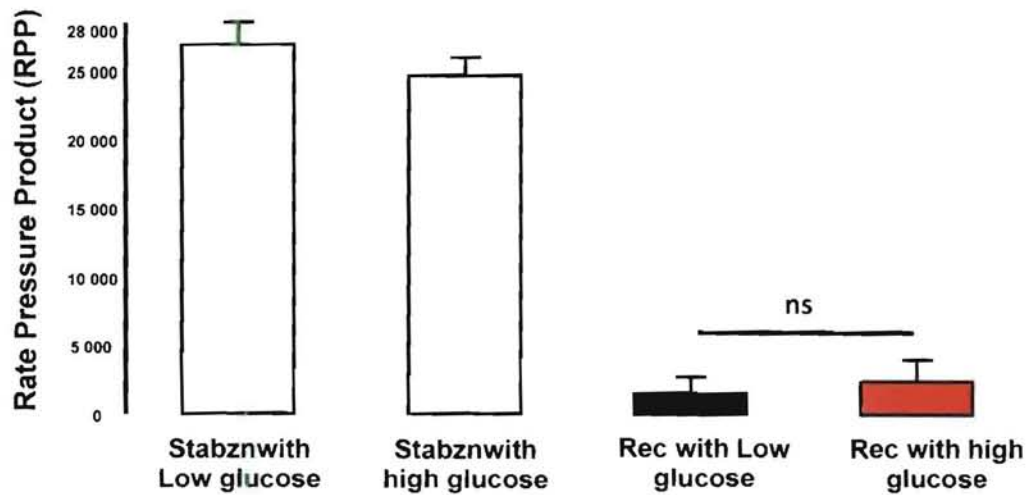


**Figure 5-6:** Effect of glucose on LVDP in model 2; ns- not significant vs. Rec with low glucose; Tukey-Kramer Multiple Comparisons Test

The stabilization value of LVDP for hearts with low and high glucose was:  $87.8 \pm 2.6$  and  $89.0 \pm 2.8$  mmHg respectively. (Fig 5-6) After treatment with low glucose in recovery phase, the LVDP was  $24.9 \pm 12.7$  mmHg while the LVDP after treatment with high glucose was  $39.0 \pm 16.9$  mmHg. These results between the groups in the recovery phase were statistically insignificant.

### **Effect of high glucose on rate pressure product**

The mean RPP in stabilization for low glucose hearts was  $26924 \pm 1493$  and that for hearts treated with high glucose was  $24681 \pm 1330$ . (Fig 5-7) The RPP after treatment with low glucose was  $1559 \pm 1234$  while that after treatment with high glucose was  $2493 \pm 1550$ . These data in the recovery phase were statistically insignificant.



**Figure 5-7:** Effect of glucose on RPP in model 2; ns- not significant vs. Rec with low glucose; Tukey-Kramer Multiple Comparisons Test

**Table 5-3:** Effect of glucose on systolic, diastolic pressures and coronary flow in model 2

Systolic Pressure (mmHg)		
	Low glucose	High glucose
<b>Stabilization</b>	89.6±1.8	87.3±3.0
<b>Recovery</b>	57.1±18.6	69.2±17.3
Diastolic Pressure (mmHg)		
	Low glucose	High glucose
<b>Stabilization</b>	1.7±2.6	0.0±1.7
<b>Recovery</b>	32.3±7.9**	30.2±7.5**
Coronary flow (ml)		
	Low glucose	High glucose
<b>Stabilization</b>	9.4±0.3	9.3±0.4
<b>Recovery</b>	10.7±0.4	11.1±0.4*

**Table 5-3:** \*\*-  $p < 0.01$  vs. Respective stabilization values; \*-  $p < 0.05$  vs. Respective stabilization value

The effect on diastolic pressure with both high and low glucose treatment in the recovery phase increased diastolic pressure as significantly ( $p<0.01$ ) as compared to its respective stabilization phase. The treatment had no significant change on systolic pressures. The coronary flow in the high glucose recovery phase was significantly ( $p<0.05$ ) increased over the stabilization phase. (Table 5-3)

### **Summary**

The treatment with high glucose in model 2 had no effect on recovery. The proposed reason for this is the presence of added adrenaline in AHF phase which may have promoted the uptake of FFA as substrates despite the availability of glucose.

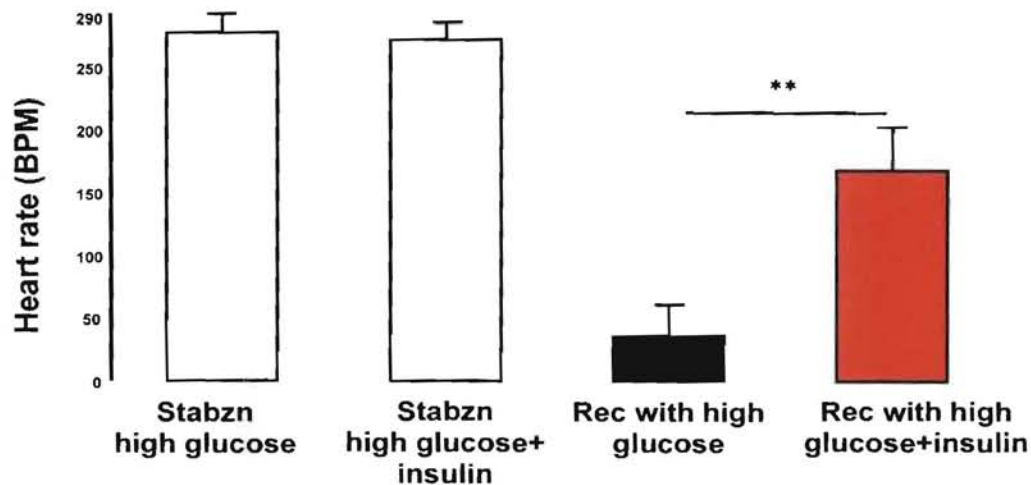
## **RESULTS**

### ***Effect of glucose and insulin in recovery phase of model 2***

Insulin and glucose have been shown to be cardioprotective in the ischemic-reperfusion injury due to the activity of insulin that promotes the uptake of glucose in the myocardium.<sup>125</sup> Hence we decided to test insulin and glucose in our model with adrenaline in AHF phase (model 2). We hypothesize that treatment with insulin and high (11.1mmol/L) glucose improves the functional recovery vs. high glucose in the recovery phase.

### ***Effect of high glucose + insulin treatment on heart rate***

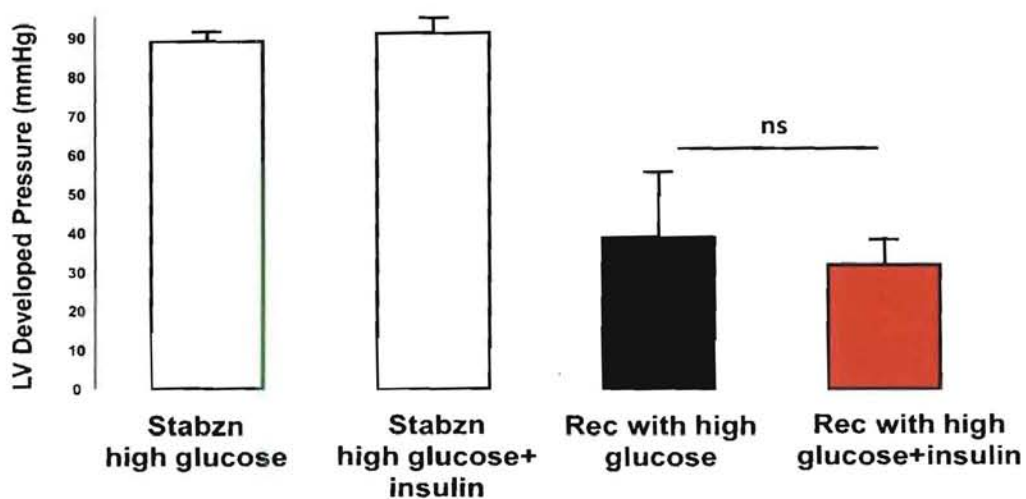
The HR in stabilization phase of hearts treated with high glucose and hearts treated with high glucose plus insulin were (Fig 5-8):  $277.6\pm14.8$  and  $272.0\pm14.5$  BPM respectively. In recovery phase, hearts with just high glucose had a recovery HR of  $36.7\pm25.0$  BPM while addition of insulin to the heart improved the HR to  $168.5\pm34.5$  BPM. This improvement in HR was statistically significant with  $p<0.01$  vs. recovery with high glucose.



**Figure 5-8:** Effect of glucose + insulin on HR in model 2; \*\*  $p < 0.01$  vs. Rec with high glucose; Tukey-Kramer multiple comparison test

**Effect of high glucose + insulin treatment on left ventricular developed pressure**

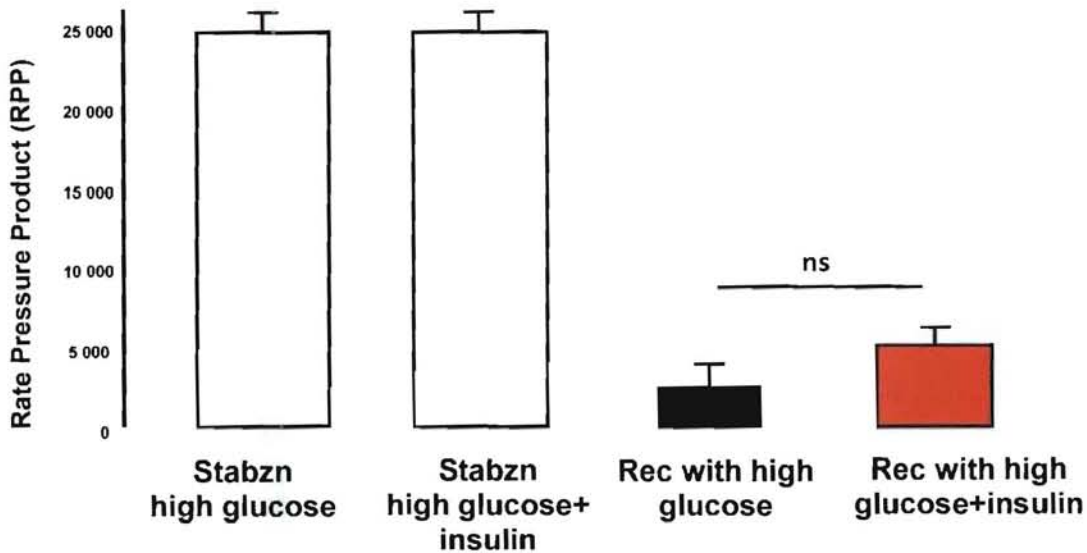
Hearts treated with high glucose had stabilization phase LVDP of  $89.0 \pm 2.8$  while the hearts treated with high glucose+ insulin had stabilization LVDP of  $91.2 \pm 4.1$  mmHg. (Fig 5-9) After subjecting hearts to model 2, the hearts treated with just high glucose had a LVDP of  $39.0 \pm 17.0$  mmHg while those treated with insulin + high glucose had a LVDP of  $31.9 \pm 7.2$  mmHg. These values in the recovery phase between groups were statistically insignificant.



**Figure 5-9:** Effect of glucose + insulin on LVDP in model 2; ns- not significant vs. Rec with high glucose; Tukey-Kramer multiple comparison test

**Effect of high glucose + insulin treatment on rate pressure product:**

High glucose hearts in the stabilization phase had a RPP of  $24681 \pm 1330$  and those treated with high glucose+ insulin had stabilization RPP of  $24765 \pm 1330$ . (Fig 5-10) Treatment with high glucose in recovery phase had a RPP of  $2493 \pm 1550$  while insulin combined with high glucose had a recovery RPP of  $5176 \pm 1058$ . Despite the slight improvement, these values are statistically insignificant.



**Figure 5-10:** Effect of glucose + insulin on RPP in model 2; ns- not significant vs. Rec with high glucose; Tukey-Kramer multiple comparison test

**Table 5-4:** Effect of glucose+ insulin on systolic, diastolic pressures and coronary flow in model 2

Systolic Pressure (mmHg)		
	High glucose	High glucose + insulin
<b>Stabilization</b>	$87.3 \pm 3.0$	$95.17 \pm 3.2$
<b>Recovery</b>	$69.2 \pm 17.3$	$33.4 \pm 7.5^*$
Diastolic Pressure (mmHg)		
	High glucose	High glucose + insulin
<b>Stabilization</b>	$0.0 \pm 1.7$	$3.8 \pm 1.1$
<b>Recovery</b>	$30.7 \pm 7.5$	$1.5 \pm 3.3^{**}$

Coronary flow (ml)		
	High glucose	High glucose + insulin
<b>Stabilization</b>	9.3±0.4	9.5±0.2
<b>Recovery</b>	11.1±0.4	10.0±0

**Table 5-4:** \*-  $p < 0.05$  vs. High glucose recovery; \*\*-  $P < 0.01$  vs. High glucose recovery

Addition of insulin decreased systolic pressure in the recovery phase vs. stabilization ( $p < 0.05$ ). It also reduced the diastolic pressure significantly when compared to HG recovery ( $p < 0.01$ ). The coronary flow was had no changes between groups. (Tab 5-4)

### **Summary**

The addition of insulin does improve the functional recovery of isolated hearts subjected to severe model of *de novo* AHF. This improvement however, is attributed to the increase in heart rate only but not contractility. Addition of insulin also significantly improved relaxation of the ventricles by reducing diastolic pressure.

## **RESULTS**

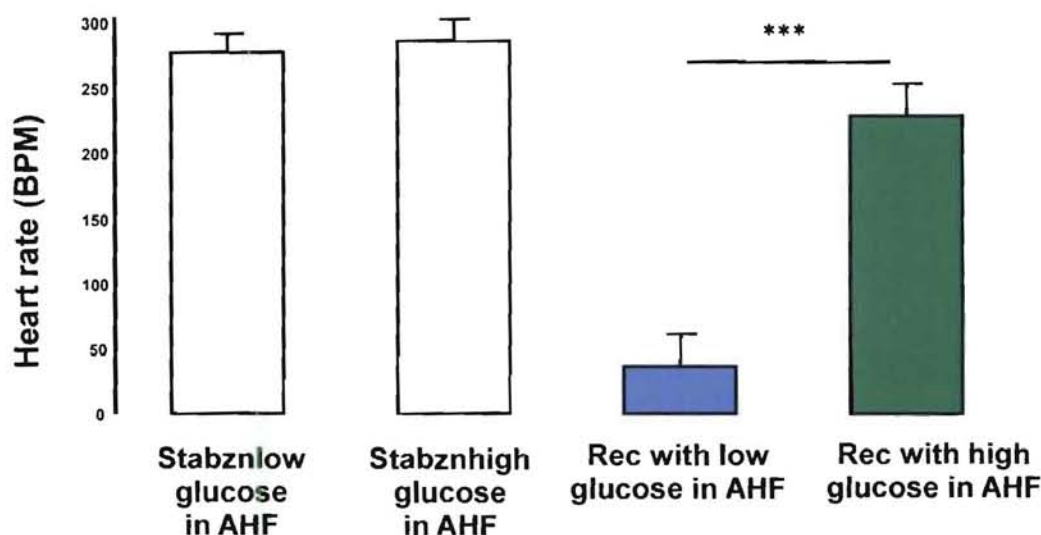
### ***Effect of glucose in AHF phase in model 2***

To test if the provision of glucose in the AHF phase is cardioprotective, we compared the effects of high (11.1mmol/L) or low (2.5mmol/L) glucose in the AHF phase followed by high glucose in recovery phase for both groups. These experiments were done in the model 2.

### ***Effect of glucose in AHF phase in model 2 on heart rate***

In the stabilization phase, hearts perfused with low glucose had a HR of  $277.6 \pm 14.8$  BPM and the hearts perfused with high glucose in AHF had a stabilization HR of

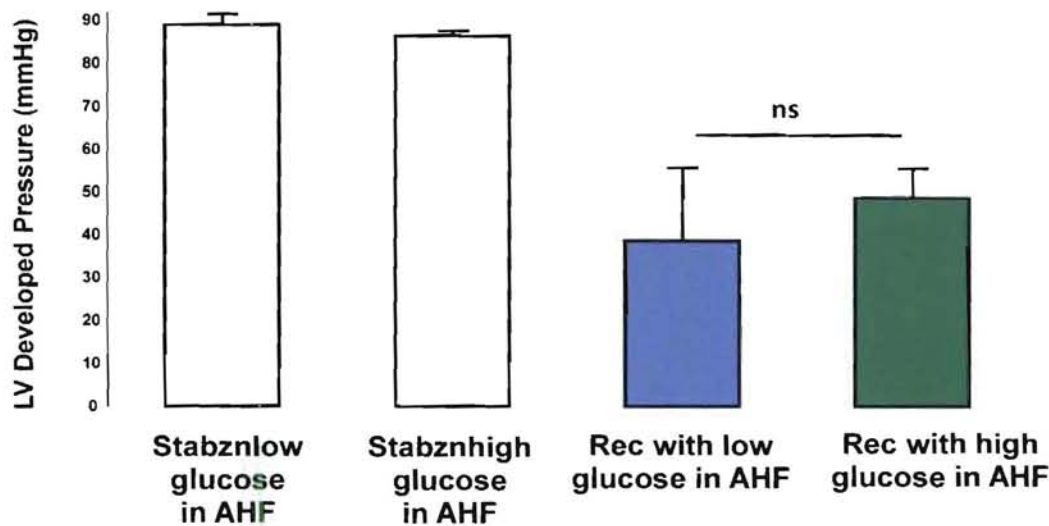
287.1±17.2 BPM. (Fig 5-11) At the end of recovery phase, hearts that had been treated with low glucose had a HR of 36.7±25.0 BPM while those that were treated with high glucose in the AHF phase had a recovery HR of 229.5±25.6 BPM. This increase was very significant with the p value being less than 0.001 vs. hearts treated with low glucose.



**Figure 5-11:** Effect of glucose in AHF phase on heart rate in model 2; \*\*\*-  $p < 0.001$  vs. Rec with low glucose in AHF; Tukey-Kramer multiple comparison test

**Effect of glucose in AHF phase in model 2 on left ventricular developed pressure**

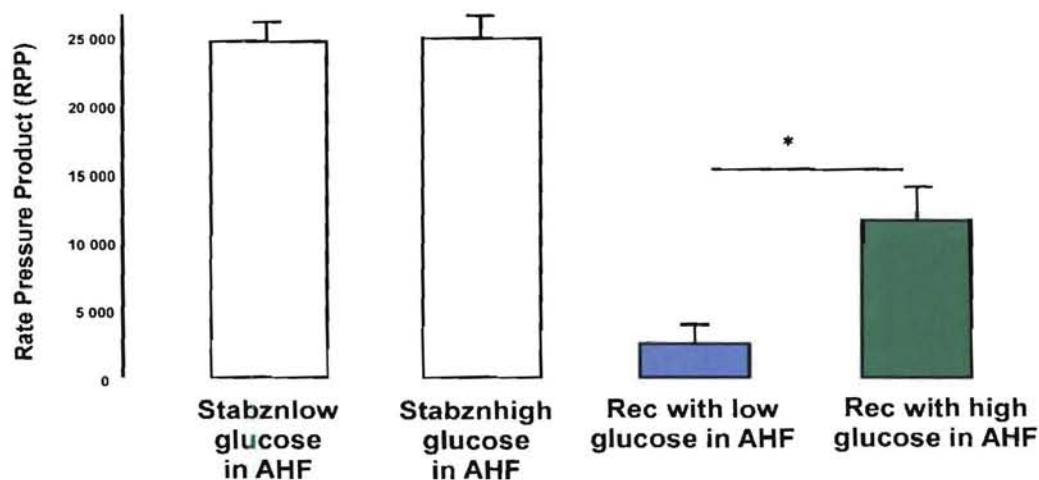
Stabilization LVDP for hearts treated with low glucose was 89.0±2.8 mmHg and that for the high glucose in AHF hearts was 86.7±1.0mmHg. (Fig 5-12) In recovery phase, hearts that were treated with low glucose in AHF had a LVDP of 38.9±16.9 mmHg while those treated with high glucose in AHF phase had a recovery LVDP of 49.2±7.0 mmHg. Addition of glucose in AHF had no significant effect on LVDP of the hearts.



**Figure 5-12:** Effect of glucose in AHF phase on LVDP in model 2; ns- not significant vs. rec with low glucose in AHF; Tukey-Kramer multiple comparison test

**Effect of glucose in AHF phase in model 2 on rate pressure product**

Stabilization values of RPP for hearts treated with low glucose in AHF and hearts treated with high glucose in AHF were:  $26481 \pm 1330$  and  $24949 \pm 1627$  respectively. (Fig 5-13) The hearts treated with low glucose in AHF phase showed a recovery RPP of  $2493 \pm 1550$ . The hearts treated with high glucose in AHF phase however, had RPP of  $11677 \pm 2447$ . This increase was almost 5 times the hearts with low glucose in AHF. The data between groups in recovery phase were statistically significant with the  $p < 0.01$  vs. recovery with low glucose in AHF.



**Figure 5-13:** Effect of glucose in AHF phase on RPP in model 2; \* -  $p < 0.05$  vs. Rec with low glucose in AHF; Tukey-Kramer multiple comparison test

**Table 5-5:** Effect of high glucose in AHF phase on systolic, diastolic pressure and coronary flow in model 2

Systolic Pressure (mmHg)		
	Low glucose in AHF	High glucose in AHF
<b>Stabilization</b>	87.3±3.0	93.2±1.5
<b>Recovery</b>	69.2±17.3	67.4±14.7
Diastolic Pressure (mmHg)		
	Low glucose in AHF	High glucose in AHF
<b>Stabilization</b>	0.0±1.7	6.4±1.1
<b>Recovery</b>	30.2±7.5	6.1±1.2***
Coronary flow (ml)		
	Low glucose in AHF	High glucose in AHF
<b>Stabilization</b>	9.3±0.4	10.1±0.4
<b>Recovery</b>	11.1±0.4	12.7±0.3

**Table 5-5:** \*\*\*-  $p < 0.001$  vs. Recovery with low glucose in AHF

Treatment with high glucose in the AHF phase in model 2 significantly ( $p < 0.001$ ) reduced diastolic pressure when compared against low glucose in AHF, thus improving relaxation. There was no significant change in the systolic pressures between groups. The coronary flow was increased in the recovery phases versus the respective stabilization phases ( $p < 0.05$  vs. Low glucose in AHF;  $p < 0.001$  vs. High glucose in AHF) (Table 5-5)

### **Summary**

These experiments show that glucose is protective when administered in the AHF phase as it improves the functional recovery by improving heart rate and reducing diastolic pressure. There was no effect on the LVDP.

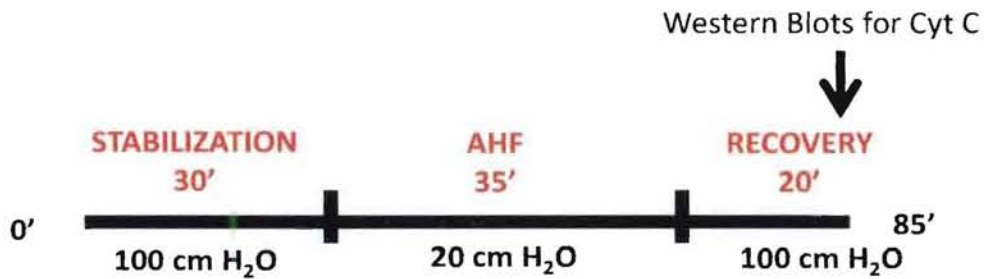
## **RESULTS**

### ***Effect of metabolic therapy on cell death***

To test whether metabolic therapy against de novo AHF would reduce the cell death; we performed Western blots using model 2 of AHF for cytochrome C (Cyt C) which is a marker for apoptosis. Five groups were studied: hearts with no AHF, hearts treated with low glucose (LG) in recovery phase, hearts treated with low glucose+ insulin (LG+Ins) in recovery phase, hearts treated with high glucose (HG) in recovery phase and hearts treated with high glucose+ insulin (HG+ Ins) in recovery. Lowry assay<sup>126</sup> was used to quantify the proteins. After running the gels and transfer, the membranes was probed using a CytC antibody and bands were recorded under the GeneGnome and then analyzed using ImageJ software.

## Protocol

The hearts were perfused as follows



The insulin, high glucose, high glucose+ insulin were perfused in the recovery phase. Lowry protein quantification was performed to analyze the amount of protein, as described in the methods chapter.

## RESULTS

### Effects of metabolic therapy on cell death

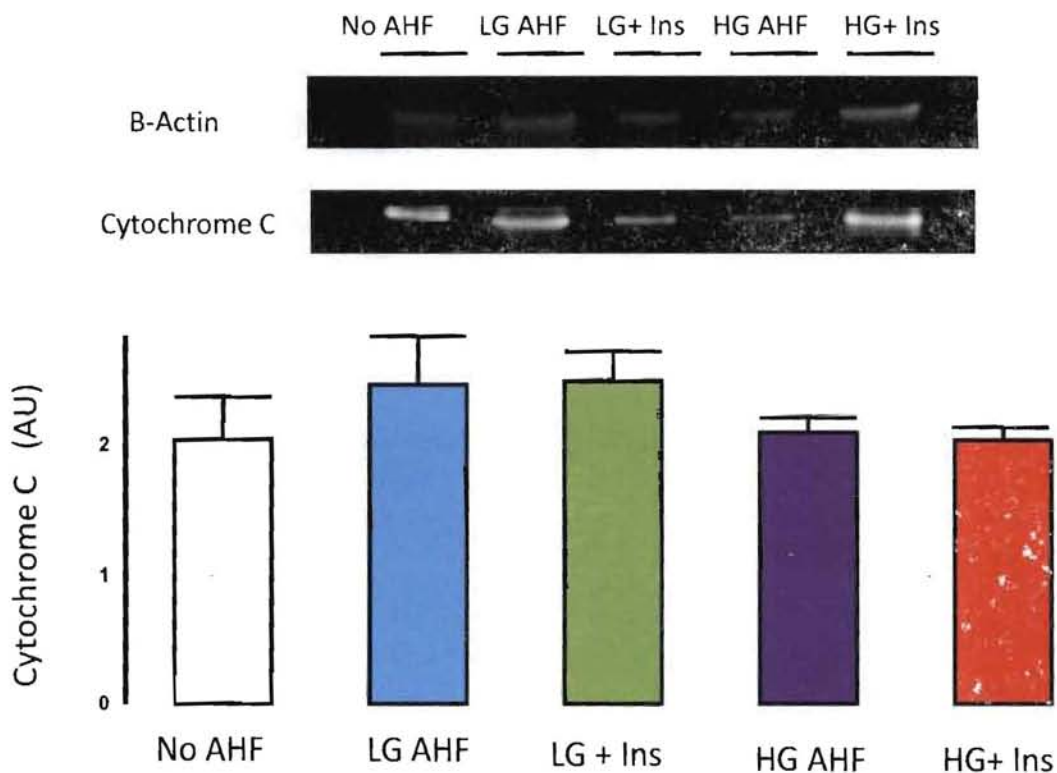


Figure 5-14: Western blots and effect of metabolic treatment on cytochrome C

## Summary

Metabolic treatment with glucose and insulin combined or individually had no effect on the expression of cyt C when hearts were exposed to AHF.

## **DISCUSSION**

In our model of *de novo* acute heart failure, provision of glucose improved functional recovery when administered in the recovery phase of model 1 in absence of hyperadrenergic stimulus. We propose that this improvement is due to the increase of glucose oxidation after acute failure. In a chronically failing heart, glucose oxidation is reduced due to elevated fatty acid metabolism<sup>27</sup>. From our experiments, we propose that infusion of glucose-insulin should be tested as a therapy in acute heart failure patients. Glucose reduces the elevated diastolic pressure, thereby improving relaxation of the ventricles. Addition of adrenaline in the AHF phase reduced the effect of glucose, suggesting that adrenaline promotes fatty acid uptake by reducing glucose oxidation, but this proposal merits further work. One way to confirm this hypothesis would be to measure the glucose and fatty acid oxidation in isolated hearts during the AHF phase.

Our preliminary experiments shed light on the effect of adrenaline on recovery from AHF in our model. Addition of insulin to glucose when AHF was induced by addition of adrenaline improved functional recovery by increasing heart rate and reducing diastolic pressure. This effect on increased heart rate suggests stimulation of the sinus node due to glucose and insulin. Previously, Senges *et al*<sup>127</sup> studied the effect of glucose-insulin-potassium (GIK) infusion in patients with sinus node dysfunction and found that GIK improved sinus node function. This improvement was, however, attributed to glucose and insulin and not potassium. Glucose was found to regulate the slow inward current which caused the acceleration of the sinus rate in patients with sinus node dysfunction, improvement of the SA nodal function and reduction of post-stimulatory sinus node recovery time. Our experiments are the first to provide insight into the effect of glucose and insulin on the sinus node of an acutely failing heart. The future work for experiments in the metabolic group could involve the examination of sinus node electrophysiology in an acutely failing heart and treating with glucose and insulin and to assess recovery.

Insulin as a pharmacological agent activates the survivor activating factor enhancement (SAFE) pathway<sup>48</sup> which reduces infarct size after ischaemic-reperfusion injury. Future work in our experiments could be to examine the mechanisms involved in the protective effect of glucose and insulin. Heart failure has metabolic implications, hence alterations in the metabolism would be considered as a viable treatment. Studies to regulate metabolism in chronic heart failure (CHF) patients have shown promising results,<sup>116,128,129</sup> but there are no published data on metabolism and acute heart failure. Our promising data indicate that reduction in fatty acid metabolism and elevation of protective glycolysis could be exploited to develop therapies against acute heart failure patients.

Administration of glucose in the AHF phase of our experiments improved the functional recovery despite the presence of adrenaline. These data indicate that glucose in the acute heart failure phase is cardioprotective. This is relevant to the situation of patients coming into the Emergency Room with clinical AHF in a hyperadrenergic state and receiving a glucose-insulin drip. Glucose administered in the AHF phase should also have an effect on the sinus node and hence improve heart rate if there is sinus bradycardia.

The provision of glucose and insulin and the subsequent improvement in the heart rate in failing hearts corresponds to improved sinus node activity. Our data suggests this improvement is caused by increasing glycolysis. This could be clarified by the measurement of glycolysis in the acutely failing hearts and merits future work.

### **Metabolic agents and cell death**

We hypothesized that the functional recovery improvement in hearts treated with glucose and insulin would reduce cell death by reducing the expression of Cyt C which is released by the mitochondria in response to apoptosis. Our data showed no significant reduction in levels of Cyt C when treated with glucose, insulin or a combination of the two. Cyt C is involved in the early stages of the initiation of apoptosis in myocytes. Cyt C, once released by the mitochondria, forms a part of an assembly called as apoptosome which has other components including apoptotic protease activating factor-1 (APAF-1), procaspase 9 and dATP.<sup>130</sup> The assembly activates the caspases which then cause cell death. However, apoptosis is not the sole mechanism of death of the cells in the myocardium. Other mechanisms of cell

death including necrosis and autophagy need further research in our model of acute heart failure. Also, instead of cyt C, observing the activation of other pro-apoptotic molecules like caspases will help us understand the mechanism of cell death in acute heart failure.

## **CONCLUSION**

In absence of an adrenergic stimulus, provision of glucose (11.1mmol/L) in the perfusate improved functional recovery by increasing the heart rate.

However, this cardioprotective outcome of glucose was abrogated in the presence of adrenaline in the AHF phase. Addition of insulin combined with glucose in the recovery phase improved the recovery.

Administration of glucose individually or in combination with insulin had no effect on the cell death after AHF.

## *Chapter 6*

### **TESTING PHARMACOLOGICAL**

### **DRUGS FOR RECOVERY**

### **FOLLOWING DE NOVO ACUTE**

### **HEART FAILURE**

#### **INTRODUCTION**

AHF may be associated with multiple conditions like acute coronary syndromes, arrhythmias, hypertension, existing chronic heart failure and kidney dysfunction. In acute cases, time is limited; hence the need for pharmacological agents to stabilize the cardiovascular system in such patients arises. Many patients with AHF receive oxygen, diuretics and vasodilators.<sup>19</sup> These agents help stabilize the patient. However, due to the heterogeneity of clinical presentations associated with AHF, there is a need for more therapeutic pharmacological agents and for the understanding of their underlying mechanisms. Sphingosine-1-phosphate (S1P) is one such molecular agent which reduces infarct size and improves functional recovery after ischaemic-reperfusion injury.<sup>95</sup> S1P is cardioprotective by activating the Survivor Activating Factor Enhancement (SAFE)<sup>84</sup> pathway which involves the activation of signal transducer and activator of transcription factor 3 (STAT3). Activation of STAT3 improves mitochondrial function by inhibiting the opening of the mitochondrial permeability transition pore (mPTP). There is however no study examining the effects and molecular mechanisms involved in cardioprotection by S1P in an acute heart failure setting. Hence, we decided to test S1P in our model of *de novo* AHF. We also propose that the SAFE pathway is involved in the

cardioprotective effect of S1P in AHF and that it prevents cell death by inhibiting cytochrome C release from the mitochondria to improve mitochondrial integrity.

AG490 is an inhibitor of the JAK/STAT3 pathway and reduces the phosphorylation of STAT3, thus attenuating the SAFE pathway. In ischaemic hearts, AG490 abrogates protective SAFE pathway and promotes cell death by increasing the number of apoptotic cells.<sup>48</sup>

Ranolazine is an anti-anginal drug that reduces cardiac troponin-T and infarct size in rats subjected to regional ischaemia. Its metabolic effects include partial suppression of fatty acid oxidation, hence promoting glycolysis, which should help generate ATP in a failing heart.<sup>27</sup> Ranolazine ameliorates calcium and sodium homeostasis in the heart without any deleterious effects on heart rate or systolic pressure.<sup>131,132</sup> It is cardioprotective against diastolic heart failure.<sup>132</sup> However, there are no studies that report the effect of administration of ranolazine in *de novo* AHF. We tested whether ranolazine administered in our model of *de novo* AHF improves the functional recovery.

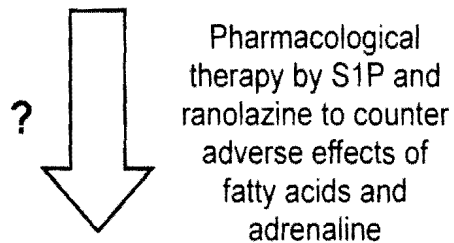
**Models used:** These experiments were done in two separate models: model 1 and model 2. AHF in model 1 was induced by changing the substrate concentration (2.5mmol/L glucose and 1.3mmol/L fatty acids) and reducing the perfusion pressure. Both S1P and ranolazine were administered in the AHF phase. To test the involvement of SAFE pathway activated by S1P in our model of *de novo* acute heart failure, AG490 was administered in the AHF phase. The recovery phase of these hearts also had the same substrate concentration as the AHF phase. The next set of experiments were performed using model 2 of AHF where adrenaline was added in the AHF phase to make the heart failure more severe in addition to the other changes in model 1 (lowering the perfusion pressure and changing the substrate concentration in the perfusate). S1P was added in the AHF as well as in the recovery phase. The fatty acid and glucose concentrations were maintained as in model 1 in the AHF and recovery phases.

Ranolazine was tested in model 1 and was administered in AHF phase. The concentration used was 5µM, based on studies done by Maruyama *et al.*<sup>133</sup>

## HYPOTHESIS

We hypothesized that provision of the pharmacological agents sphingosine-1-phosphate and ranolazine either during an episode of AHF or in the recovery phase would improve functional recovery and reduce cell death.

### ACUTE HEART FAILURE



### IMPROVED FUNCTIONAL RECOVERY

*Figure 6-1: Hypothesis diagram for pharmacological agents*

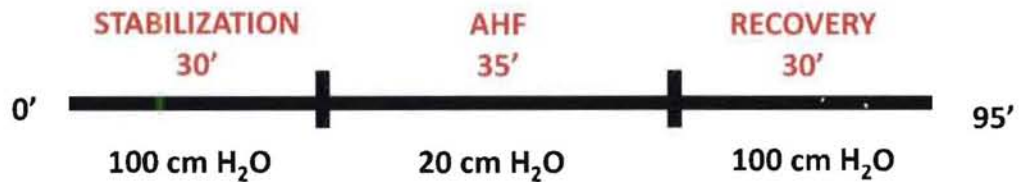
## MATERIALS AND METHODS

### Animals

As previously described in chapter 4.

### Isolated rat heart perfusions

*Protocols for isolated heart perfusions for functional recovery and Western blotting in this section*



	Stabilization	AHF	Recovery
Model 1	11.1mmol/L Glucose; no FFA	2.5mmol/L Glucose; 1.3mmol/L FFA; 10nM S1P	2.5mmol/L Glucose; 1.3mmol/L FFA
Model 2	11.1mmol/L Glucose; no FFA	2.5mmol/L Glucose; 1.3mmol/L FFA; 10 <sup>-8</sup> M Adrenaline; 10nM S1P	2.5mmol/L Glucose; 1.3mmol/L FFA
Model 2	11.1mmol/L Glucose; no FFA	2.5mmol/L Glucose; 1.3mmol/L FFA; 10 <sup>-8</sup> M Adrenaline	2.5mmol/L Glucose; 1.3mmol/L FFA; 10nM S1P

The experiments in this section focused on examining the following:

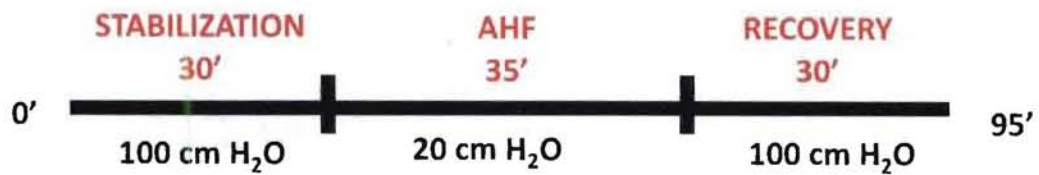
***Is S1P cardioprotective in AHF?***

The preliminary experiments in this group were performed to assess if provision of S1P would improve functional recovery of acutely failing hearts. S1P was tested in both model 1 (without adrenaline in AHF) and model 2 (adrenaline in AHF phase).

***Mechanisms involved in cardioprotection: Is the SAFE Pathway involved?***

The Survivor Activating Factor Enhancement (SAFE) pathway protects the heart against ischaemic-reperfusion injury by the activation of janus kinase (JAK) which belongs to the family of tyrosine kinases, involved in the transduction of signals from the cytosol to the nucleus<sup>48</sup> and signal transducer and activator of transcription 3 (STAT3) which confers cardioprotection by reducing infarct size and improving the functional recovery in an ischaemic heart.<sup>48</sup>

**Protocol**

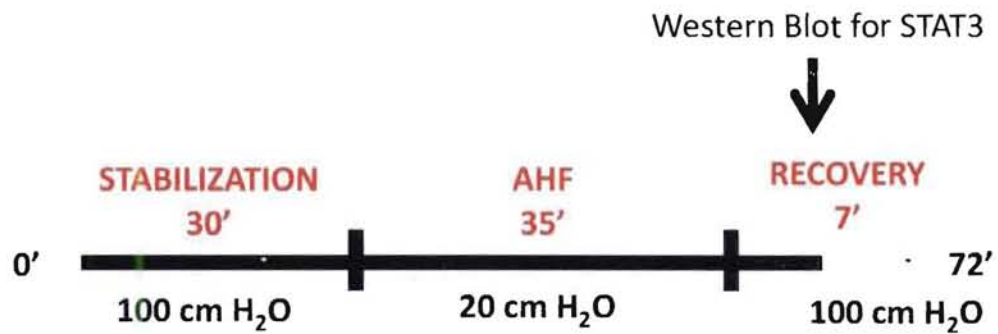


	Stabilization	AHF	Recovery
Model 2	11.1mmol/L Glucose; no FFA	2.5mmol/L Glucose; 1.3mmol/L FFA; 10 <sup>-8</sup> M Adrenaline	2.5mmol/L Glucose; 1.3mmol/L FFA
Model 2	11.1mmol/L Glucose; no FFA	2.5mmol/L Glucose; 1.3mmol/L FFA; 10 <sup>-8</sup> M Adrenaline; 100nM AG490	2.5mmol/L Glucose; 1.3mmol/L FFA
Model 2	11.1mmol/L Glucose; no FFA	2.5mmol/L Glucose; 1.3mmol/L FFA; 10 <sup>-8</sup> M Adrenaline	2.5mmol/L Glucose; 1.3mmol/L FFA; 10nM S1P
Model 2	11.1mmol/L Glucose; no FFA	2.5mmol/L Glucose; 1.3mmol/L FFA; 10 <sup>-8</sup> M Adrenaline; 100nM AG490	2.5mmol/L Glucose; 1.3mmol/L FFA; 10nM S1P

(AG490- inhibitor of STAT3)

**Western blots for determination of STAT3 in model 2 of AHF**

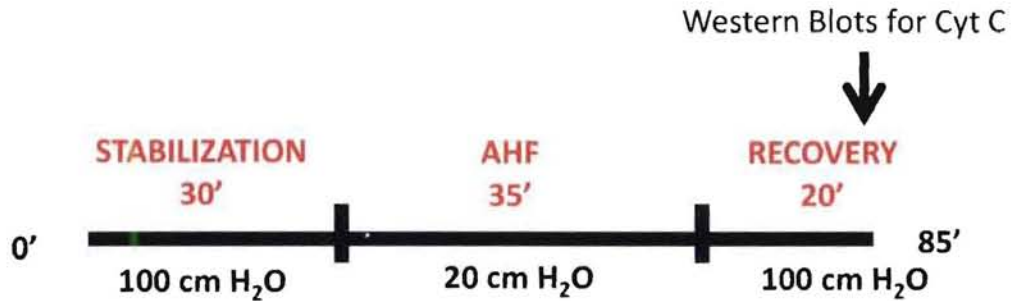
**Protocol for isolated heart perfusion**



	Stabilization	AHF	Recovery
Model 2	11.1mmol/L Glucose; no FFA	2.5mmol/L Glucose; 1.3mmol/L FFA; 10 <sup>-8</sup> M Adrenaline	2.5mmol/L Glucose; 1.3mmol/L FFA
Model 2	11.1mmol/L Glucose; no FFA	2.5mmol/L Glucose; 1.3mmol/L FFA; 10 <sup>-8</sup> M Adrenaline; 100nM AG490	2.5mmol/L Glucose; 1.3mmol/L FFA
Model 2	11.1mmol/L Glucose; no FFA	2.5mmol/L Glucose; 1.3mmol/L FFA; 10 <sup>-8</sup> M Adrenaline	2.5mmol/L Glucose; 1.3mmol/L FFA; 10nM S1P
Model 2	11.1mmol/L Glucose; no FFA	2.5mmol/L Glucose; 1.3mmol/L FFA; 10 <sup>-8</sup> M Adrenaline; 100nM AG490	2.5mmol/L Glucose; 1.3mmol/L FFA; 10nM S1P

**Effect of S1P on cell death in model 2 of AHF**

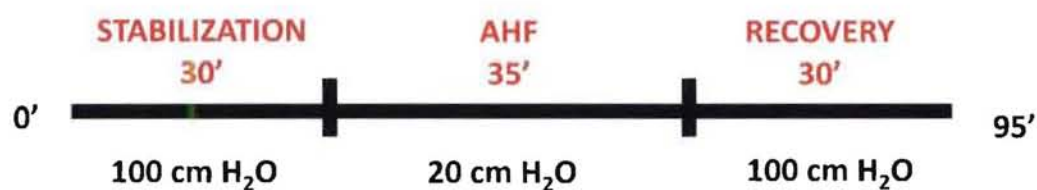
**Protocol for isolated heart perfusion**



	Stabilization	AHF	Recovery
Model 2	11.1mmol/L Glucose; no FFA	2.5mmol/L Glucose; 1.3mmol/L FFA; 10 <sup>-8</sup> M Adrenaline	2.5mmol/L Glucose; 1.3mmol/L FFA
Model 2	11.1mmol/L Glucose; no FFA	2.5mmol/L Glucose; 1.3mmol/L FFA; 10 <sup>-8</sup> M Adrenaline; 100nM AG490	2.5mmol/L Glucose; 1.3mmol/L FFA
Model 2	11.1mmol/L Glucose; no FFA	2.5mmol/L Glucose; 1.3mmol/L FFA; 10 <sup>-8</sup> M Adrenaline	2.5mmol/L Glucose; 1.3mmol/L FFA; 10nM S1P
Model 2	11.1mmol/L Glucose; no FFA	2.5mmol/L Glucose; 1.3mmol/L FFA; 10 <sup>-8</sup> M Adrenaline; 100nM AG490	2.5mmol/L Glucose; 1.3mmol/L FFA; 10nM S1P

## To examine the effect of Ranolazine on functional recovery in model 1 of AHF

### *Protocol for isolated hearts*



	Stabilization	AHF	Recovery
Model 1	11.1mmol/L Glucose; no FFA	2.5mmol/L Glucose; 1.3mmol/L FFA	2.5mmol/L Glucose; 1.3mmol/L FFA
Model 1	11.1mmol/L Glucose; no FFA	2.5mmol/L Glucose; 1.3mmol/L FFA; 5µM Ranolazine	2.5mmol/L Glucose; 1.3mmol/L FFA

### Western blots to examine the effect of metabolic therapy on cell death

Lowry's method of protein quantification was used to assess the quantity of proteins in the samples and the Western blots were run as described in the methods section in chapter 2.

### End points

As previously described in chapter 4.

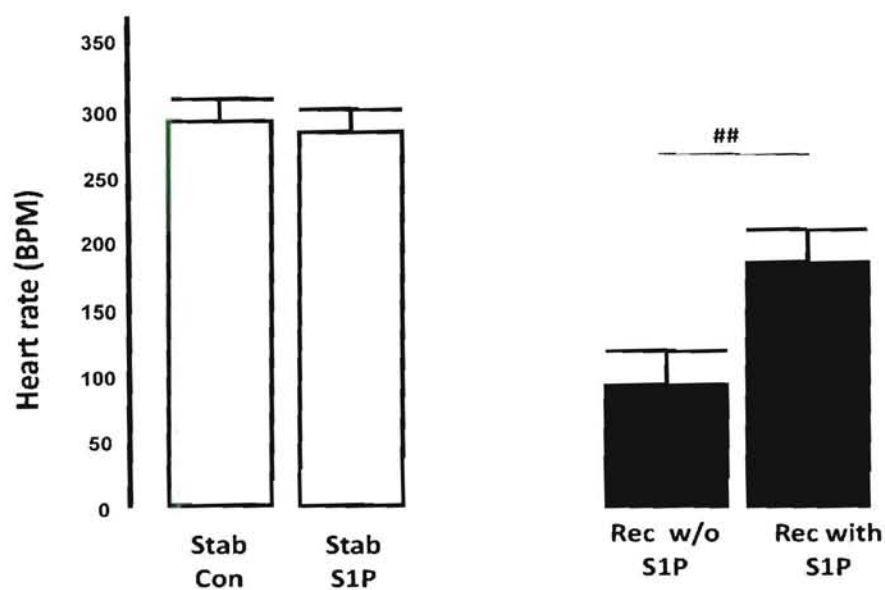
### Statistical analysis

As previously described in chapter 4.

## RESULTS

### Is S1P cardioprotective?

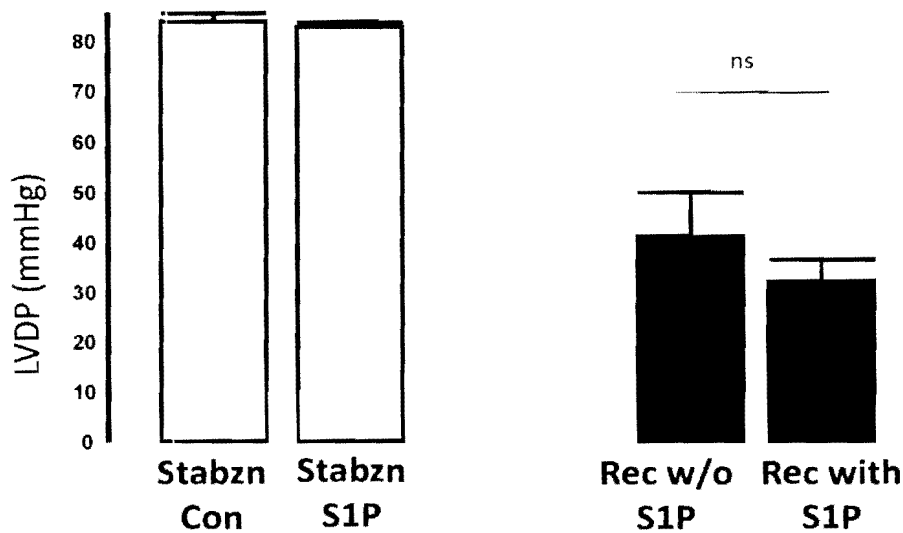
#### *Effect of S1P in model 1 of AHF on heart rate*



**Figure 6-2:** Effect of sphingosine-1-phosphate (S1P) on HR in model 1 (Stabzn- Stabilization phase; Rec- Recovery phase; S1P- sphingosine 1 phosphate); ##  $p < 0.01$  Rec w/o S1P vs. Rec with S1P; Tukey- Kramer Multiple Comparison Test

After 30' of stabilization, heart rates of in both groups had no significant difference. (Fig 6-2) However, at the end of recovery phase, hearts which were treated with S1P in the AHF phase had a mean HR of  $182.5 \pm 25.2$  vs.  $91.1 \pm 25.7$  BPM of the control hearts (##  $p < 0.01$  vs. recovery without S1P).

**Effect of S1P in model 1 of AHF on left ventricular developed pressure (LVDP)**



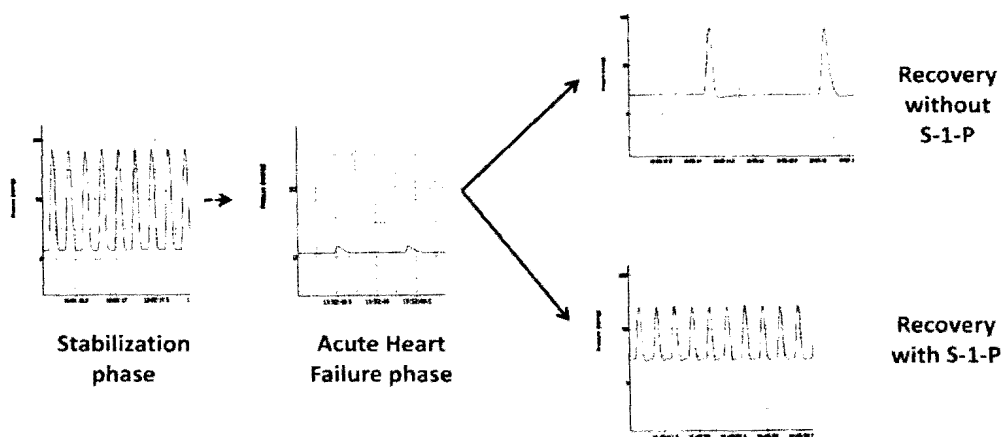
**Figure 6-3:** Effect of S1P on LVDP in model. (Stabzn- Stabilization phase; Rec- Recovery phase; S1P- sphingosine 1 phosphate; LVDP- left ventricular developed pressure); ns- not significant vs. rec with S1P; Tukey- Kramer Multiple Comparison Test

The recovery between the two groups was statistically insignificant. The mean stabilization LVDP for controls and S1P hearts were 84.0±1.6 mmHg and 82.8±1.0 mmHg respectively. (Fig 6-3) On treatment with S1P in AHF phase the recovery LVDP was 32.0±5.3 mmHg vs. 41.1±8.8 mmHg for control hearts.

**Effect of S1P in model 1 of AHF on rate pressure product**

The changes in rate pressure product between the two groups were statistically insignificant despite the treatment with S1P. The stabilization values for control and S1P group were 22903±990 and 24599±1310 respectively. The recovery values for the same groups were 3605±392 vs. 5557±691 respectively. (Fig 6-4)

There were no significant differences between systolic pressure, diastolic pressure and coronary flow between groups in the recovery phase.



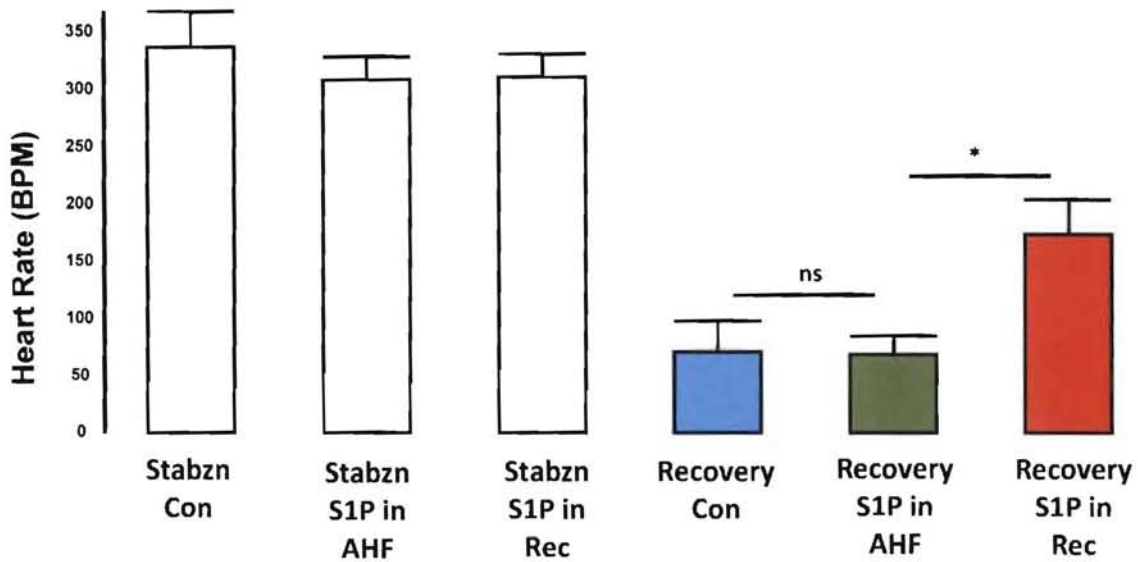
**Figure 6-5:** Example of pressure traces in hearts without and with S1P

### **Summary**

In AHF induced without adrenaline (model 1), S1P treatment improved heart rate but not the LV developed pressure. There was no reduction in diastolic pressure, thus suggesting impaired relaxation

## RESULTS: S1P ACTIVITY IN MODEL 2

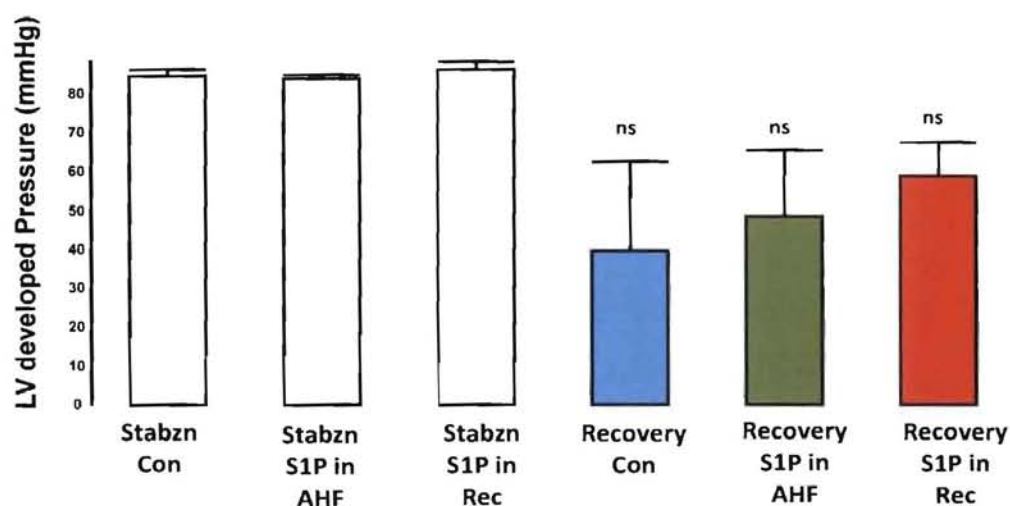
### S1P in model 2 of AHF on heart rate



**Figure 6-6:** Effect of S1P on HR in model 2 (Stabzn- Stabilization phase; Rec- Recovery phase; S1P- sphingosine 1 phosphate; Con- Control); \*-  $p < 0.05$  vs. Recovery S1P in AHF; ns- not significant; Tukey-Kramer Multiple Comparisons Test

The stabilization values for control hearts, hearts that were treated with S1P in AHF phase and hearts that had S1P treatment in recovery phase were:  $338.2 \pm 31.0$ ,  $310.4 \pm 20.0$  and  $313.0 \pm 20.2$  BPM respectively. (Fig 6-6) Recovery when S1P was administered with adrenaline in the AHF phase was  $69.6 \pm 15.7$  while S1P administered in the recovery phase had a HR of  $175.17 \pm 30.7$ ; the controls had a recovery HR of  $71.6 \pm 27.4$  BPM. Heart rate was increased significantly ( $p < 0.05$ ) when S1P was administered in the recovery phase vs. when S1P was administered in the AHF phase.

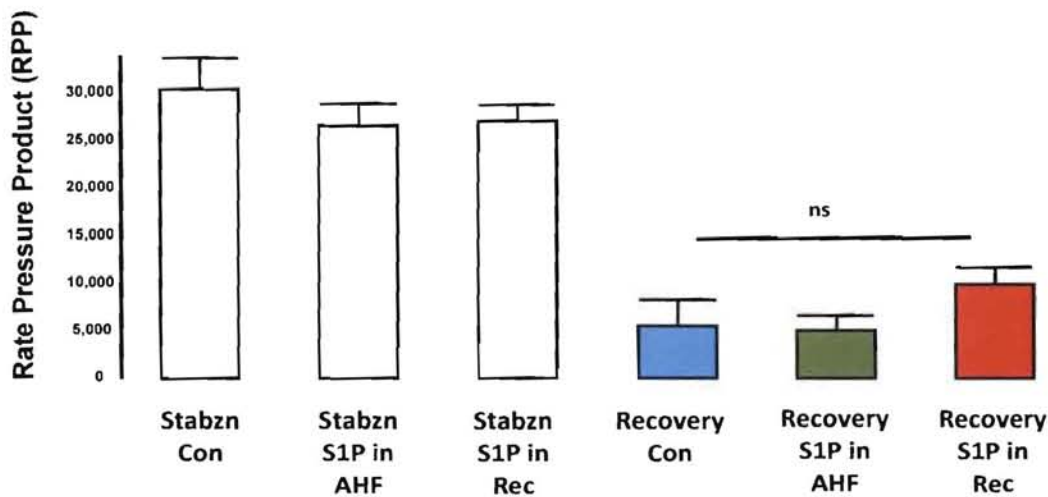
### Effect of S1P on left ventricular developed pressure



**Figure 6-7:** Effect of S1P on LVDP in model 2. (Stabzn- Stabilization phase; Rec- Recovery phase; S1P- sphingosine 1 phosphate; Con- Control); ns- no significance between different groups in recovery phase; Tukey-Kramer Multiple Comparisons Test

The change in LVDP was not significant amongst groups in the recovery phase.(Fig 6-7) The left ventricular developed pressures (LVDP) for the stabilization phase of the three groups were:  $84.8 \pm 1.6$ ,  $84.3 \pm 1.0$  and  $86.7 \pm 1.9$  mmHg for control, hearts with S1P in AHF phase and hearts with S1P in recovery phase respectively. After 35' of AHF and 30' of recovery phase, control hearts had a recovery LVDP of  $39.9 \pm 23.1$  mmHg while hearts treated with S1P in AHF phase showed LVDP of  $49.0 \pm 16.9$  mmHg. The hearts that were treated with S1P in the recovery phase showed LVDP of  $59.3 \pm 8.5$  mmHg.

**Effect of S1P in model 2 of AHF on rate pressure product**



**Figure 6-8:** Effect of S1P on RPP in model 2. (Stabzn- Stabilization phase; Rec- Recovery phase; S1P- sphingosine 1 phosphate; Con- Control); ns- no significance between different groups in recovery phase; Tukey-Kramer Multiple Comparisons Test

Rate pressure products (RPP) for the three groups in the stabilization phase were:  $30597 \pm 3488$  for controls,  $26656 \pm 2384$  for hearts that were then treated with S1P in the AHF phase and  $27134 \pm 1807$  for hearts that were treated with S1P in the recovery phase. (Fig 6-8)

The recovery RPP for these groups were:  $5603 \pm 2901$  for controls;  $5125 \pm 1611$  for hearts after treatment with S1P in AHF phase and  $9979 \pm 1765$  for hearts treated with S1P in recovery phase. There were no significant differences between groups in the recovery phase. One of the control hearts and one of the S1P in AHF hearts had zero recovery and hence all hearts done on that day were excluded from the study.

**Table 6-2:** Effect of S1P treatment in AHF and recovery phase on systolic, diastolic pressures and coronary flow

<b>Systolic pressure (mmHg)</b>			
	<b>Control</b>	<b>S1P in AHF</b>	<b>S1P in Rec</b>
<b>Stabilization</b>	84.8±1.1	88.1±2.0	93.2±2.8
<b>Recovery</b>	118.6±31.8	113.0±10.7	104.9±12.1
<b>Diastolic Pressure (mmHg)</b>			
	<b>Control</b>	<b>S1P in AHF</b>	<b>S1P in Rec</b>
<b>Stabilization</b>	0.0±1.5	3.8±1.7	6.5±1.8
<b>Recovery</b>	78.7±23.7	63.9±14.1	45.6±7.2
<b>Coronary flow (ml)</b>			
	<b>Control</b>	<b>S1P in AHF</b>	<b>S1P in Rec</b>
<b>Stabilization</b>	9.5±0.5	8.5±0.2	9.2±0.4
<b>Recovery</b>	10.6±0.6	10.3±0.3	11.0±0.3

### **Summary**

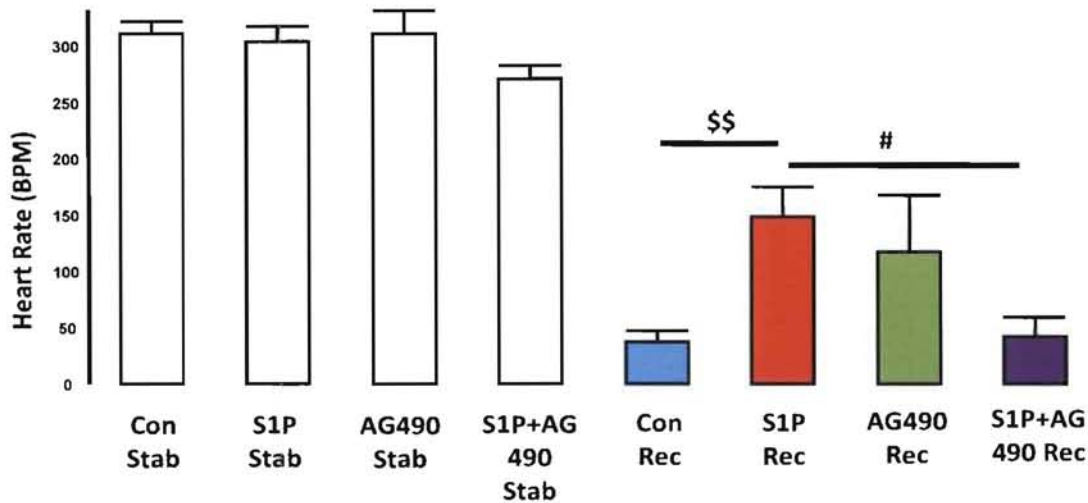
These data suggest that S1P administered in AHF had significantly low recovery versus S1P administered in recovery phase when tested in model 2. These experiments indicate that S1P improved functional recovery by improving heart rate only but not contractility. We chose to investigate the role of the SAFE pathway in this protection by S1P.

## **RESULTS**

### **To examine the involvement of SAFE in AHF by comparing heart rate**

Our data showed that the addition of AG490 to S1P-induced protection abolished the cardioprotective effect of SAFE pathway. (Fig 6-9) The control, S1P, AG490 and S1P+AG490 hearts had baseline stabilization HR values of 311.1±11.1, 304.1±13.9, 310.7±20.7 and 271.1±11.8 BPM respectively. After 30' recovery phase, control hearts exhibited HR of 37.9±9.7 BPM while those treated with S1P had a recovery

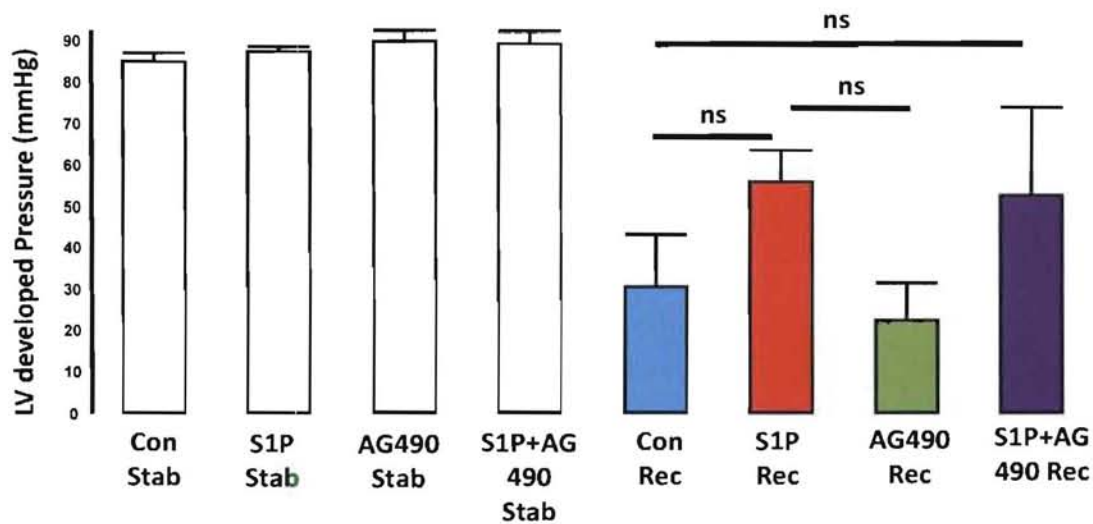
HR of  $148.8 \pm 26.4$ . AG490 treatment showed a recovery HR of  $117.3 \pm 50.4$ . However, a combination of S1P and AG490 had a significantly reduced HR of  $42.3 \pm 17.1$ . This shows that the addition of AG490 to S1P induced protection abolished the cardioprotective effect of SAFE pathway.



**Figure 6-9:** Effect of S1P, AG490 and S1P+AG490 on HR. (Stab - Stabilization phase; Rec- Recovery phase; S1P- sphingosine 1 phosphate; Con- Control); \$\$-  $p < 0.01$  vs Con Rec; #-  $p < 0.05$  vs S1P+AG490 Rec; Tukey-Kramer Multiple Comparisons Test

**To examine the involvement of SAFE in AHF by comparing left ventricular developed pressure**

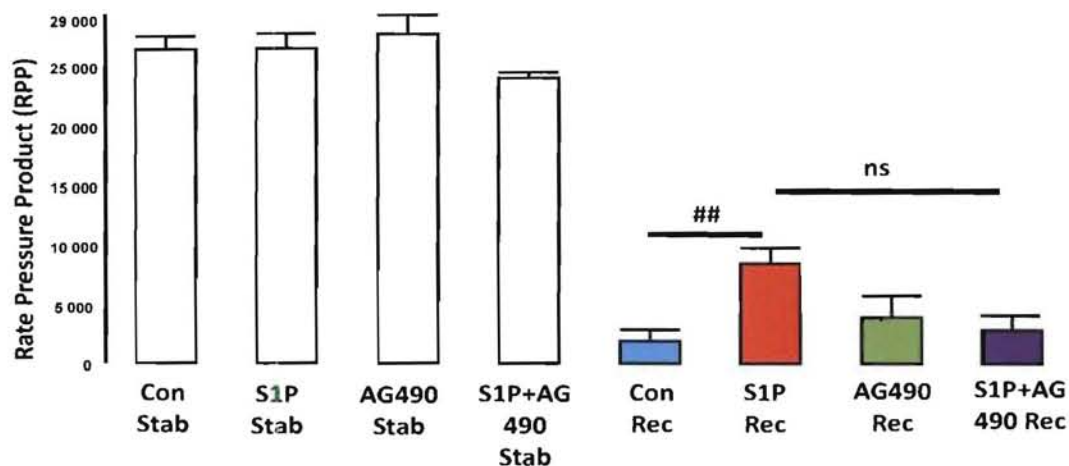
Left ventricular developed pressure in the stabilization phase of the 4 groups was  $84.9 \pm 2.0$ ,  $87.1 \pm 1.1$ ,  $89.7 \pm 2.4$  and  $89.0 \pm 2.8$  for control, S1P, AG490 and S1P+AG490 hearts respectively. (Fig 6-10) The recovery values after 35' of AHF and 30' of recovery phase were:  $30.4 \pm 12.6$ ,  $55.6 \pm 7.7$ ,  $22.3 \pm 9.0$  and  $52.3 \pm 21.1$  mmHg respectively for control, S1P, AG490 and S1P+AG490. There was no statistical significance between groups in the recovery phase.



**Figure 6-10:** Effect of S1P, AG490 and S1P+AG490 on LVDP. (Stab -Stabilization phase; Rec- Recovery phase; S1P- sphingosine 1 phosphate; Con- Control); ns- not significant between groups; Tukey-Kramer Multiple Comparisons Test

**To examine the involvement of SAFE in AHF by comparing rate pressure product**

Rate pressure product for the different groups in stabilization phase was  $26433 \pm 1112$ ,  $26508 \pm 1273$ ,  $27752 \pm 1604$  and  $24013 \pm 535$  for control, S1P, AG490 and S1P+AG490 respectively. (Fig 6-11) At the end of the recovery phase, these groups had RPP values of  $1941 \pm 987$ ,  $8461 \pm 1350$ ,  $3975 \pm 1832$  and  $2871 \pm 1268$  for control, S1P, AG490 and S1P+AG490 respectively. Statistically, there was no difference between S1P and S1P+AG490 but, S1P improved RPP vs. controls.



**Figure 6-11:** Effect of S1P, AG490 and S1P+AG490 on RPP. (Stab- Stabilization phase; Rec- Recovery phase; S1P- sphingosine 1 phosphate; Con- Control); ##-  $p < 0.01$  vs Con Rec; ns- not significant vs S1P+AG490 Rec; Tukey-Kramer Multiple Comparisons Test

**Table 6-3:** Effect of S1P, AG490 and S1P+AG490 treatment on systolic, diastolic pressures and coronary flow

Systolic pressure (mmHg)				
	Control	S1P	AG490	S1P+AG490
Stabilization	88.7±2.6	89.8±2.0	92.2±3.1	94.3±2.5
Recovery	96.5±16.5	100.2±7.6	110.1±9.6	105.7±17.6
Diastolic Pressure (mmHg)				
	Control	S1P	AG490	S1P+AG490
Stabilization	3.7±1.4 <sup>###</sup>	2.6±1.8 <sup>###</sup>	2.5±2.9 <sup>###</sup>	5.3±2.1 <sup>##</sup>
Recovery	66.0±12.7	44.5±5.1 <sup>**</sup>	87.7±10.5	53.4±9.8
Coronary flow (ml)				
	Control	S1P	AG490	S1P+AG490
Stabilization	8.7±0.2	9.0±0.2	8.6±0.3	9.1±0.3
Recovery	10.9±0.3	10.9±0.3	10.1±0.3	11.0±0.6

**Table 6-3:** <sup>###</sup>-  $p < 0.001$  vs. Respective recovery groups; <sup>##</sup>-  $p < 0.01$  vs. Respective recovery group; <sup>\*\*</sup>-  $p < 0.01$  vs. AG490 recovery

The stabilization phase diastolic pressures were significantly increased in recovery phase for all groups (###-  $p < 0.001$  and ##-  $p < 0.01$  vs. S1P+AG490 rec). Treatment with S1P had a significantly lower ( $p < 0.01$ ) diastolic pressure as compared to AG490 in the recovery phase. (Table 6-3)

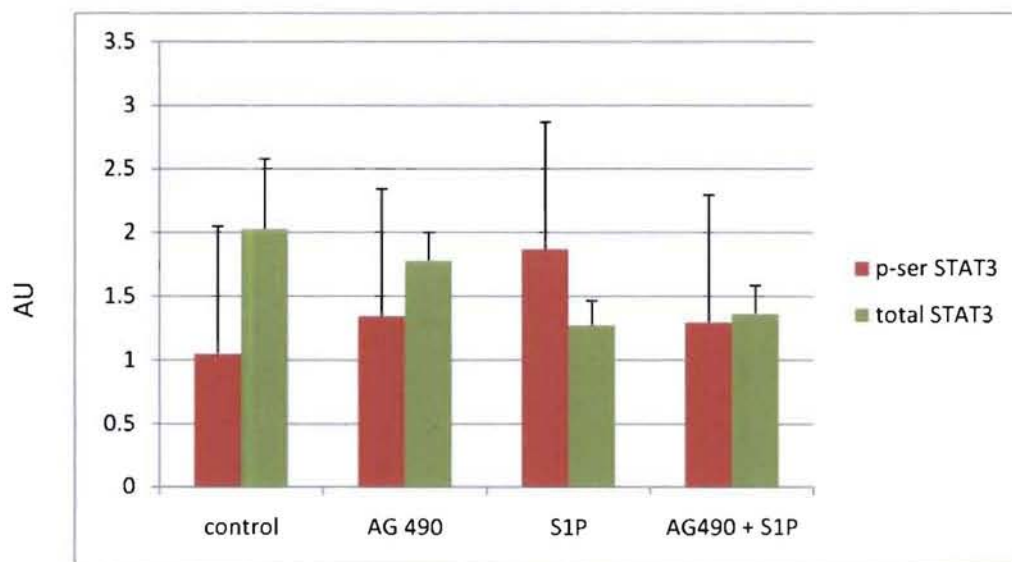
### Summary

Our data suggest that the protective action of S1P was indeed abrogated by the addition of the Janus kinase inhibitor, AG490. These are preliminary data that suggests that the SAFE pathway might be involved in the cardioprotective mechanism of S1P against *de novo* acute heart failure.

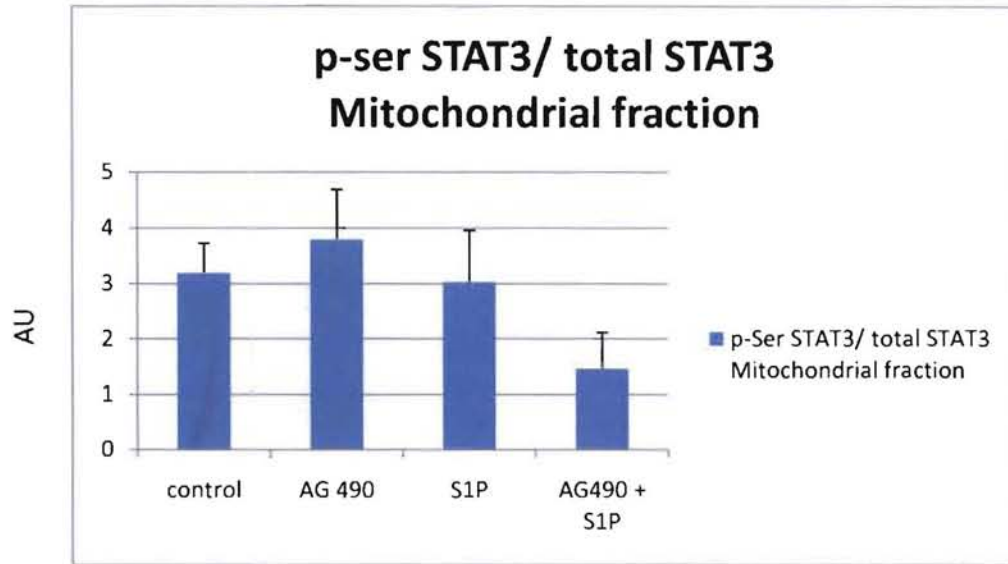
## RESULTS

### Western blots for determination of STAT3 in AHF:

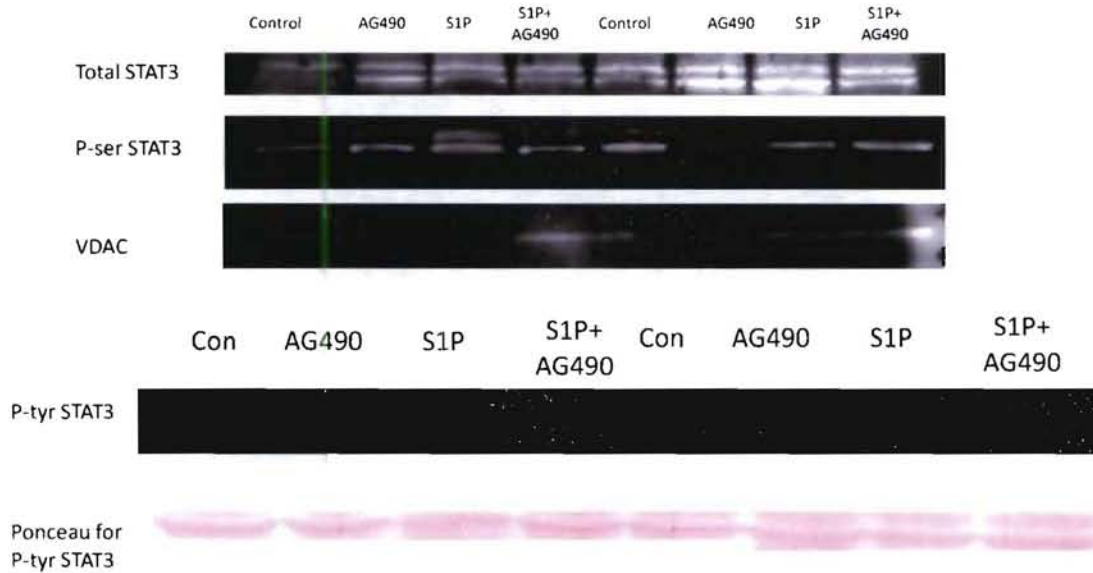
We measure the expression of phosphorylated STAT3 when it was bound to its tyrosine residue and serine residue. We saw expression of STAT3 when it was bound to its serine residue only. We did not see any expression of phosphorylated STAT3 in the tyrosine residue and hence the graphs are not shown here.



**Figure 6-12:** Phosphoserine STAT3 and Total STAT3 in mitochondrial fraction

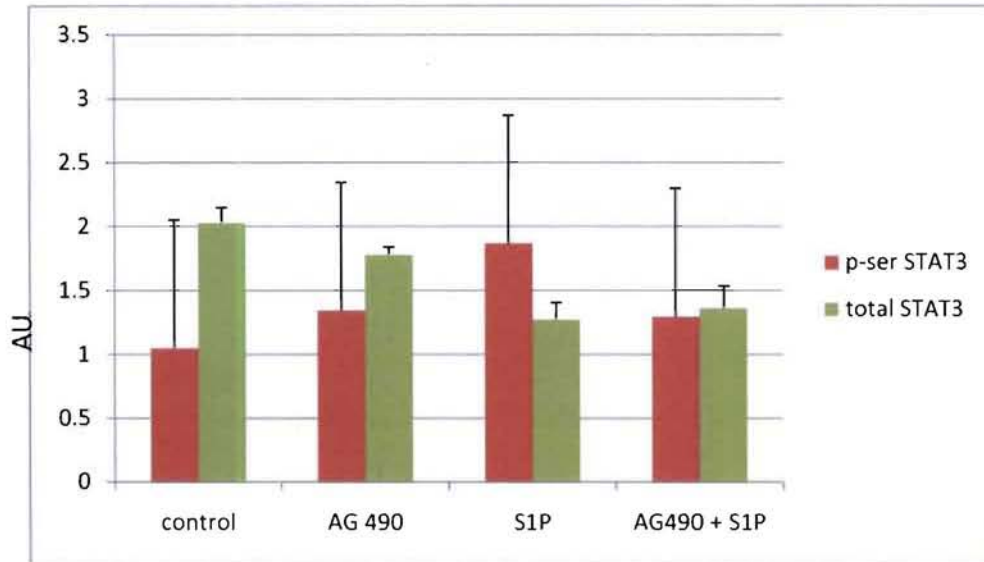


**Figure 6-13:** Ratio of p-ser STAT3/total STAT3 in mitochondrial fraction

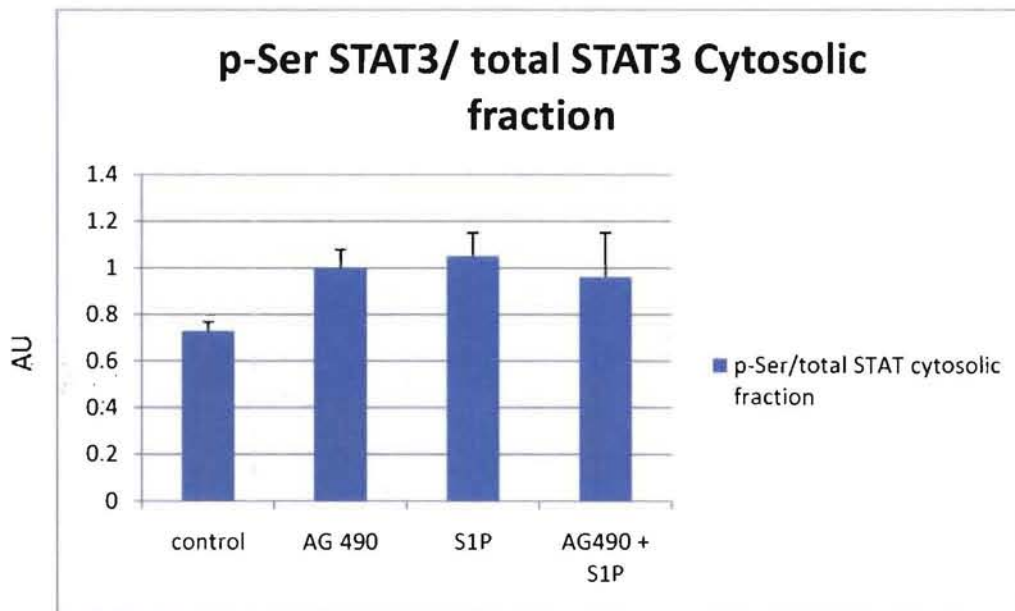


**Figure 6-14:** Western blots for STAT3 in the mitochondrial fraction

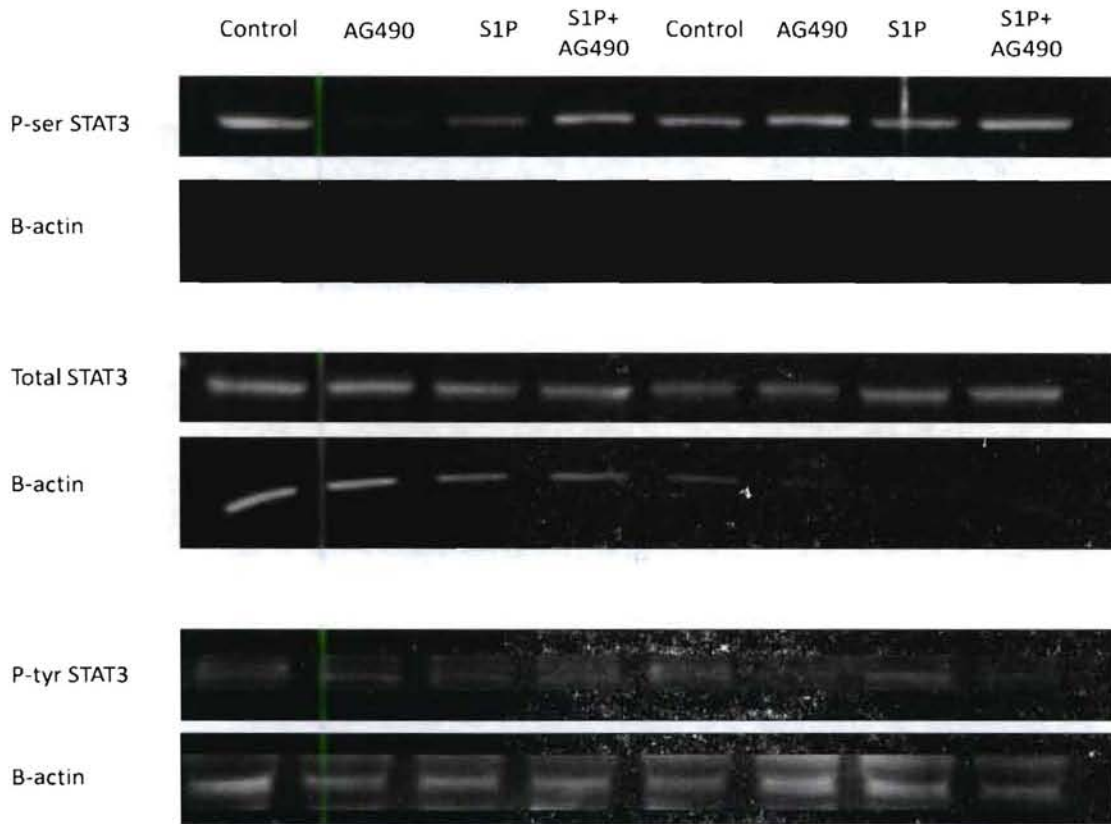
In the mitochondrial fraction of the hearts subjected to AHF and 7' recovery, there was no significant difference in the amount of phosphor-ser STAT3. This suggests that the phosphorylated STAT3 was not translocated to the mitochondria.



**Figure 6-15:** Phosphoser STAT3 and Total STAT3 in cytosolic fraction

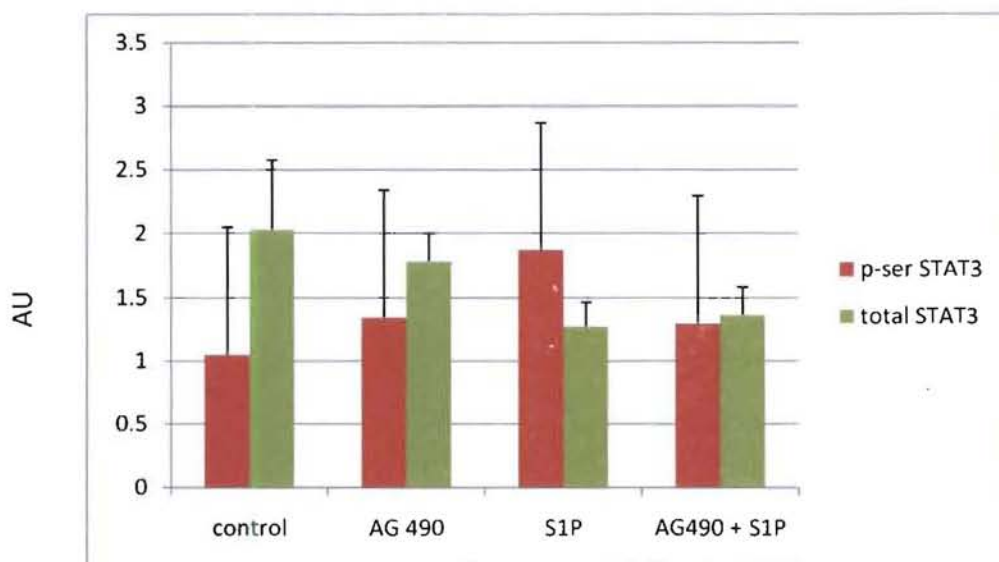


**Figure 6-16:** Ratio of p-ser STAT3/ total STAT3 in cytosolic fraction

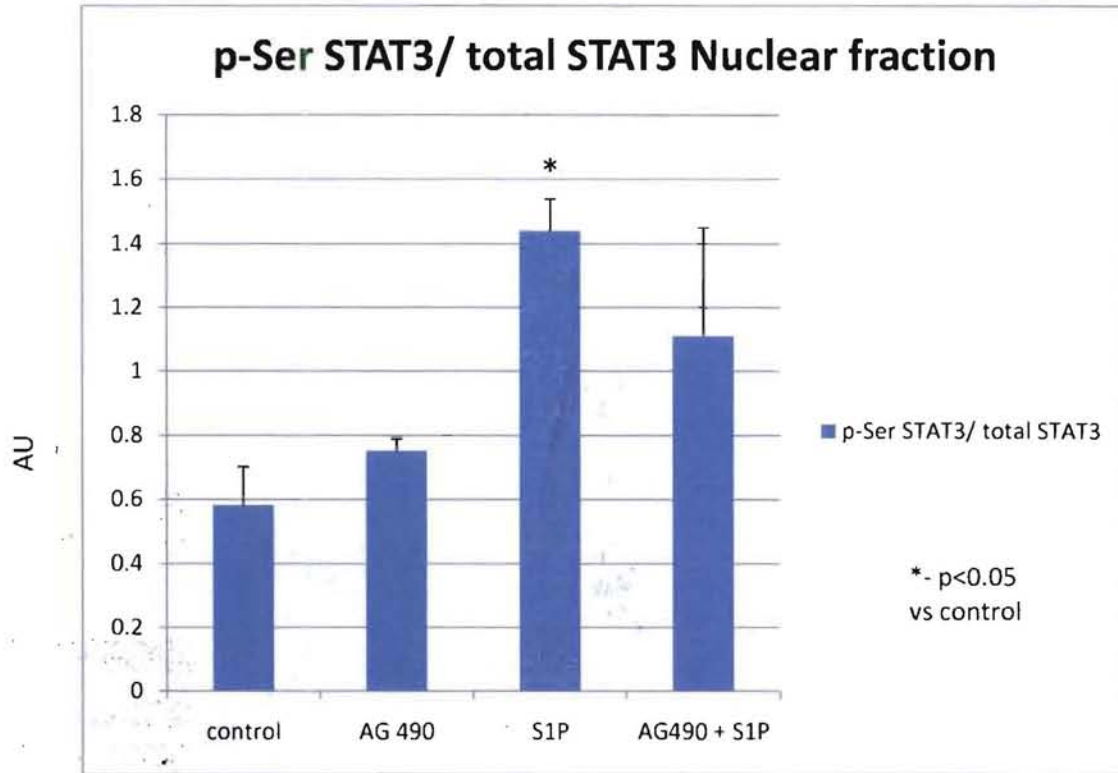


**Figure 6-17:** Western blots for STAT3 in the cytosolic fraction

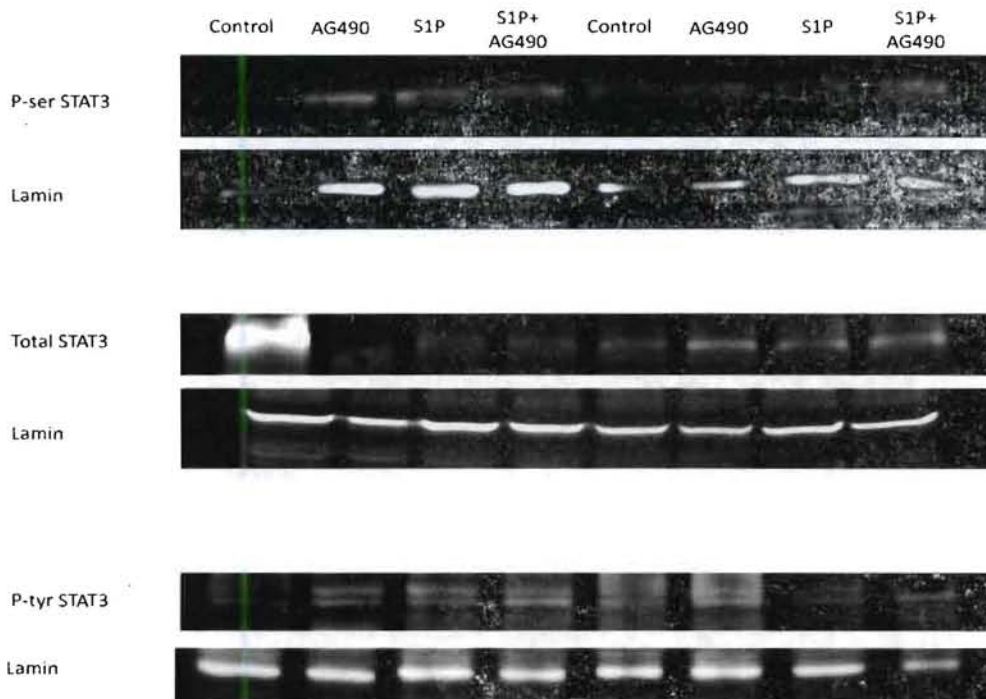
Observing the cytosolic fraction, S1P increased phosphor-ser STAT3 when compared to controls but this effect was not attenuated by the inhibitor AG490.



**Figure 6-18:** Phosphoser STAT3 and Total STAT3 in nuclear fraction



**Figure 6-19:** Ratio of p-ser STAT3/ total STAT3 in nuclear fraction



**Figure 6-20:** Western blots for STAT3 in the nuclear fraction

In the nuclear fraction, we observed significant increase in phosphorylation of STAT3 when compared vs. controls thus suggesting that the activity of phosphorylated STAT3 was via the serine residue and mediated in the nucleus.

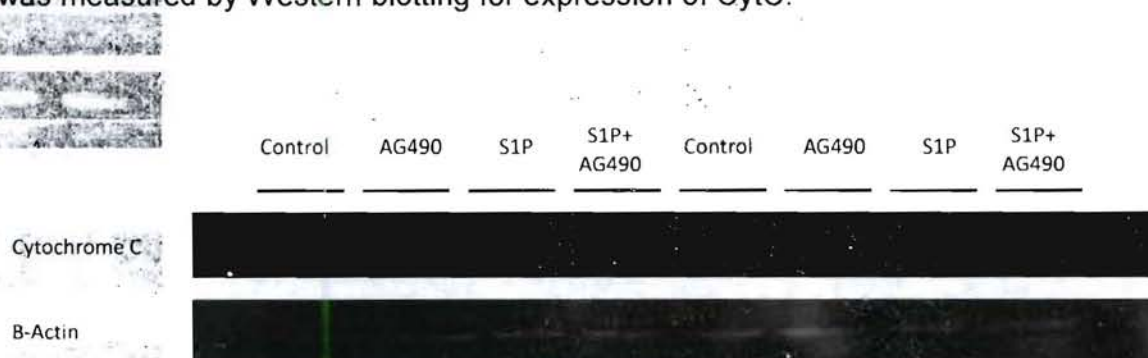
### **Summary**

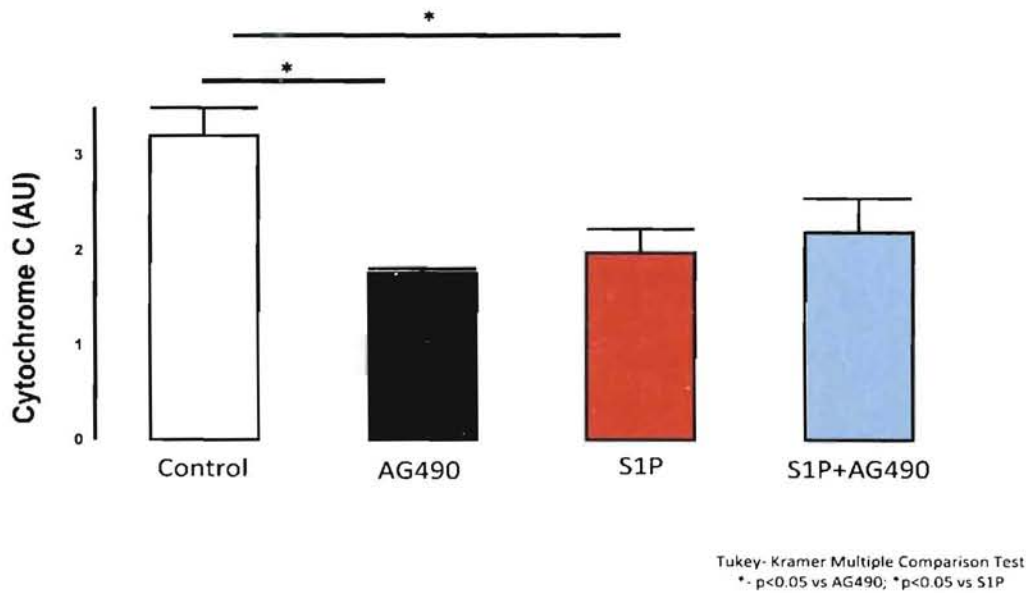
The Western blots for STAT3 suggest that after AHF, treatment with S1P increases the amount of activated STAT3 in the nucleus which might contribute to the protective effect of S1P.

## **RESULTS**

### **Effect of S1P on cell death**

Expression of cytochrome C (CytC) was analysed in hearts subjected to AHF. The protein quantification was done using Lowry's method<sup>126</sup> as previously described and subsequently the gels were run and probed for CytC. Treatment with S1P significantly reduced the expression of Cyt C after AHF as did AG490. Cell death was measured by Western blotting for expression of CytC.





**Figure 6-21:** Effect of pharmacological treatment on expression of cytochrome C

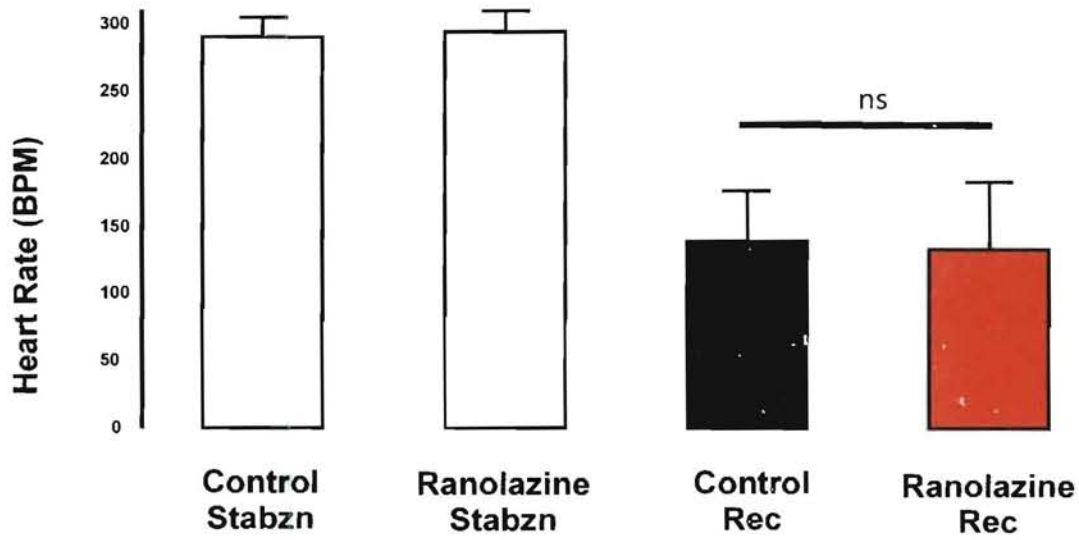
Reduction of CytC expression by treatment with S1P was not abrogated by the addition of AG490, suggesting that this protection was not via the SAFE pathway. The same endpoints as the ones described above were monitored for these experiments. The glucose concentration in the stabilization phase was 11.1mmol/L with no fatty acids in the perfusate, the AHF and recovery phase saw reduced glucose (2.5mmol/L) and high fatty acids (1.3mmol/L) in the perfusate.

## RESULTS

### ***Effect of ranolazine administration in the AHF phase***

Isolated hearts were perfused with ranolazine in the AHF phase in the absence of adrenaline (model 1). Besides the reduction of perfusion pressure, glucose concentration in the perfusate was lowered to 2.5mmol/L and FFA (1.3mmol/L) was introduced in this phase.

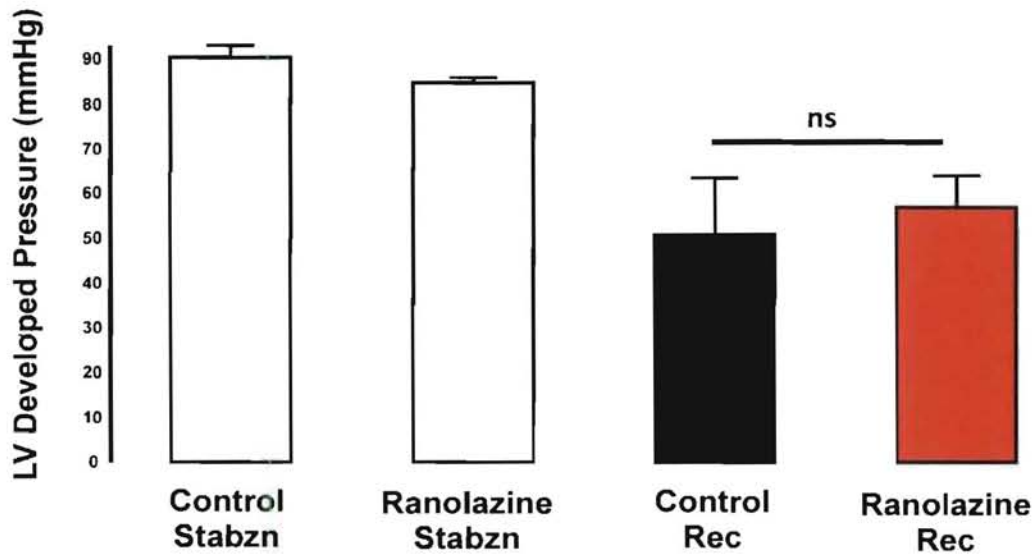
**Effect of ranolazine treatment on heart rate**



**Figure 6-22:** Effect of ranolazine on HR in model 1. (Stabzn- Stabilization phase; Rec- Recovery phase); ns- not significant vs. control rec; Tukey- Kramer Multiple Comparison Test

There was no significant change in the heart rate after treatment with ranolazine. After 30 minutes of stabilization phase, control hearts had a mean HR of 291.9±13.7 BPM and ranolazine hearts had a mean HR of 296.0±15.2 BPM. (Fig 6-22) However, the recovery HR in hearts without ranolazine treatment was 140.2±36.5 and those that were treated with ranolazine had a heart rate of 134.5±50.3 BPM.

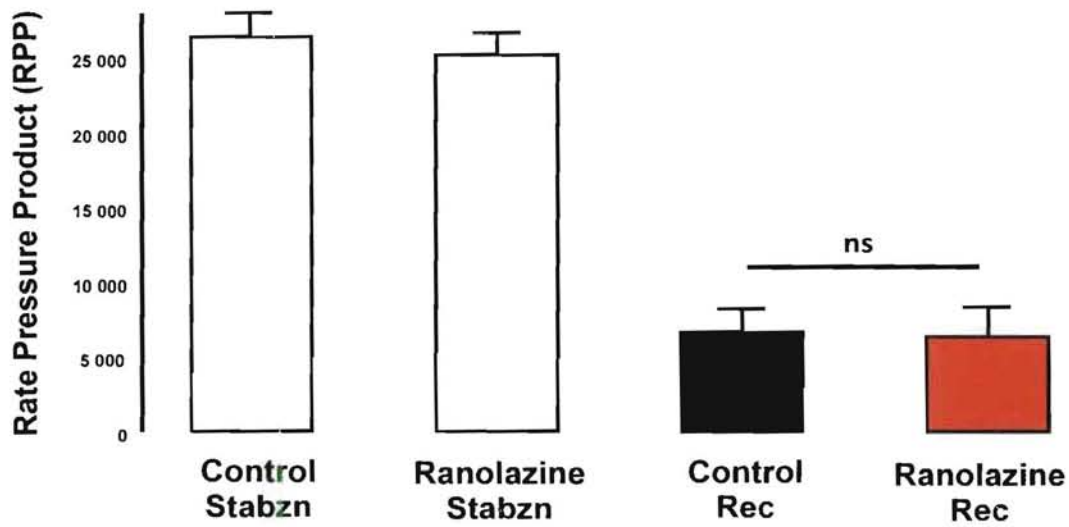
**Effect of ranolazine treatment on left ventricular developed pressure**



**Figure 6-23:** Effect of ranolazine on LVDP in model 1. (Stabzn- Stabilization phase; Rec- Recovery phase); ns- not significant vs. control rec; Tukey- Kramer Multiple Comparison Test

Treatment with ranolazine in the AHF phase had no significant improvement in LVDP of these hearts. (Fig 6-23) The left ventricular developed pressures (LVDP) for control and ranolazine hearts in the stabilization phase were:  $90.4 \pm 2.5$  and  $84.9 \pm 0.8$  mmHg. However, hearts without ranolazine treatment had a LVDP of  $51.0 \pm 12.5$  mmHg while those with ranolazine treatment in the AHF phase had  $57.2 \pm 6.9$  mmHg.

**Effect of ranolazine treatment on rate pressure product**



**Figure 6-24:** Effect of ranolazine on RPP in model 1. (Stabzn- Stabilization phase; Rec- Recovery phase); ns- not significant vs. control rec; Tukey- Kramer Multiple Comparison Test

The treatment with ranolazine had no effect on RPP. Rate pressure products (RPP) at the end of stabilization for control and ranolazine groups were 26409±1479 and 25180±1471 respectively. (Fig 6-24) Hearts that were not treated with ranolazine had recovery RPP of 6666.6±1604.6 while those with ranolazine treatment had an RPP of 6345±1979.

**Table 6-4:** Effect of ranolazine treatment on systolic pressure, diastolic pressure and coronary flow

<b>Systolic pressure (mmHg)</b>		
	<b>Control</b>	<b>Ranolazine</b>
<b>Stabilization</b>	91.4±2.4	84.3±0.3
<b>Recovery</b>	63.9±12.8	95.4±7.9*
<b>Diastolic Pressure (mmHg)</b>		
	<b>Control</b>	<b>Ranolazine</b>
<b>Stabilization</b>	0.9±0.4	0.0±0.6
<b>Recovery</b>	12.8±4.6	38.2±8.7**
<b>Coronary flow (ml)</b>		
	<b>Control</b>	<b>Ranolazine</b>
<b>Stabilization</b>	9.0±0.3	9.0±0.2
<b>Recovery</b>	10.5±0.4	10.2±0.3

**Table 6-4:** \*-  $p < 0.05$  vs. Control; \*\*-  $p < 0.01$  vs Controls

Ranolazine increased systolic pressure significantly ( $p < 0.05$  vs. controls) but had worse diastolic pressure recovery. The treatment with ranolazine in the AHF phase did not improve relaxation in the recovery phase which was significantly higher as compared to controls ( $p < 0.01$ )(Table 6-4)

### **Summary**

These data showed no statistical significance as we did not see any improvement with ranolazine when used as a cardioprotective agent in the AHF phase. Hence, we discontinued ranolazine as a pharmacological agent in our model of mild *de novo* AHF.

## DISCUSSION

### 1. *Cardioprotection by sphingosine-1-phosphate*

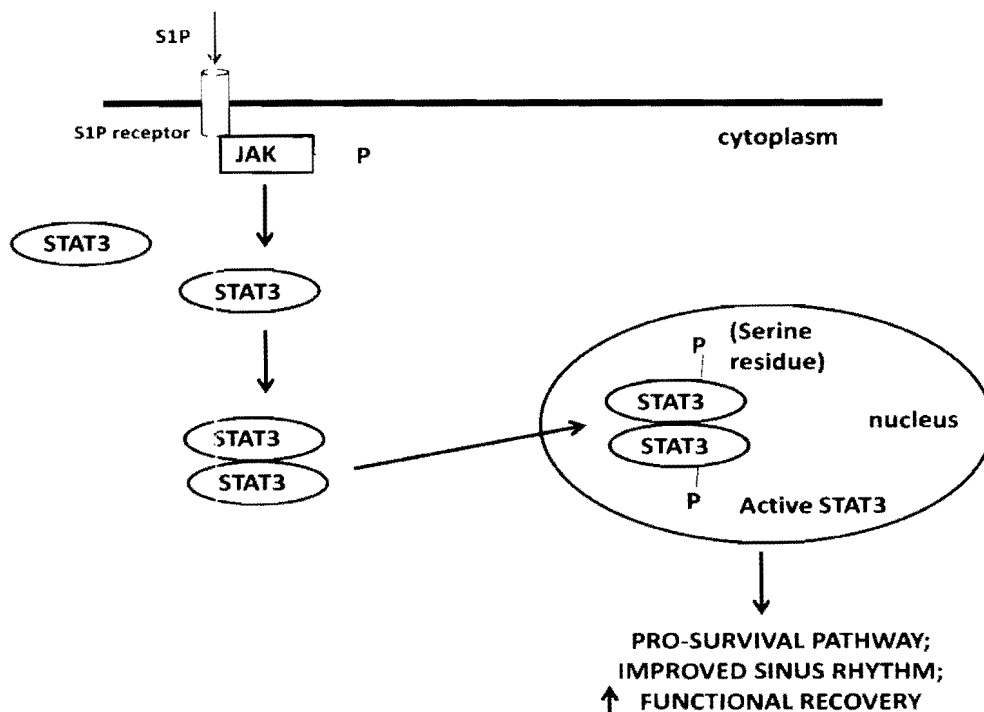
Treatment with S1P administered in the AHF phase increased the heart rate, but not the contractility. When S1P was administered in the absence of adrenaline, it was cardioprotective. There was, however, no improvement in systolic pressures in the treated hearts. S1P treatment did not reduce diastolic pressure, indicating impaired relaxation. However, in the presence of adrenaline, S1P had no effect when tested in the AHF phase but improved heart rate when administered in the recovery phase. These data suggest that cardioprotection by S1P is via the increased activity of the sinus node. S1P asserts its activity in the heart by either binding to one of the three receptors or as an intracellular agent, however there is no published data for the latter.<sup>83</sup> The three S1P receptors in the heart i.e. S1P1, S1P2 and S1P3, are involved in myocyte survival, inflammation pathways, vascular resistance and heart rate either individually or in conjugation. In experiments by Sanna *et al*<sup>87</sup>, examining the effects of various S1P receptors, S1P receptor 3 was shown to be responsible to control sinus rhythm.

### 2. *Mechanisms involved in cardioprotection by S1P*

The Reperfusion Injury Salvage Kinase (RISK)<sup>134</sup> and Survivor Activating Factor Enhancement (SAFE)<sup>48,135</sup> pathways are the main pro-survival pathways discovered in the heart which protect from lethal reperfusion injury. S1P is a preconditioning agent which reduces infarct size and improves functional recovery after an ischaemic insult. The action of S1P against ischaemic-reperfusion injury involves the partial activation of the RISK pathway.<sup>102,104,136</sup> S1P acts as a mediator between both RISK and SAFE pathways when administered at the onset of reperfusion in the ischaemia-reperfusion model. In studies by Somers *et al*<sup>102</sup> cardioprotection by S1P was abrogated both in the presence of inhibitors of SAFE and RISK pathways suggesting that cardioprotection via S1P acts as a mediator between the two pathways. Both SAFE and RISK pathways deactivate FOXO-1 which is an important mediator in the apoptosis cascade.<sup>102</sup> Our data suggests that the SAFE pathway is involved in the cardioprotective effect of S1P as we find up-regulation of STAT3 in the nuclear fraction of the cells. In our model, we propose that the activation of STAT3 by virtue

of S1P binding to its G- protein coupled receptors (GPCR's) causes STAT3 to translocate to the nucleus where the phospho-serine residue dimerises and initiates survival in the form of inactivation of pro-apoptotic factors or the propagation and expression of proteins that are involved in cardioprotection (such as Bcl, VEGF and others). We collected the tissue from the hearts at seven minutes into the recovery phase in the same manner as did Somers *et al.*<sup>102</sup> They studied the role of S1P induced cardioprotection in the SAFE and RISK pathways using isolated mouse hearts to induce a global ischaemia on a Langendorff perfusion system and found different amounts of phosphorylated STAT3 in mitochondria and cytosolic fractions when hearts were harvested at seven minutes and fifteen minutes. At seven minutes, they observed that phosphorylated STAT3 was significantly increased in the mitochondria, but this was reduced when hearts were harvested at fifteen minutes of reperfusion. In the SAFE pathway, STAT3 is first phosphorylated at the tyrosine residue which then dimerises and translocates to the nucleus, where it is phosphorylated again at the serine residue and then activates survival pathways.<sup>48</sup>

In our experiments to evaluate the effect of STAT3 in AHF, despite multiple attempts, we did not see any STAT3 expression in our phospho-tyrosine residue of STAT3. However, after treatment with S1P and harvesting the hearts at seven minutes into the recovery phase, we saw significant elevation of the phospho-serine residue of STAT3, which suggests that STAT3 might be translocated directly to the nucleus and that the phosphorylation on the serine residue might be the key to induce its protective effect. We speculate that this increase in phosphorylated STAT3 is a time dependent outcome similar to that found by Somers *et al.*<sup>102</sup> We anticipate that further experiments at different time points will help elucidate the mechanism of cardioprotection by STAT3 and its various cellular fractions.



**Figure6-25:** Proposed scheme for cardioprotective effect of S1P by activation of SAFE pathway. (adapted from Lecour S, 2009)<sup>48</sup>(S1P- sphingosine-1-phosphate; JAK- Janus kinase; STAT3- signal transducer and activator of transcription 3)

### 3. S1P and cell death

S1P was protective against cell death in our experiments. It significantly reduced the expression of cytochrome C in hearts subjected to *de novo* acute heart failure. We speculate about the effect of the SAFE pathway in the protection against cell death in our model. The hearts treated with AG490 also showed reduced cell death, which also suggests that the protective effect of S1P in AHF is mediated via the nucleus. Cytochrome C is involved in the early stages of the pro-apoptotic pathway and we propose that inhibition of molecules like caspases would provide more conclusive proof of S1P's anti-apoptotic properties.

### 4. Ranolazine

Treatment with ranolazine did not show any significant improvement in our model of heart failure. Ranolazine acts by reducing the cellular acetyl-CoA quantities by inhibiting the  $\beta$ -oxidation of fatty acids and activation of pyruvate dehydrogenase.<sup>137,138</sup> In our experiments with ranolazine, when ranolazine was

administered in the AHF phase of the mild model, it did not improve the functional recovery. It did, however; improve the systolic pressure when compared to control hearts. Besides increasing systolic pressure, hearts treated with ranolazine also had elevated diastolic pressures, indicating impaired relaxation.

One possible drawback of our experiments that might contribute to the failure of ranolazine in this model might be the low concentration of glucose in the perfusate. We used 2.5mmol/L of glucose in the perfusate which is pathologically low. Ranolazine promotes glucose oxidation by partially inhibiting fatty acid oxidation and most likely needs physiological or higher levels of glucose to exert its cardioprotective effects.

## **CONCLUSION**

Treatment with S1P when administered in the AHF phase in the absence of adrenaline (model 1) greatly improves the heart rate. However, there was no change in the contractility of the heart.

In the presence of adrenaline (model 2), S1P treatment in the AHF phase had no significant improvement on the functional recovery, but S1P administered in the recovery phase improved the heart rate only.

This protection by S1P is mediated by the Survivor Activating Factor Enhancement (SAFE) pathway and exerts its effects via the nucleus.

In our model of *de novo* AHF, the low concentration of glucose in the perfusate, coupled with the high free fatty acids, probably prevented ranolazine from having any cardioprotective effects.

*Chapter 7*

**COMBINATION OF**

**PHARMACOLOGICAL AND**

**METABOLIC AGENTS FOR**

**RECOVERY FOLLOWING DE**

**NOVO ACUTE HEART FAILURE**

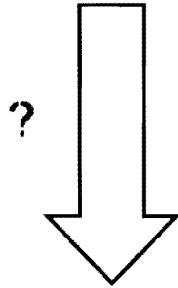
**INTRODUCTION**

Our previous experiments have shown sphingosine-1-phosphate (S1P), glucose and insulin individually to be cardioprotective against our models of *de novo* acute heart failure. However, these agents improve functional recovery by increasing the heart rate only. There was no improvement in the contractility of the left ventricle. Hence, we hypothesized that the combination of metabolic and pharmacological agents will further add to the cardioprotective properties of these agents and that these agents will improve the mitochondrial respiration following an episode of acute heart failure.

**HYPOTHESIS**

The previous experiments with metabolic and pharmacological agents individually improved functional recovery by increasing the heart rate. We hypothesize that the combination of these agents would have an additive effect in the improvement of functional recovery from AHF and improve the mitochondrial respiration as well (Fig. 7-1)

**ACUTE HEART FAILURE  
(AHF)**



Combination of therapy by  
metabolic and pharmacological  
agents to counter adverse effects  
of fatty acids and adrenaline

**IMPROVED FUNCTIONAL  
RECOVERY; IMPROVED  
MITOCHONDRIAL  
RESPIRATION**

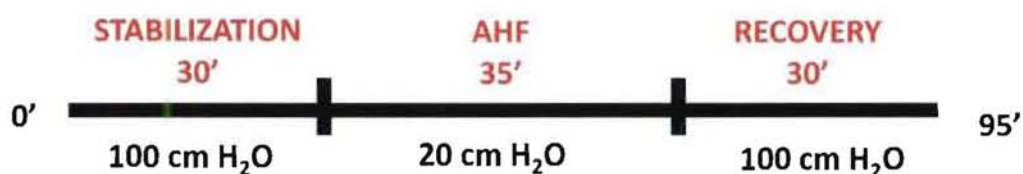
***Figure 7-1:** Hypothesis that treatment with metabolic + pharmacological agents improves recovery after AHF*

## MATERIALS AND METHODS

### Animals

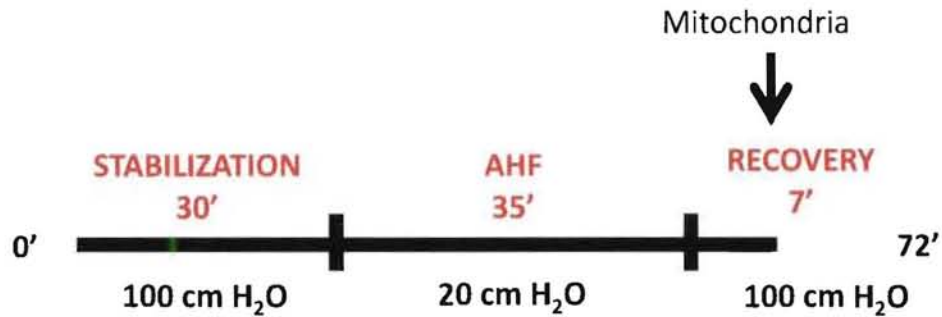
As previously described in chapter 4.

### *Protocols for isolated heart studies*



	Stabilization	AHF	Recovery
Model 2	11.1mmol/L Glucose; no FFA	2.5mmol/L Glucose; 1.3mmol/L FFA; 10 <sup>-8</sup> Adrenaline	11.1mmol/L Glucose; 1.3mmol/L FFA
Model 2	11.1mmol/L Glucose; no FFA	2.5mmol/L Glucose; 1.3mmol/L FFA; 10 <sup>-8</sup> Adrenaline	11.1mmol/L Glucose; 1.3mmol/L FFA; 10nM S1P
Model 2	11.1mmol/L Glucose; no FFA	2.5mmol/L Glucose; 1.3mmol/L FFA; 10 <sup>-8</sup> Adrenaline	11.1mmol/L Glucose; 1.3mmol/L FFA; 0.3mU Insulin
Model 2	11.1mmol/L Glucose; no FFA	2.5mmol/L Glucose; 1.3mmol/L FFA; 10 <sup>-8</sup> Adrenaline	11.1mmol/L Glucose; 1.3mmol/L FFA; 10nM S1P; 0.3mU Insulin

## Mitochondrial studies



	Stabilization	AHF	Recovery
Model 2	11.1mmol/L Glucose; no FFA	11.1mmol/L Glucose; no FFA; (no AHF)	11.1mmol/L Glucose; no FFA
Model 2	11.1mmol/L Glucose; no FFA	2.5mmol/L Glucose; 1.3mmol/L FFA; $10^{-8}$ Adrenaline	11.1mmol/L Glucose; 1.3mmol/L FFA
Model 2	11.1mmol/L Glucose; no FFA	2.5mmol/L Glucose; 1.3mmol/L FFA; $10^{-8}$ Adrenaline	11.1mmol/L Glucose; 1.3mmol/L FFA; 10nM S1P
Model 2	11.1mmol/L Glucose; no FFA	2.5mmol/L Glucose; 1.3mmol/L FFA; $10^{-8}$ Adrenaline	11.1mmol/L Glucose; 1.3mmol/L FFA; 0.3mU Insulin
Model 2	11.1mmol/L Glucose; no FFA	2.5mmol/L Glucose; 1.3mmol/L FFA; $10^{-8}$ Adrenaline	11.1mmol/L Glucose; 1.3mmol/L FFA; 10nM S1P; 0.3mU Insulin

## Mitochondrial respiration studies

The hearts were harvested at 7' in the recovery phase and the mitochondria were isolated and respiration measured on the Clark-type electrode as described in chapter 2.

### End points

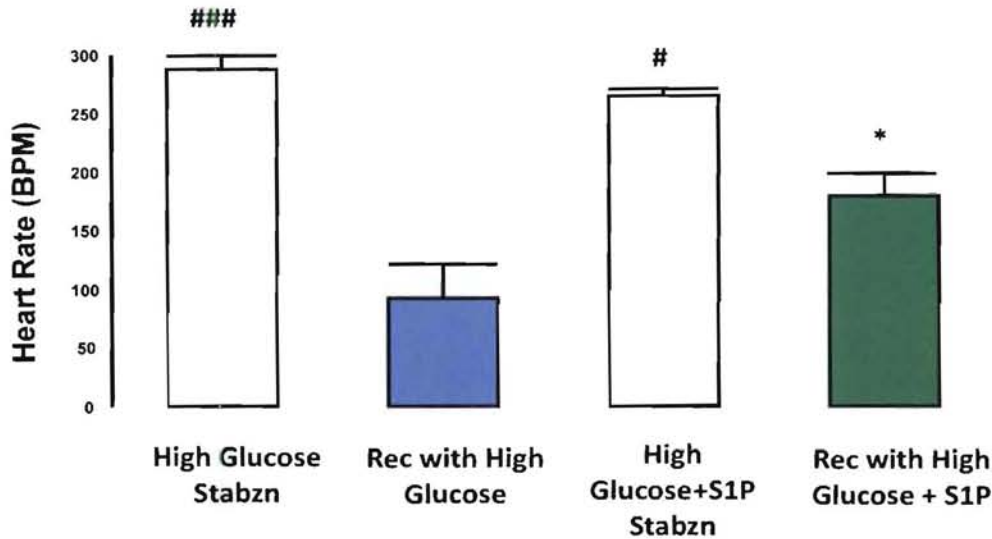
As previously described in chapter 4.

### Statistical analysis

As previously described in chapter 4.

## RESULTS

### *Effect of high glucose + S1P on heart rate*



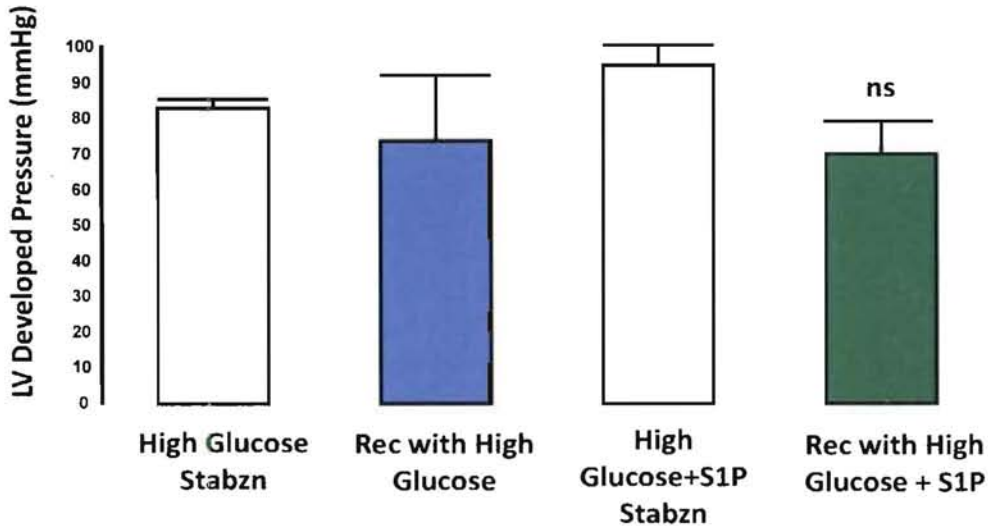
**Figure 7-2:** *Effect of glucose + S1P on HR in model 2. (Stabzn- Stabilization phase; S1P- sphingosine-1-phosphate; Rec: Recovery phase); ### p<0.001 vs. Rec with high glucose; # p<0.05 vs. Rec with high glucose+S1P; \*p<0.05 vs. Rec with high glucose; Tukey- Kramer Multiple Comparison Test*

Treatment with S1P and glucose significantly improved the heart rate in these hearts ( $p<0.01$  vs. recovery with high glucose). (Fig 7-2) In the stabilization phase, control hearts had a HR of  $288.2\pm 11.7$  BPM and the HG+S1P hearts had a stabilization HR of  $265.8\pm 5.8$  BPM. After 30' recovery phase, hearts perfused with high glucose showed a HR of  $92.7\pm 28.6$  BPM while those treated with high glucose+ S1P had a HR of  $179.7\pm 18.2$  BPM.

### ***Effect of high glucose + S1P on left ventricular developed pressure (LVDP)***

Addition of S1P to glucose did not alter the LVDP at recovery. (Fig 7-3) In the stabilization phase, control hearts showed LVDP of  $83.1\pm 2.5$  mmHg while the high glucose+ S1P hearts had stabilization LVDP as  $95.0\pm 5.6$  mmHg. After 30' of recovery, control hearts had an LVDP of  $73.9\pm 18.7$  mmHg while the high glucose+

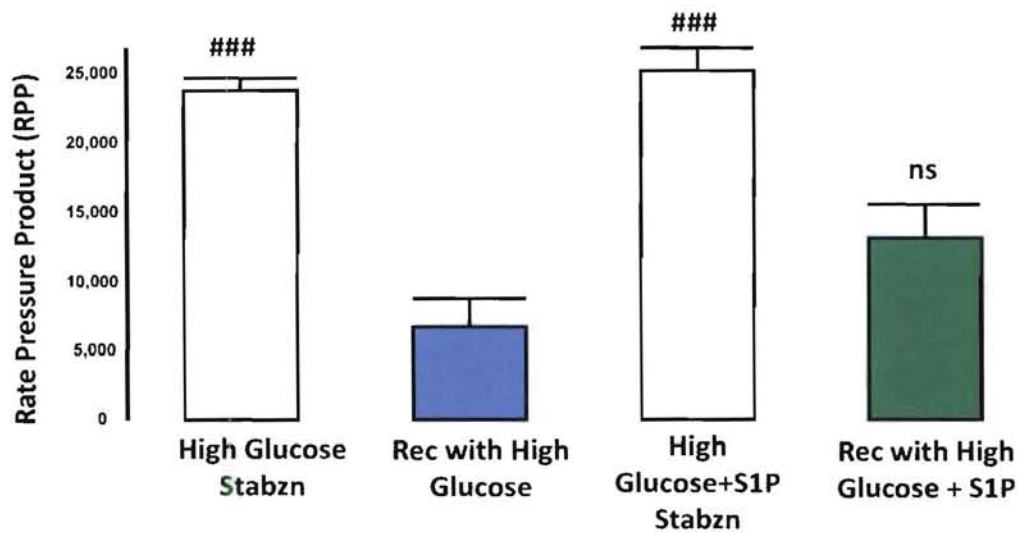
S1P hearts had a recovery LVDP of  $70.1 \pm 9.1$  mmHg. It was statistically insignificant between the groups.



**Figure 7-3:** Effect of glucose + S1P on LVDP. (Stabzn- Stabilization phase; S1P- sphingosine-1-phosphate; Rec: Recovery phase); ns- not significant vs. Rec with high glucose; Tukey- Kramer Multiple Comparison Test

**Effect of high glucose + S1P on rate pressure product (RPP)**

The stabilization values of RPP of control and HG+S1P hearts were  $23903 \pm 870$  and  $25272 \pm 1625$  respectively. (Fig 7-4) Treatment with high glucose (in the recovery phase) alone had a recovery RPP of  $6762 \pm 2084$  while the combination of S1P and high glucose had a recovery RPP of  $13153 \pm 2436$ . The stabilization phase values of both groups were very significant vs. their respective recovery groups ( $p < 0.001$ ). However, treatment with high glucose plus S1P had no significant improvement in the RPP of these hearts.



**Figure 7-4:** Effect of glucose + S1P on RPP. (Stabzn- Stabilization phase; S1P- sphingosine-1-phosphate; Rec: Recovery phase); ###  $p < 0.001$  vs. respective recovery groups; ns- not significant vs. Rec with high glucose; Tukey- Kramer Multiple Comparison Test

**Table 7-1:** Effect of high glucose+ S1P on systolic and diastolic pressures and coronary flow in model 2:

Systolic pressure (mmHg)		
	High glucose	High glucose + S1P
Stabilization	89.0±2.1	96.7±5.8
Recovery	85.8±18.8	77.8±9.8
Diastolic Pressure (mmHg)		
	High glucose	High glucose + S1P
Stabilization	5.9±1.6	1.6±1.2
Recovery	11.9±3.8	7.6±3.0
Coronary flow (ml)		
	High glucose	High glucose + S1P
Stabilization	9.1±0.2	8.8±0.3
Recovery	10.8±0.2**	10.5±0.3**

**Table 7-1:** \*\*-  $p < 0.01$  vs. Respective stabilization values

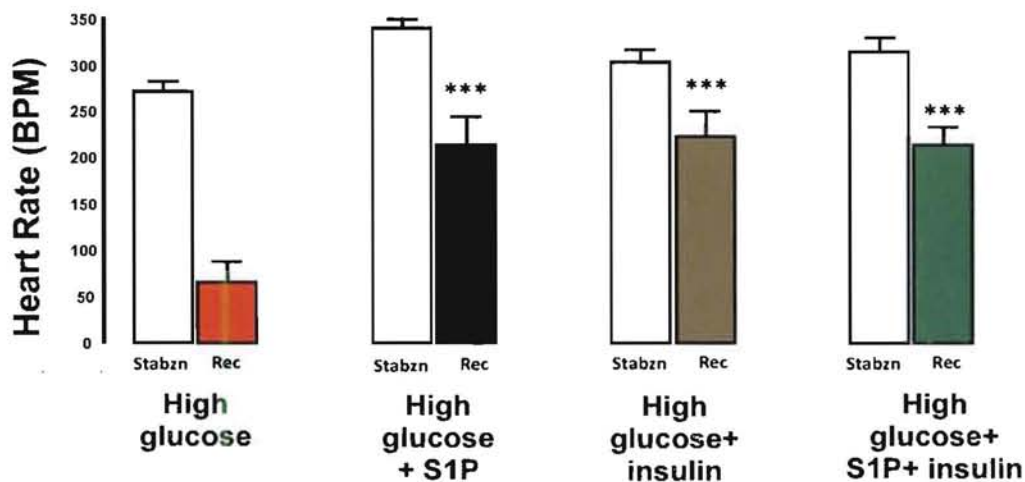
There were no statistical differences between groups in systolic and diastolic pressures. However, both groups had significantly ( $p < 0.01$ ) higher coronary flow rates in the recovery phase vs. respective stabilization phases. (Table 7-1)

### **Summary**

A combination of high glucose and sphingosine-1-phosphate improved the functional recovery of the hearts after an episode of severe *de novo* acute heart failure on our system. This recovery was only due to the improvement in the heart rate. There was no significant improvement in the contractility of the heart as showed by the insignificant improvement in the left ventricular developed pressure.

## **RESULTS**

***Effect of combination of metabolic+ pharmacological treatment on heart rate:***



**Figure 7-5:** Effect of glucose+ insulin + S1P on HR. (Stabzn- Stabilization phase; S1P- sphingosine-1-phosphate; Rec: Recovery phase); \*\*\*-  $p < 0.001$  vs. High glucose in recovery phase; Tukey- Kramer Multiple Comparison Test

Treatment with metabolic agents glucose and insulin as well as molecular agent S1P increased the heart rate significantly vs. hearts treated with glucose only. (Fig 7-5) Stabilization HR for high glucose (HG), HG+S1P, HG+ insulin and HG+S1P+insulin hearts was 272.2±10.5, 340.8±11.3, 303.4±14.1 and 314.8±17.0 BPM respectively;

HG plus insulin had a recovery HR of  $223.4 \pm 27.6$ ; HG+S1P had a recovery HR of  $214.5 \pm 30.7$ ; HG+S1P+Insulin had a recovery HR of  $214.4 \pm 19.2$ ; HG hearts had a recovery HR of  $66.5 \pm 22.6$  BPM.

**Effect of combination of metabolic+ pharmacological treatment on left ventricular developed pressure**



**Figure 7-6:** Effect of glucose+ insulin + S1P on LVDP. (Stabzn- Stabilization phase; S1P- sphingosine-1-phosphate; Rec: Recovery phase); \*\*\*-  $p < 0.001$  vs. all groups in recovery phase; Tukey- Kramer Multiple Comparison Test

The combination of metabolic and molecular agents had a significant improvement vs. all groups, displaying improved contractility of the hearts. (Fig 7-6)Stabilization LVDP values for HG, HG+S1P, HG+ insulin and HG+S1P+ insulin were  $83.2 \pm 1.2$ ,  $83.8 \pm 0.6$ ,  $86.3 \pm 1.2$  and  $91.6 \pm 2.4$  mmHg respectively. Treatment with HG+Insulin had a recovery LVDP of  $42.5 \pm 8.5$ ; HG+S1P had a recovery LVDP of  $66.5 \pm 2.2$ ; HG+S1P+Insulin treatment had a recovery LVDP of  $177.2 \pm 38.6$ mmHg which was significant over all other groups in the recovery phase ( $p < 0.001$ ); HG hearts however, had a recovery of  $20.5 \pm 8.3$  mmHg.

**Effect of combination of metabolic+ pharmacological treatment on rate pressure product comparison**



**Figure 7-7:** Effect of glucose+ insulin + S1P on RPP. (Stabzn- Stabilization phase; S1P- sphingosine-1-phosphate; Rec: Recovery phase); \*\*\*-  $p < 0.001$  vs. all groups in recovery phase; Tukey- Kramer Multiple Comparison Test

The improvement in heart rate and LV developed pressure significantly improved the rate pressure product ( $p < 0.001$ ) in the hearts treated with the combination of molecular and metabolic agents when compared against all groups. (Fig 7-7) Stabilization values of RPP were 22651±796 for HG, 28605±1139 for HG+S1P, 26167±2226 for HG+ insulin and 28880±1795 for HG+ S1P + insulin; In the recovery phase, treatment with HG+ insulin had a RPP of 9263±2226; and HG+S1P had a recovery RPP of 14003±1742; while HG+S1P+Insulin improved recovery to 40568±11054 which was significant over all other groups in the recovery phase ( $p < 0.001$ ) and HG hearts had a recovery RPP of 2064±984.

**Table 7-2:** Effect of combination of metabolic+ pharmacological treatment on systolic, diastolic pressures and coronary flow in model 2:

Systolic pressure (mmHg)				
	High glucose	High glucose + S1P	High glucose + insulin	High glucose + S1P + insulin
<b>Stabilization</b>	92.9±1.2	92.5±0.6	96.5±1.5	96.8±3.0
<b>Recovery</b>	69.3±11.0	97.3±4.6	99.1±2.6	251.7±39.0***
Diastolic Pressure (mmHg)				
	High glucose	High glucose + S1P	High glucose + insulin	High glucose + S1P + insulin
<b>Stabilization</b>	9.5±1.0	8.8±1.0	10.1±0.3	5.1±2.2
<b>Recovery</b>	48.8±13.5*	30.8±5.1	56.6±9.6**	74.5±13.6***/##
Coronary flow (ml)				
	High glucose	High glucose + S1P	High glucose + insulin	High glucose + S1P + insulin
<b>Stabilization</b>	9.1±0.3	9.4±0.2	8.9±0.3	9.1±0.4
<b>Recovery</b>	10.6±0.2	12±0.4**	10.5±0.5	10.6±0.3

**Table 7-2:** \*\*\*-  $p < 0.001$  vs. all recovery groups in systolic pressure; \*-  $p < 0.05$  vs. High glucose stabilization diastolic pressure; \*\*-  $p < 0.01$  vs. HG+insulin stabilization diastolic pressures; \*\*\*-  $p < 0.001$  vs. HG+insulin+ S1P stabilization diastolic pressure; ##  $p < 0.01$  vs. HG+ Insulin recovery diastolic pressure; \*\*-  $p < 0.01$  vs. HG+S1P stabilization coronary flow.

The combined treatment with pharmacological and metabolic agents significantly increased systolic pressure ( $p < 0.001$ ) in the recovery phase as compared to all other groups in the recovery phase. (Table 7-2) This treatment also increased diastolic pressures in the recovery groups when compared to HG+S1P alone. All diastolic pressure values in the recovery were significantly elevated compared to their

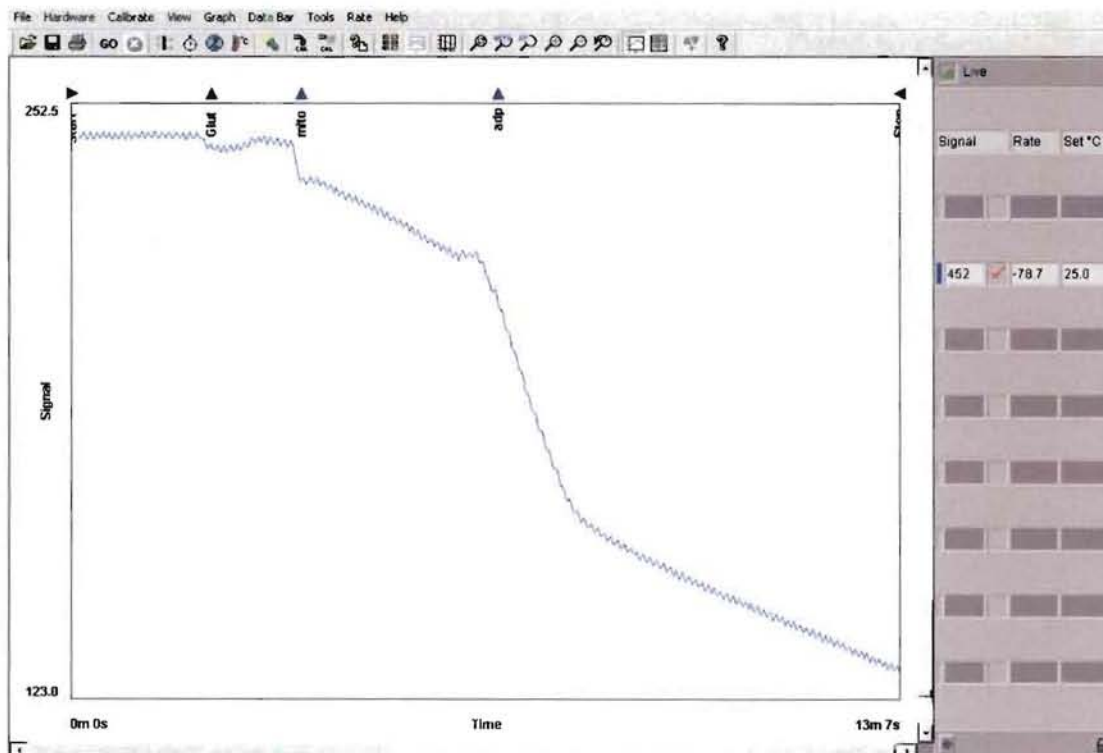
stabilization values ( $p < 0.05$  vs HG stabilization;  $p < 0.01$  vs. stabilization with HG+ insulin and  $p < 0.001$  vs. HG+ S1P+ insulin). Comparing the coronary flow, only HG+S1P in recovery phase had a significantly ( $p < 0.01$ ) higher flow as compared to its stabilization phase.

### **Summary**

The combination of metabolic and pharmacological agents not only improved heart rate, but LVDP also. The improvement in LVDP has not been seen in any of the other experiments where only either metabolic or pharmacological agents were used. Also, the decrease in LVEDP was seen when high glucose was combined with S1P. Hence, we conclude suggesting that a combination of metabolic and pharmacological therapy is essential in the treatment of AHF.

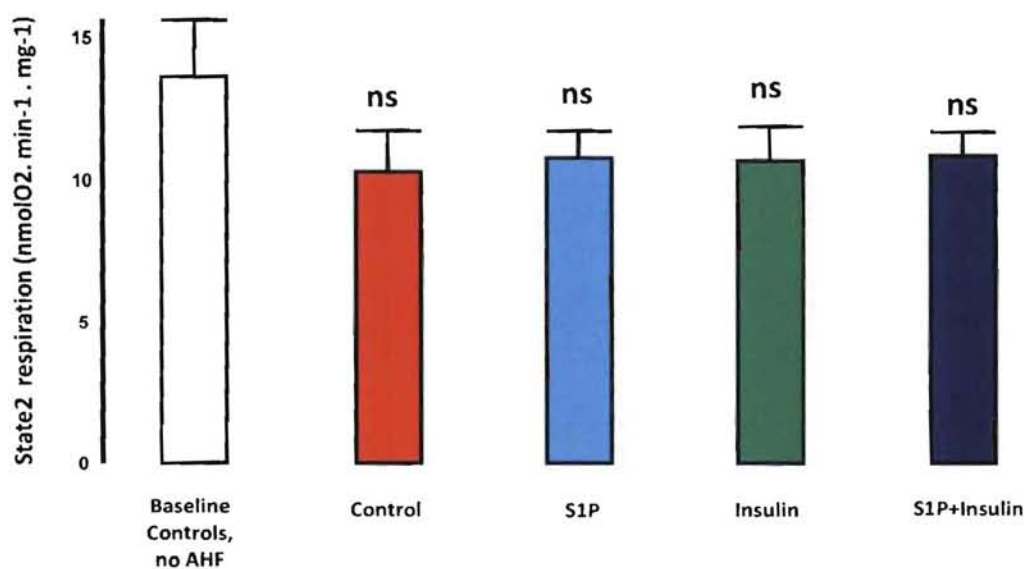
## **RESULTS**

### ***Mitochondrial respiration studies***



**Figure7-8:** Example of a trace on the Clarke type electrode measuring mitochondrial respiration

### State2 respiration comparison

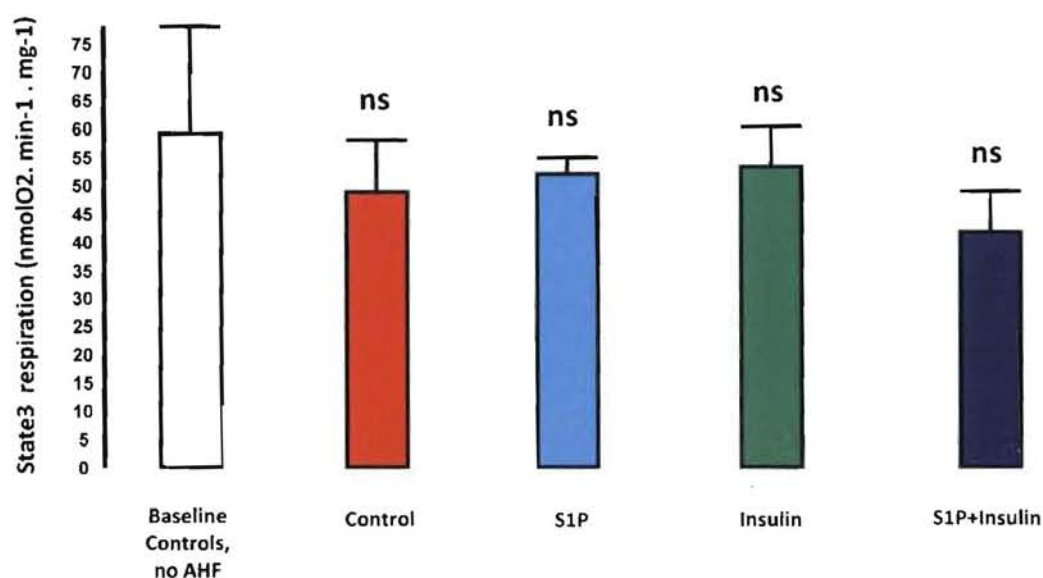


**Figure 7-9:** Effect of metabolic and pharmacological agents on State2 respiration; ns- not significant between groups and vs. baseline controls; Bonferroni Multiple Comparisons Test

Baseline controls (no AHF) showed a state2 respiration of  $13.6 \pm 1.9 \text{ nmolO}_2 \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ ; Control hearts (AHF) showed state2 respiration values of  $10.3 \pm 1.4 \text{ nmolO}_2 \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ ; hearts treated with S1P had a state2 respiration value of  $10.8 \pm 0.9 \text{ nmolO}_2 \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ ; hearts treated with Insulin had a state2 respiration value of  $10.7 \pm 1.2 \text{ nmolO}_2 \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ ; while hearts with HG+S1P+Insulin had a state2 respiration value of  $10.9 \pm 0.7 \text{ nmolO}_2 \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$

No significance amongst groups was observed suggesting that there was no change in the ability of the mitochondria to utilize substrate. (Fig 7-9)

### State3 respiration comparison



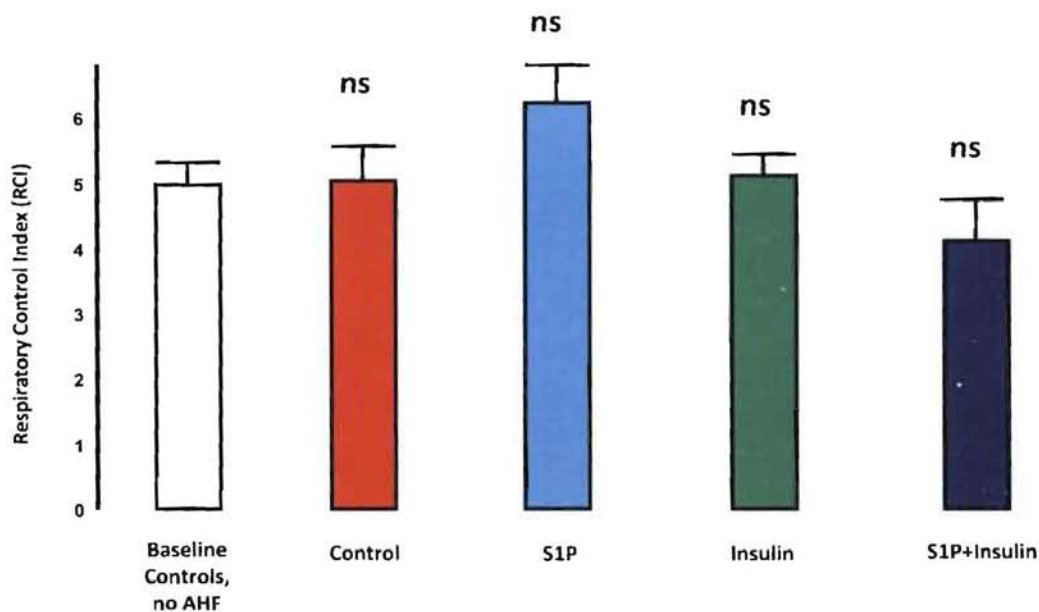
**Figure 7-10:** Effect of metabolic and pharmacological agents on State3 respiration; ns- not significant between groups and vs. baseline controls; Bonferroni Multiple Comparisons Test

Baseline controls (no AHF) had a state3 respiration value of  $59.1 \pm 18.9 \text{ nmolO}_2 \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ ; Controls (with AHF) had a state3 value of  $48.6 \pm 9.3 \text{ nmolO}_2 \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ ; hearts treated with S1P had a state3 respiration value of  $51.9 \pm 2.8 \text{ nmolO}_2 \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ ; Insulin treated hearts had a state3 respiration value of  $53.1 \pm 7.0 \text{ nmolO}_2 \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ ; After the S1P+Insulin treatment, the observed state3 respiration values were  $41.5 \pm 7.2 \text{ nmolO}_2 \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$

No statistical significance was observed between groups suggesting that the rate of conversation of ATP to ADP was insignificant between groups. (Fig 7-10)

### Respiratory control index (RCI) comparison

Baseline controls had an RCI of  $4.9 \pm 0.3 \text{ AU}$ ; controls with AHF had an RCI of  $5.0 \pm 0.5 \text{ AU}$ ; S1P treatment increased RCI to  $6.2 \pm 0.5 \text{ AU}$ ; Insulin treatment had an RCI of  $5.1 \pm 0.3 \text{ AU}$  and S1P+Insulin had an RCI of  $4.1 \pm 0.6 \text{ AU}$ . There was no statistical significance between various groups. (Fig 7-11)



**Figure 7-11:** Effect of metabolic and pharmacological agents on RCI; ns- not significant between groups and vs. baseline controls; Bonferroni Multiple Comparisons Test

## DISCUSSION

### 1. Outcome of functional recovery:

In our previous experiments, treatment with either metabolic or pharmacological agents displayed improved recovery only due to the increase of heart rate. The recovery in contractility in those experiments was very poor. The failing heart not only has a compromised heart rate, but also compromised contractility. In the previous experiments, treatment with glucose and insulin significantly reduced the diastolic pressure, indicating improved relaxation. The aim of experiments in this chapter was to examine if the combination of metabolic and molecular agents not only improve the heart rate, but the contractility also.

When S1P was combined with glucose, the effect was seen on only heart rate meaning that this effect was also mediated by the sinus node, as seen previously. However, when S1P was coupled with glucose and insulin, it improved HR as well as LVDP. We propose the following explanation for this effect:

S1P has a metabolic effect where it promotes the uptake of glucose by insulin. In experiments done in pancreatic beta cells, exposure to high glucose up-regulated the activity of sphingosine kinase which increased S1P levels and that stimulated the secretion of insulin.<sup>96</sup> When insulin and glucose were perfused in the recovery phase of our previous experiments, the resultant effect was observed on the heart rate only. However, with the addition of S1P to this combination of glucose and insulin, we propose that S1P promotes glucose metabolism by inhibiting fatty acid oxidation in these hearts.

Another explanation of this increase of LVDP might be the effect of insulin as a pharmacological agent. Insulin is one of the activators of the SAFE pathway and promotes survival. We propose that the combination of S1P and insulin also stimulates the SAFE pathway and the phosphorylation of STAT3 takes place in the nucleus which elucidates protection. These experiments possibly display interplay of activities of S1P and insulin, but more work is needed to confirm this hypothesis.

## ***2. Effect of metabolic and pharmacological therapy on mitochondrial respiration:***

The combination of these agents had no effect on the mitochondrial respiration when hearts were harvested from the Langendorff apparatus at 7' into the recovery phase. We used this time point to extract mitochondria because at 7' Somers *et al*<sup>102</sup> saw elevated phosphorylation of STAT3. We failed to see any change in the phosphorylated STAT3 contents when treated with S1P and similarly, when a combination was tested, we did not see a difference in mitochondrial respiration. This also indicates that the protective pathway involved in AHF is the SAFE pathway but this does not involve the mitochondria to exert its effect. This pathway is mediated via the nucleus since we observed the elevated levels of phosphorylated STAT3 in the nuclear fraction at 7'.

## **CONCLUSION**

The combination of glucose and S1P administered in the recovery phase of model 2 (AHF with adrenaline) increased the heart rate but not the contractility. However, the combination of S1P, insulin and glucose administered in the recovery phase (of model 2) not only increased heart rate, but also LV developed pressure.

The combination of metabolic and pharmacological agents did not have a statistically significant effect on the mitochondrial respiration. The individual agents also had no effect on the recovery of mitochondrial function.

# *Chapter 8*

## **CONCLUSIONS,**

## **LIMITATIONS and**

## **FUTURE WORK**

The experiments in this thesis describe data obtained with a new inexpensive isolated heart model of *de novo* acute heart failure (AHF). This model is easily reproducible, simple to perform and allows us to study the effect of acute heart failure (AHF) on the heart in isolation. To our knowledge, this is the first *ex-vivo* model of AHF using isolated rat hearts perfused in a modified Langendorff retrograde system. We tested both the metabolic agents glucose and insulin and the molecular agent sphingosine-1-phosphate (S1P) as therapeutic agents on two different experimental groups, all with AHF.

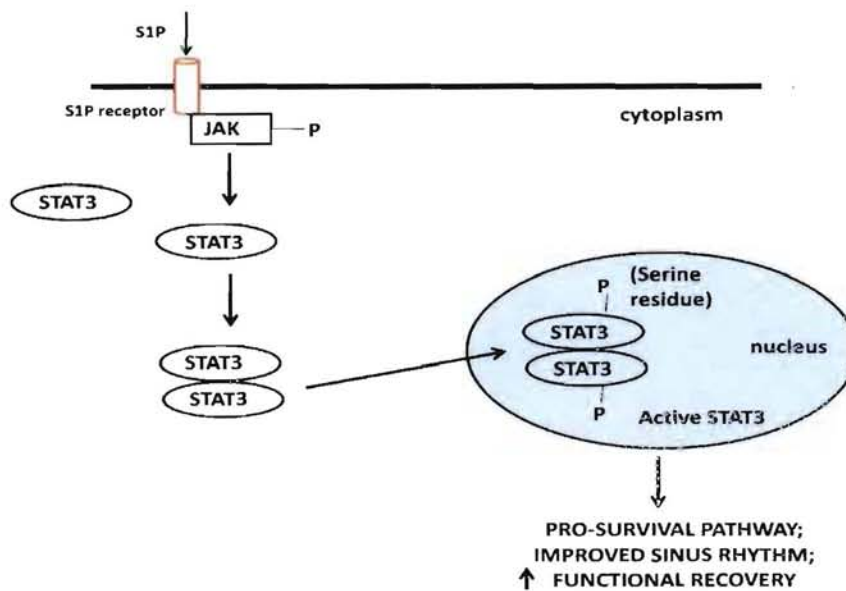
- Model 1 where AHF was induced by hypotension and substrate changes only, no adrenaline was added and
- Model 2 where AHF was characterized by substrate changes along with adrenaline and reduced perfusion pressure.

The two models were used to determine functional recovery and cell death by apoptosis by measuring the expression of cytochrome C. S1P is cardioprotective in ischaemic-reperfusion injury and reduces infarct size.<sup>74,84,102,139,140</sup> Provision of glucose is protective to the metabolically threatened cell because glucose generates membrane protective ATP both via glycolysis and Krebs cycle activity<sup>141</sup> that counters stress situations such as AHF.

In model 1, glucose in the recovery phase improved heart rate and reduced diastolic pressure, hence improving recovery. S1P administered in the AHF phase also increased heart rate but not the contractility.

In model 2, addition of adrenaline, which mimics the adrenergic stimulus associated with AHF, abrogated the effect of glucose alone. However, heart rate increased and diastolic pressure was reduced when insulin was added in combination with glucose. In other experiments, provision of glucose in the AHF phase with adrenaline also improved recovery. There was no effect on expression of cytochrome C after treatment with glucose and insulin.

Treatment with S1P in the recovery phase of model 2 also increased heart rate but had no effect on contractility. This improvement in function by S1P is mediated via the survivor activating factor enhancement (SAFE) pathway which involves the activation of signal transducer and activator of transcription factor 3 (STAT3) (Fig 8-1). STAT3 phosphorylation was significantly up-regulated in the nucleus. Treatment with S1P also significantly reduced the expression of cytochrome C which is a marker for apoptosis.

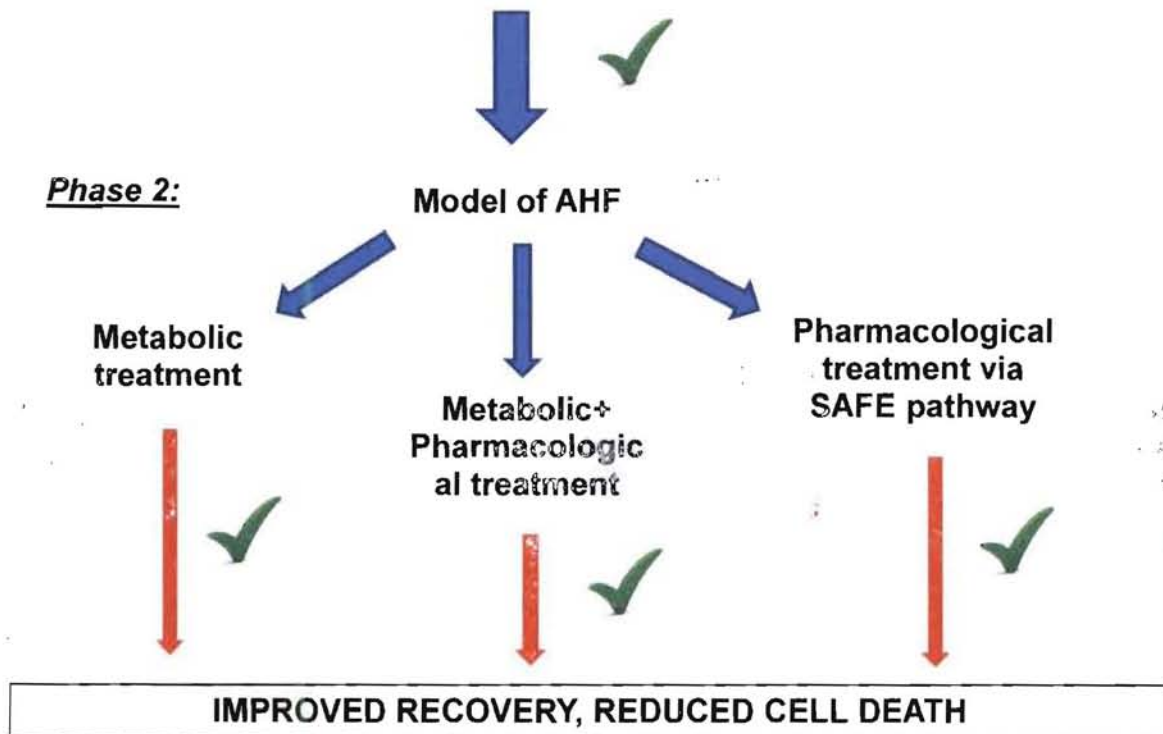


**Figure 8-1:** Schematic of the SAFE pathway (adapted from Lecour S)<sup>48</sup>. SAFE pathway is activated by S1P receptors which in turn activate the Janus kinases which on phosphorylation activate signal transducers and activators of transcription 3 (STAT3) molecules. The STAT3 molecules dimerize and translocate to the nucleus thereby activating the pro-survival pathway. STAT3 belongs to a family of transcription factors which are capable of forming homo and heterodimers which influence gene transcription in the nucleus. (SAFE- Survivor Activating Factor Enhancement pathway; JAK- Janus kinase; STAT3- signal transducer and activator of transcription 3; TNF- tumor necrosis factor)

When both pharmacological and metabolic agents were used in combination, glucose+insulin+ S1P not only increased heart rate, but contractility also. There was, however, no change in mitochondrial respiration. The expression of phosphorylated STAT3 was elevated significantly in the nucleus, suggesting that the SAFE pathway is involved and exerts its protective effects via the nucleus. The increase in heart rate in all our experiments with pharmacological and metabolic agents suggests that these agents work on the sinus node and improve sinus rhythm, hence improving recovery.

**Phase 1:**

**Validation of an acute heart failure (AHF) model using isolated rat hearts**



**Figure 8-2:** Treatment with either metabolic or pharmacological or a combination of the two improves recovery after AHF

### **Limitations of this study**

The first and most important limitation of our study is the use of isolated hearts. Despite the advantages of isolated heart preparations, the separation of hearts from the body is non-physiological. Heart failure is a systemic disease and a failing heart is the outcome of multiple factors. Isolation of the heart does not do justice to the effect of other systems in the body on the heart or vice-versa. The function of an isolated heart diminishes over time with an almost 5-10% per hour reduction of contractile function and heart rate.<sup>142</sup> Studies within these time points help generate useful data but the limitation of time is nonetheless a limitation with this technique.

The second limitation of our study is the use of non-physiological levels of substrates in our perfusate. Glucose at 2.5mmol/L is not clinically physiological and fatty acids at elevated in high adrenergic situations are pathological. Despite being an excellent substitute for blood in isolated heart system, one of the major limitations of the Krebs- Henseleit buffer is reduced oxygen carrying capacity in comparison to blood. The maintenance of temperature in the normal perfused heart is not difficult, but in conditions like reduced pressure, it drops rapidly and needs constant monitoring to maintain the system at 37°C.

Another limitation of the study is the applicability of our model in clinical situations. Hypotensive acute heart failure is indicative of Takotsubo cardiomyopathy and is very rare. Most acute heart failure is caused due to the worsening of chronic heart failure and our model focuses on a very specialized hypotensive condition.

### **S1P and AHF – future work**

The activity of S1P is mediated via its receptors.<sup>17,143</sup> Future work shall involve the delineation of the specific S1P receptors and their functions in an AHF setting. S1P receptor 3 is mainly involved in the regulation of heart rate, and since the improvement in our hearts is mainly in the heart rates, we strongly speculate the involvement of S1P receptor 3 in AHF. Besides regulating heart rate, S1P exerts its effects in various other cardiovascular entities like vascular tone and resistance, inflammation and cell survival in other models of cell death.<sup>88</sup> S1P is a component of high density lipoprotein (HDL) cholesterol and is thought to contribute to the

cardioprotective effect of HDL.<sup>87,144,145</sup> We would like to measure plasma HDL levels in patients with acute heart failure and measure the S1P content in these molecules.

Our experiments with S1P demonstrate three interesting outcomes:

- S1P improves functional recovery of the acutely failing heart by activating the SAFE pathway.
- The cytoprotective role of S1P against apoptosis is via an unknown mechanism which merits further work.
- Combination of S1P with the metabolic agents not only increased the heart rate, but the contractility also.

S1P is an enigmatic molecule. Despite the fact that S1P activates the SAFE pathway to elucidate cardioprotection in a heart subjected to ischaemic-reperfusion injury, S1P also acts as an agent facilitating a crosstalk between SAFE and RISK paths.<sup>102</sup> The mechanism of this cardioprotection is by phosphorylation of STAT3 which translocates to the nucleus and to the mitochondria as well and reduces cell death by reducing infarct size.<sup>102</sup>

In our model of *de novo* AHF, when S1P was administered in the AHF phase in absence of adrenaline, it was protective by increasing the heart rate. This protection was abrogated when adrenaline was added in the AHF phase. However, S1P in recovery phase improved heart rate despite the presence of adrenaline. This activity of S1P was mediated by the SAFE as shown by the increased levels of phosphorylated STAT3 in the nucleus, but not in the mitochondrial fraction. Reduction of cytochrome C by S1P treatment was independent of SAFE pathway in our experiments.

### **Proposed mechanisms for activity of S1P**

These data indicate more than one mechanisms involved in protection by S1P. One possible mechanism is that S1P acts as an intracellular agent to reduce the cell death. Ceramide and S1P belong to the same family, even though their cellular functions are opposite. While ceramide promotes apoptosis, S1P is an anti-

apoptotic.<sup>85,88</sup> This delicate balance of ceramide and S1P acts as a rheostat and increased concentrations of intracellular S1P promote cell survival by its action on mobilization of intracellular calcium and activation of phospholipase D.<sup>146</sup> We suspect that in an acutely failing heart, S1P exerts its anti-apoptotic effects as an intracellular messenger. This hypothesis requires further investigation.

The findings of this thesis also suggest a possible metabolic effect of S1P when used in combination with glucose and insulin. S1P is shown to be responsible for the regulation of glucose stimulated insulin secretion in the pancreatic cells. However, the effects of S1P and GI have never been tested in the heart. In the pancreatic cells, high concentration of glucose stimulates the activity of sphingosine kinase, thus, producing S1P.<sup>99</sup> In our model, when administered individually, S1P, glucose and insulin only improved the heart rate, thus suggesting that they all work on the sinus node. However, when given together, they not only increased the heart rate, but LV developed pressure as well. We propose that S1P facilitates the uptake of glucose in presence of insulin and is energy efficient and hence the contractility is improved. This promising hypothesis requires more work.

### **Future work with metabolic agents**

As Thorstein Veblen<sup>1</sup> said, “The outcome of any serious research can only be to make two questions grow where only one grew before.” We started this project with the idea of setting up and validating a model of acute heart failure but we ended up with not just a model, but information about agents involved in the recovery of AHF and their possible mechanisms as well. Our experiments examined the effect of metabolic and pharmacological agents on recovery against AHF and opened the field to many new questions and fascinating opportunities.

Glucose and insulin improved recovery by elevating the heart rate in our acutely failing hearts. The first aim of our future work will be to examine the sinus node in AHF and test the effect of glucose and insulin on recovery. Insulin acts via the SAFE pathway to provide protection<sup>147</sup>; we are keen to see if the metabolic pathways also activate the SAFE pathway. In order to measure the uptake of glucose and fatty

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<sup>1</sup> TV: 1857-1929. American economist and Sociologist

acids in AHF, we shall also measure rates of glycolysis and fatty acid oxidation in the AHF phase, both in the presence and absence of adrenaline.

We used hypotension and extreme substrate changes with an adrenergic stimulus to induce acute heart failure, but very few patients present with such harsh complications. In sub-Saharan Africa, hypertension is the leading cause of acute heart failure and is independent of gender.<sup>36</sup> The future work should involve establishing and validating two models of hypertensive AHF. First, using the isolated hearts, where AHF is induced by acute hypertension, we will raise the height of the perfusion column to 170 cm H<sub>2</sub>O. This shall be coupled with changes in substrates as described in this thesis and the addition of adrenaline. The effect of increasing the height of the perfusion column that induced failure has been studied by Opie<sup>72</sup> in 1965, they found that above a perfusion pressure of 180mmHg, isolated hearts went into irreversible failure. The second model shall be *in vivo* to study the effects on the entire organism. To validate an *in vivo* model, we propose to utilize spontaneously hypertensive rats that we shall monitor by echocardiography to confirm chronic heart failure and then subject these hearts to AHF on the Langendorff system. Or we could use haemorrhage as a model to induce hypotension to test the systemic effects of hypotensive AHF in the whole organism. The measurement of biomarkers like B-type natriuretic peptide (BNP), pro-BNP, tumor necrosis factor (TNF)  $\alpha$ , interleukin-6 (IL-6), troponin etc will also help us to better understand the progression of the disease.

### **Ketones as important fuels (and a possible therapy) against AHF**

Ketones, like amino acids, are substrates for the heart, but their utilization to generate ATP is very low due to their low concentration in blood. In a chronically failing heart, concentration of blood ketones is increased and this elevation is proportional to the severity of dysfunction and neurohormonal activation.<sup>37</sup> Ketones work by inhibiting the oxidation of both glucose and fatty acids and the processes involved in this activity are not very well understood. The suppression of fatty acid oxidation by ketones appears to be independent of malonyl Co-A activity, which normally binds to carnitinepalmitoyltransferase1 to inhibit fatty acid oxidation.<sup>53,56</sup> In working hearts, addition of ketone bodies to glucose increased the efficiency by 36% and increased acetyl CoA almost 18-fold.<sup>148</sup> Ketones also minimally reduce the

oxygen consumption and are therefore good substrates.<sup>29</sup> Ketones are the most efficient fuels in adverse conditions, based on the ATP generated per molecule of oxygen consumed.<sup>29</sup> In working rat hearts, ketones perfused with glucose showed no difference in cardiac function when compared to glucose alone; however, the rate of glucose oxidation was reduced.<sup>18</sup> There are no data that examine the effect of ketones as substrates in an acutely failing heart. We propose that in AHF where glucose metabolism is suppressed by elevated catecholamines and fatty acids, an addition of ketones to glucose and insulin will increase functional recovery by suppressing fatty acids.

In advanced CHF, hearts have impaired substrate uptake to generate ATP.<sup>149,150</sup> Ketone metabolism is preserved in the myocardium while it is diminished in the skeletal muscle.<sup>150</sup> Given the efficiency of ketones to generate ATP, we propose that administration of ketones in an acutely failing heart (*de novo* or acute decompensated) could be a promising novel therapy in the emergency room to salvage the myocardium.

### **SUMMARY OF EXPERIMENTS AND FUTURE WORK**

**Table 8-1:** Summary of outcomes of experiments and future work

	<b>Possible treatments</b>	<b>Outcome of treatment</b>
<b>Acute heart failure (AHF)</b>	Glucose-Insulin (GI) infusion	Heart rate (HR) improvement, reduction of LVEDP, energy efficient fuel, reduces damage caused by catecholamines
	Sphingosine-1-phosphate (S1P)	HR improvement by possible stimulation of S1P receptor 3, reduces apoptosis
	Combination of GI+S1P	HR improvement, improves contractility by increasing LV developed pressure

	Ketones	More efficient in generation of ATP than glucose or fatty acids, future work
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(HR- heart rate; LVEDP- left ventricular end diastolic pressure; S1P- sphingosine-1-phosphate; ATP- adenosine triphosphate)

### **Future potential benefits of glucose, insulin and S1P on AHF**

AHF is the most common cause of cardiovascular events leading to hospitalization in the elderly.<sup>23,43</sup> AHF can either be the exacerbation of chronic heart failure or may be *de novo*. However, the most common cause of AHF admissions is the worsening of CHF.<sup>19,39</sup> Monitoring of AHF clinically can be broadly divided into three phases: immediate treatment of symptoms and stabilization of patient, in-hospital management and post-discharge period where the risk of re-hospitalization is very high.<sup>151</sup> AHF is a clinically complex disease and despite the numerous trials,<sup>66</sup> the outcome for patients appears dismal. A subtype of *de novo* AHF, Takotsubo cardiomyopathy which is also called stress cardiomyopathy or “broken heart syndrome” was first described in Japan, but has grown since and many cases are now reported globally.<sup>45,46</sup> This is characterized by acute transient left ventricular failure caused by acute emotional stress.<sup>46</sup> One of the major reasons of the lack of new therapies and treatments is the lack of basic research coupled with the uncertainty regarding the metabolism and physiological pathways involved in the progression and recovery of AHF, mostly in *de novo* AHF.

The understanding of *de novo* AHF and the metabolism involved may be explained using a hypothetical example of an early humanoid hunter in the plains of Africa who had to endure many stressful circumstances including extreme physical exercise, high adrenergic stress and often, loss of blood. The extreme surroundings may have led to the development of protective metabolic pathways to counter the adverse effects of catecholamines and fatty acids.<sup>6</sup> AHF similarly is a stress situation which would ideally elevate the levels of catecholamines which in turn are deleterious to the heart as they promote the up-regulation of fatty acid oxidation. Our model of *de novo* AHF follows this example and we induced AHF by lowering the perfusion pressure and increasing FFA in the perfusate while simultaneously lowering the

glucose concentration. We explore specific conditions which we believe are imperative in the metabolism for progression and treatment of the disease.

The metabolic experiments described in this thesis strongly point to the cardioprotective effects of administration of glucose-insulin (GI). The addition of adrenaline in the AHF phase of our experiments had deleterious effects on the recovery and can correlate with a possible catecholamine spike in patients despite the lack of actual measurements in the clinical setting. AHF, whether *de novo* or acute decompensation of CHF is a stress stimulus, should ideally elevate the blood concentration of catecholamines leading to the up-regulation of fatty acid oxidation. Based on the experiments in this thesis, the addition of GI might be protective to the acutely failing heart. In Takotsubo cardiomyopathy which is a sub-type of AHF, catecholamines are known to be supra-physiological which is believed to cause acute dilation of the left ventricle.<sup>46,152</sup> The mechanisms leading to this dilation are still unknown however, several hypotheses have been suggested,<sup>45</sup> the most promising of them being the myocardial stunning as a direct effect of sympathetic activity, causing a calcium overload and leading to contraction band necrosis.<sup>41</sup>

### **Reasons why metabolic therapy could work**

Visualizing AHF as metabolic abnormalities where impaired substrate utilization in presence of increased sympathetic drive may help us design new therapies and markers to predict outcomes in patients. J Cohn in 1984 clearly showed that plasma concentration of epinephrine was a better marker of prognosis in patients of CHF.<sup>153</sup> Elevated catecholamines promoting uptake of FFA in CHF prompted to the discovery of various drugs like trimetazidine which partially shift the metabolism from fatty acids to glucose in a failing heart to improve ATP production.<sup>27</sup> Data from our studies point to the unfavorable effects of adrenaline which are partially countered by the addition of GI to the heart. Testing a drug like trimetazidine in the post discharge “high risk” period may be beneficial as it could improve the heart function by altering the metabolism, especially in patients with acute decompensated HF. However, more work needs to be done before such theories are tested in patients. Besides the ATP production, the infusion of GI also improves the activity of sinus node, as observed by Senges *et al* in 1982 where it was the glucose- insulin that improved the

sinus node activity and not potassium.<sup>127</sup> Data from our studies also show an improvement in sinus node activity by increasing heart rate in presence of glucose-insulin.

The possible effect of S1P in protection elucidated by GI makes it a potential metabolic mediator, besides being an anti-apoptotic molecule. Since S1P is a prime component of HDL, monitoring the HDL levels in patients with AHF might be a useful diagnostic measure for the clinicians.

Regarding potential application to patients, this thesis has successfully presented the first ever *de novo* AHF animal model and points to certain factors which may help in the clinical setting. Blood pressure, heart rate and monitoring in an Intensive Care Unit are already standard measures for patients with AHF. Intravenous catecholamine therapy with agents such as dobutamine, dopamine, and milrinone, is often used to support the failing left ventricle<sup>154</sup> but such agents may have side-effects such as oxygen-wastage that are linked to the catecholamine-elevated blood FFA levels. The data presented in this thesis suggest investigation of alternate modes of stimulating depressed sinus node activity in AHF,<sup>49</sup> such as the administration of intravenous glucose-insulin or S-1-P.

In summary, this PhD Project has used the well-established Langendorff perfusion system to study acute heart failure. It defines the basic principles of future molecular and metabolic approach which could potentially not only help in the better understanding of the disease mechanism but also in the development of novel treatments. This thesis has explored aspects of the metabolic and molecular therapy of acute heart failure using a simple reproducible isolated heart model, and the author is optimistic that future work will bring further success in this field.

# **PUBLICATIONS**

## **Papers:**

- 1) Deshpande G, McCarthy J, Mardikar H, Lecour S and Opie L. (2010) Effects of Sphingosine-1-Phosphate on acute contractile heart failure. *Cardiovasc Drugs Ther* 24:5, 459-460
- 2) Opie, Lionel H. , Lecour, Sandrine , Mardikar, Harshawardhan and Deshpande, Gaurang P.(2010) Cardiac survival strategies: an evolutionary hypothesis with rationale for metabolic therapy of acute heart failure', *Transactions of the Royal Society of South Africa*, 65: 3, 185 — 189

## **Conference Presentations**

- 1) SA Heart conference, Cape Town, South Africa:  
Title of poster presentation: *Glucose and insulin improve functional recovery after de novo acute heart failure by stimulating the sinus node.* Deshpande GP, Lecour S and Opie LH, SA heart journal 2013; (10) 393. February 2013.
- 2) SA Heart conference, Sun City, South Africa:  
Title of oral presentation: *Glucose and insulin: emerging metabolic therapy against acute heart failure (AHF).* Deshpande GP, Lecour S and Opie LH. SAHJ; 2012;9(3) 171. July 2012.
- 3) Society for heart and vascular metabolism (SHVM), Brussels, Belgium:  
Title of poster presentation: *Sphingosine-1-Phosphate (S-1-P) and glucose: A new therapeutic approach to acute heart failure (AHF)?* GP Deshpande, S Lecour, LH Opie. June 2011
- 4) SHVM Conference: Oxford, United Kingdom:  
Title of poster presentation: *Glucose and insulin: emerging metabolic therapy against acute heart failure (AHF).* Deshpande GP, Lecour S and Opie LH. August 2012.

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