Chapter 1 : Literature review

1.1. Anatomy of the Heart

The walls of the heart are composed of three layers comprising the endocardium, myocardium and epicardium. The myocardium contains the muscle cells or cardiomyocytes. The heart consists of four compartments: The right and left atria, right and left ventricles. The heart is oriented so that the anterior aspect is the right ventricle while the posterior aspect shows the left atrium (Williams, 1989). The atria form one unit and the ventricles another. This has special importance to the electrical function of the heart. The left ventricular free wall and the septum are much thicker than the right ventricular wall (Greenbaum *et al.*, 1981). This is logical since the left ventricle pumps blood to the systemic circulation, where the pressure is considerably higher than for the pulmonary circulation, which arises from right ventricular out-flows (Hall, 2010). The cardiac muscle fibres are oriented spirally and are divided into four groups: Two groups of fibres wind around the outside of both ventricles. Beneath these fibres a third group winds around both ventricles. Beneath these fibres a fourth group winds only around the left ventricle. The fact that cardiac muscle cells are oriented more tangentially than

radially, and that the resistivity of the muscle is lower in the direction of the fibre has importance in electrocardiography and magnetic echocardiography. The heart has four valves. Between the right atrium and ventricle lies the tricuspid valve, and between the left atrium and ventricle is the mitral valve. The pulmonary valve lies between the right ventricle and the pulmonary artery, while the aortic valve lies in the out-flow tract of the left ventricle (controlling flow to the aorta). The blood returns from the systemic circulation (Voss, 1989) to the right atrium and from there goes through the tricuspid valve to the right ventricle. It is ejected from the right ventricle through the pulmonary valve to the lungs. Oxygenated blood returns from the lungs to the left atrium and from there through the mitral valve to the left ventricle. Finally blood is pumped through the aortic valve to the aorta and the systemic circulation (Voss, 1989).

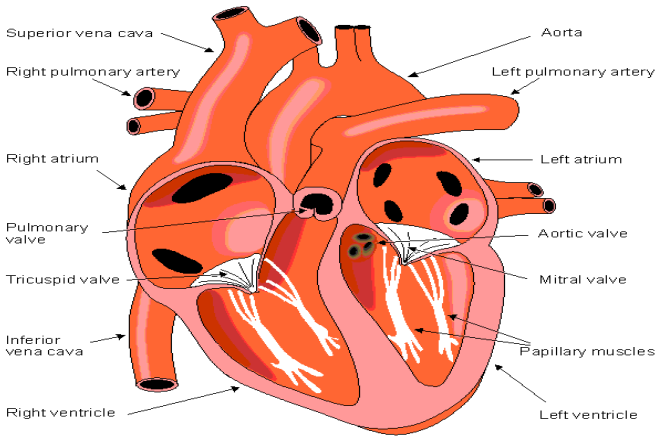


Figure 1: The anatomy of the heart and associated vessels.

1.2. Cardiovascular disease

Cardiovascular disease is the single largest cause of death by disease in Australia today (Health.A.S, 2010). Ischaemic heart disease is one of the major causes of morbidity and mortality in the world (Murray & Lopez, 1997). Because of accumulating morbidity in older people it is also the most expensive disease group in terms of direct health and care expenditure, coupled to the burden associated with disability and lowered quality of life (Health.A.S, 2010). Conventional strategies targeting a reduction in morbidity and mortality due to cardiovascular disease could be further improved by enhancing myocardial protection against oxidative stress particularly in the vulnerable aged heart (Epstein & Wei, 1992). Older patients are more likely to suffer from multiple comorbidities that increases the likelihood of myocardial damage. Advanced age also increases the risk of mitochondrial dysfunction due to reactive oxygen species [ROS] and this has become an active field in ageing research due to their possible participation in many degenerative processes (Riedel, 1998). The generation of ROS during aerobic metabolism constitutes a problem particularly in highly aerobic tissues like the heart (Apel & Hirt, 2004). Under normal conditions these ROS are effectively dealt with by endogenous antioxidants. It is only during Ischaemic reperfusion when the endogenous

antioxidants become overwhelmed that ROS can be toxic and damage the myocardial cells (Freeman & Crapo, 1982; Rao *et al.*, 1983). There are two sources contributing to ROS generation during ischaemia-reperfusion; xanthine oxidase and the mitochondrial respiratory chain. Such ROS are highly reactive and capable of damaging many biological macromolecules (Dhalla, 2000; Ide & Ichikawa, 2000; Slezak *et al.*, 1995).

1.3. Reactive Oxygen Species [ROS]

Reactive oxygen species (ROS) constitute a class of molecules derived from the metabolism of oxygen (O_2), and are characterized by high chemical reactivity. They include free radicals, such as superoxide ($O_2^{\bullet}$), superoxide anion radical ($O_2^{\bullet -}$), hydroxyl radicals ($OH^{\bullet}$), and non-radicals such as hydrogen peroxide (H_2O_2) (Bayr, 2005) and peroxynitrite (ONOO-). These ROS can damage other molecules and the cell structures of which they are a part. Among the most important of these are the actions of free radicals on the fatty acid side chains of lipids in the various membranes of the cell, especially mitochondrial membranes. Hydroxyl radicals remove a hydrogen atom in the fatty acid chain (Apel & Hirt, 2004).

1.4. The origins of oxidative stress in the heart

When the flow of blood to the heart is suddenly stopped (ischaemia) and then restarted (reperfusion), this can injure the myocardium (Manche *et al.*, 1995). The underlying pathogenesis involves calcium overload and oxidative stress. It is known that oxidative stress during ischaemia reperfusion arises from sudden changes in myocardial oxygen tension and that calcium overload is one consequence of disrupted ionic homeostasis (Mattson, 2002). Cardiac reperfusion injury occurs during various clinical procedures such as percutaneous coronary intervention, coronary artery bypass grafting, valve replacements and heart transplants where possible undesirable outcomes include reperfusion arrhythmias, microvascular damage, stunning, hibernation, necrotic and apoptotic cell death (Yao *et al.*, 2002). Cardiac reperfusion injury is associated with significant morbidity. There is therefore a pressing need to better understand the mechanisms involved in cardiac reperfusion injury and develop strategies to combat it.

1.5. Myocardial protection of the ageing heart

The main thrust of the myocardial protection studies presented in this thesis is to find a suitable method for protecting the older heart. This presents a challenge as shown in Figure 2 where increasing age with or without hypertrophy leads to deteriorating post-ischaemic performance. In addition powerful protective scenarios such as ischaemic preconditioning, is less effective in the post-infarcted-, failing- and/or aged- heart (Pantos *et al.*, 2007). Elderly patients are more likely to suffer from multiple comorbidities that increase the likelihood of myocardial damage (Jahangir *et al.,* 2007). Advanced age also increases the risk of mitochondrial dysfunction, impaired energetic reserve, elevated oxidant damage, and increased susceptibility to calcium overload (Boengler *et al.*, 2009; Boengler, 2007). Deaths caused by coronary heart disease occur in 74% of all Australian in the >75 compared to only 5% among those aged <55 (Health.A.S, 2010). This may be related to the increased vulnerability which we have seen in ageing normal and hypertrophic hearts (Figure 2). The seriousness of this problem is further highlighted by predictions that by the year 2025 >20% of the Australian population will be >60-years old (Jahangir *et al.*, 2007) compared to only about 13% aged >65 in 2006 (Health.A.S, 2010).

Thus the aim of the first study investigated in this thesis was to seek out a potential protective agent against ischaemia reperfusion in the older isolated and perfused rat heart. 36 week old rats were chosen to represent middle age in humans where cardiac problems frequently begin to make themselves known.

Figure 2 shows the decline in the recovery of the rate perfusion product (heart rate x left ventricular developed pressure) during reperfusion after global ischaemia of isolated perfused rat hearts. (Data for 4 and 9 month rats (Dr N King unpublished findings) and 18 months rats (Snoeckx *et al.*, 2001).

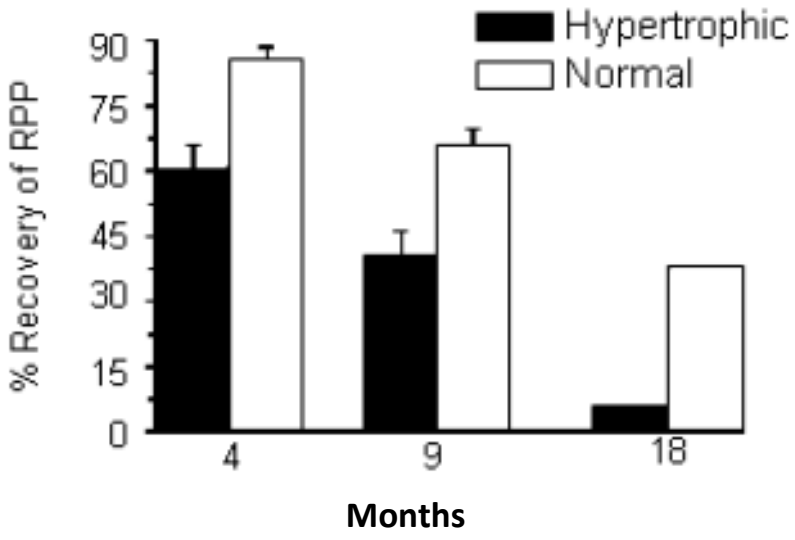
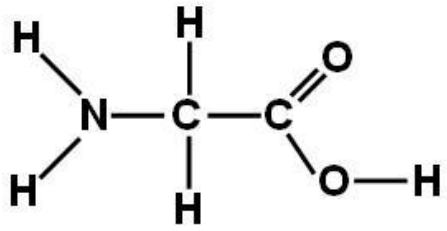


Figure 2: The recovery for rate pressure product.

1.6. Glycine

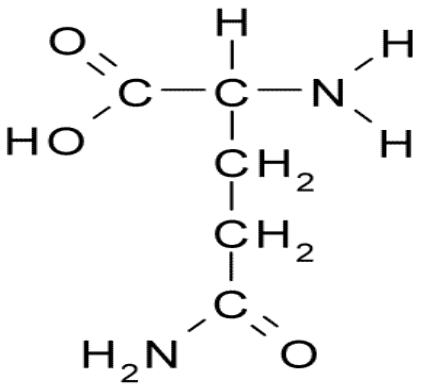
Glycine is the smallest of the 20 amino acids commonly found in proteins. Studies have recommended that application of glycine can protect tissues and organs in several pathophysiological circumstances (Gundersen *et al.,* 2005). For example, glycine attenuates ischemia/reperfusion injury to a variety of organs and tissues such as, heart, liver, skeletal muscle ,kidney, and intestine (Pan *et al.*, 2005; Weinberg *et al.*, 1997). Glycine protects against shock caused by endotoxin, sepsis and haemorrhage. So far its use has only a modest place in clinical medicine (Qi *et al.*, 2007).



Glycine chemical structure

1.7. Glutamine

Glutamine is a five-carbon amino acid. Glutamine is synthesised by the glutamine synthetase enzyme from ammonia and glutamate (Hall *et al.*, 1996). Glutamine is the most abundant free amino acid in several organs including the heart and the liver (Krebs *et al.*, 1949) and an important energy source in the gastrointestinal tract and the immune system (Melis *et al.*, 2004). Bolotin *et al* .(2007) found that glutamine restores myocardial function subsequent to ischaemia–reperfusion injury (Bolotin *et al.*, 2007) however, glutamine has low solubility and low thermostability and because of that is not administrated intravenously.



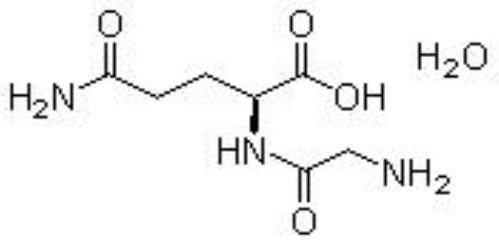
Glutamine chemical structure

1.8. Glycyl-Glutamine

The preceding discussion has outlined the cardioprotective potential of glycine and glutamine. Glycyl-glutamine (gly-gln), a dipeptide synthesised from glutamine and glycine, has higher solubility, and thermostability than either of the constituents, glutamine and glycine. This dipeptide can hydrolyse into glutamine and glycine after intravenous infusion and is extensively applied in parenteral nutrition as the transporter of glutamine (Fürst *et al.*, 1997). Gly-gln has been used to treat intestinal disease, and also affects growth performance and extensively reduces the extent of diarrhoea (Shu *et al.*, 2007; Song, 2004). Gly-gln is transported by the proton-dependent transporter the human peptide transporter 2 (PEPT2) which has been found in different organs including the heart (Lin & King, 2007).

One of the attractions of using gly-gln is that this dipeptide is readily transported by a family of proton-coupled oligopeptide transporters, comprising PEPT1, PEPT2, PEPT3 and PEPT4 (Daniel & Kottra, 2004). These transporters facilitate the proton and membrane potential dependent flux of all the possible 400 different di- and 8000 different tri- peptide combinations of the 20 proteinogenic amino acids as well as a broad range of peptidomimetic drugs, including several used in the fields of cardiac surgery and

cardiology (e.g. ACE inhibitors (Daniel & Kottra, 2004). The functional expression of PEPT2 has been demonstrated in the guinea-pig and rat hearts (Lin & King, 2007). This offers the potential for simultaneously harnessing the protective potential of glycine and glutamine by delivering them in the single dipeptide L-glycyl-L-glutamine. In spite of this there is a dearth of studies investigating the cardioprotective potential of gly-gln during ischaemia reperfusion.



Gly-gln chemical structure

1.9. Homocysteine

Cardiovascular disease is the largest specific cause of death in Australia and poses a significant burden to rural and regional communities (Health.A.S, 2010). Cardiovascular disease is also an important public-health challenge worldwide. Control, early detection and treatment of this condition should receive a high priority for research (Kearney *et al.*, 2005). An elevated blood level of homocysteine is a strong risk factor for cardiovascular

disease (Clarke *et al.*, 1991; Nygård *et al.*, 1997). Homocysteine is a thiol-containing, non–protein amino acid for which the normal plasma concentrations range from 5-15 μM. Hyperhomocysteinaemia (hHcy) arises when the plasma homocysteine concentration exceeds 15 μM (Levrand *et al.*, 2007) with an upper limit of over 400 μM such as occurs in the inherited condition, cystathionine β-synthase deficiency (Antoniades *et al.*, 2009). More modest increases in plasma homocysteine (15-100 μM) occur in approximately 5-7% of the general population and are recognised as a strong independent risk factor for coronary heart disease suggesting that elevated plasma homocysteine correlates with cardiovascular disease mortality, increased incidence of stroke, dementia, Alzheimer's disease, bone fracture, and a higher prevalence of chronic heart failure (Antoniades *et al.*, 2009; Levrand *et al.*, 2007).

The pathogenesis of hyperhomocysteinaemia is thought to involve increased thrombogenicity, greater oxidative stress, over-activation of redox-sensitive inflammatory pathways, impaired endothelial function, atherogenesis, cardiac remodelling and contractile dysfunction (Levrand *et al.*, 2007). It is somewhat surprising, therefore, that several large scale clinical trials targeting a reduction in plasma homocysteine concentration through vitamin supplementation have yielded unconvincing results,

although a recent meta-analysis suggests that the conflicting results may reflect differences between individual patients' anti-platelet therapies (Selhub, 2006). The majority of the evidence investigating the effects of homocysteine has been collected from vascular endothelial cells, smooth muscle cells and monocytes (Levrand *et al.*, 2007) with studies rarely concentrating on heart cells. Additionally, the small amount of research that has been published on the heart cells largely involved cultured cells (Mishra *et al.*, 2009; Sipkens *et al.*, 2007; Wald *et al.*, 2011; Wang *et al.*, 2012; Zhao *et al.*, 2006). Culturing is known to stimulate the expression of one of the major enzymes involved in homocysteine metabolism, namely cystathionine β-synthase (Sipkens *et al.*, 2007) and to induce changes in the electrical activity of heart cells (Mitcheson *et al.*, 1998). Homocysteine has also been implicated in augmented oxidative stress, particularly leading to a reduction in nitric oxide availability in cultured heart cells (Sipkens *et al.*, 2007; Wald *et al.*, 2011) and in isolated hearts from different experimental models of hyperhomocysteinaemia (Becker *et al.*, 2005; Suematsu *et al.*, 2007). Nitric oxide is central in enabling the coronary arteries to relax and drugs such as amyl nitrate that increase nitric oxide availability are in the frontline of treatment for angina. Oxidative stress is known to play a role in the pathogenesis associated with a

sudden ischaemia and subsequent reperfusion of the heart's blood flow with ischaemia being the cause of the pain in angina. Ischaemia reperfusion is encountered during clinical procedures for cardiovascular disease including percutaneous coronary intervention, coronary artery bypass grafting, cardiac valve replacement and heart transplants, where the undesirable consequences can include damaging changes to heart rate and rhythm, harmful alteration to blood flow and cell death (Suleiman *et al.*, 2011). The hypothesis here then can be stated thus: That modifications in the plasma concentration of homocysteine affects the health and vulnerability of freshly isolated heart cells, which in turn will implicate the risk of developing cardiovascular disease. Although the majority of the data suggests homocysteine may be damaging to heart cells, it is structurally related and involved in the same metabolic pathway as the cardioprotective agent cysteine (King, 2010; Shackebaei *et al.*, 2005).

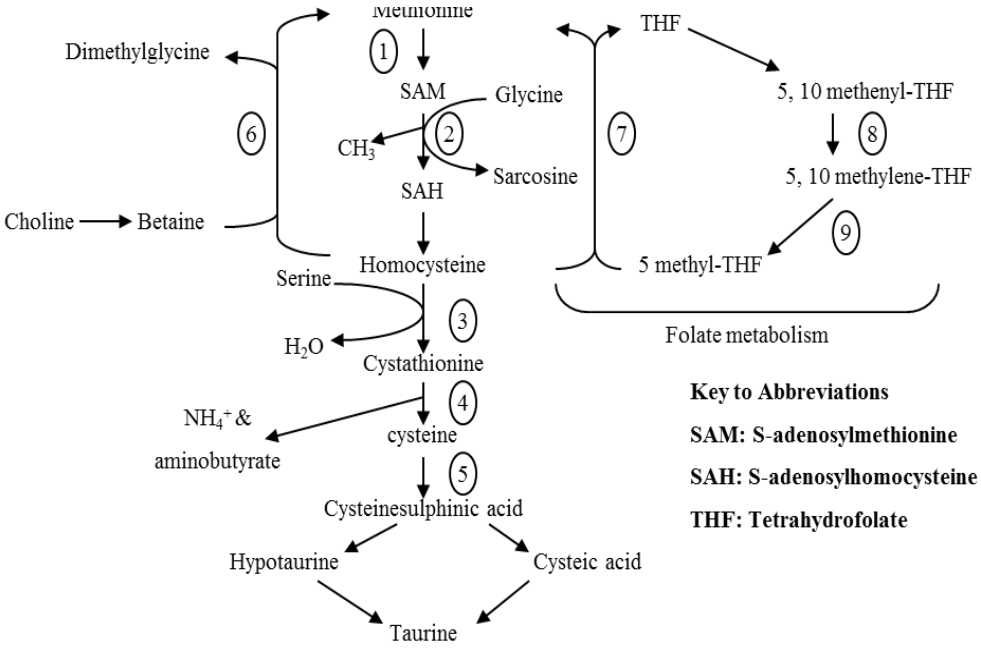
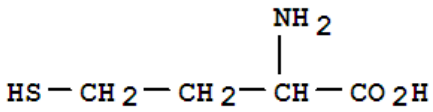


Figure 3: Metabolism of sulphur containing amino acids in heart showing the intersection of this pathway with folate and one carbon metabolism. Enzymes are numbered: 1) Methionine adenosyl-transferase; 2)Glycine N-methyltransferase; 3) Cystathionine β-synthase; 4) γ - Cystathionine synthase; 5)Cysteine sulphuric acid decarboxylase; 6) Betaine homocysteine methyltransferase; 7) Methionine synthase; 8) 5,10 methylene tetrehyrofolate dehydrogenase and 9) 5,10 methylene tetrehyrofolate reductase. Adapted from (Brosnan *et al.*, 2006).

Perfusion with cysteine during ischaemia reperfusion of isolated rat hearts led to a significant improvement in functional recovery compared to non-cysteine perfused control (Shackebaei *et al.*, 2005). This protective effect was associated with preservation of the major antioxidant glutathione (GSH). A similar effect was observed in isolated heart cells where cysteine incubation maintained the intracellular GSH concentration

during oxidative stress evoked by exposure to 0.2 mM H_2O_2 (King *et al.*, 2011). This was previously shown to be a good model of oxidative stress attended by plentiful production of hydroxyl radicals (King *et al.*, 2003). Therefore to investigate the effects of Hcy on isolated heart cells, the cells were exposed to 0.2 mM H_2O_2 in the presence/absence of Hcy. Another aim of these experiments was to evaluate the efficacy of 3 different approaches to cell isolation. These involve using normal Tyrode (King *et al.*, 2011) vs. Tyrode containing glutamate and carnitine (King *et al.*, 2003) vs. Tyrode containing the calcium buffering and antioxidant taurine (King & Suleiman, 1998). Therefore the aim of this second study in this thesis was to specifically investigate the effects of homocysteine on the functioning of freshly isolated heart cells, their response to oxidative stress and necrosis using isolated control cells and cells incubated in the presence of homocysteine, H_2O_2 and homocysteine+H_2O_2. Here we provide new insights as to how homocysteine affects the health and the vulnerability of isolated heart cells thereby adding to the knowledge of cardiovascular disease development.



Homocysteine chemical structure.

1.10. Isolated heart cells

Every single rat heart cell potentially provides a powerful tool for heart research. Over many years useful data for studies of myocardial function and metabolism has resulted from the isolated perfused rat heart. The finding of therapeutic approaches to the mitigation of human disease can be reached through research in heart cells, contributing to breakthroughs in basic science and informed treatments in the clinic with a variety of model systems, from isolated ventricular cardiomyocytes to the whole heart. These cells can offer suitable models for the pathophysiology of heart diseases in molecular detail and potentially allow for subsequent elucidation of pathogenic mechanisms (Mohler *et al.*, 2003; Salama & London, 2007). Nevertheless, the isolation of healthy cells is not easy and this imposes a significant impasse on their use in cellular studies of the heart. To overcome this problem we use the classic Langendorff technique that facilitates easy handling and the rapid aortic cannulation of the small rat heart (King *et al.*, 2003). The isolation of cardiomyocytes is characteristically reached through retrograde perfusion of hearts of a small rat with collagenase-containing solution in a Langendorff system (Bell *et al.*, 2011). The Langendorff system could be suitable for modelling cardiomyopathies such as hypertrophy/heart failure via aortic constriction. This technique offers the

capability of perfusing the heart with different buffers and enzyme solution to degrade collagen matrices that hold the cardiomyocytes in the myocardium and loosen the intercellular linkages. The duration of initial ethylene glycol-bis (2-aminoethylether)-*N, N, N′, N′*-tetraacetic acid (EGTA) perfusion affects cell quality. The complete removal of Ca^{2+} from the coronary artery was essential before the perfusion of enzymes to enhance subsequent cell separation. Enzymes, namely proteinase and collagenase were required to digest the tissue and enable single cell isolation. On the other hand if perfusion continued for too long the cells were paradoxically damaged by the Ca^{2+} free environment in EGTA and subsequently had an impaired Ca^{2+} tolerance (Daly *et al.*, 1987; Wolska & Solaro, 1996). With the system employed here it is possible to perfuse the heart without damage and without compromising the integrity of the myocardium during cardiomyocyte isolation. Competent cardiomyocytes that are tolerant to physiological levels of extracellular calcium with metabolic activity and normal morphology are desired. Due to these known difficulties three different approaches (Tyrode solution, Tyrode solution contain taurine and add glutamic acid + carnitine to the digested enzyme) to heart cell isolation were tested and assessed by the cell's subsequent ability to withstand oxidative stress.

Most of the evidence investigating the effects of homocysteine has been collected from vascular endothelial cells, monocytes and smooth muscle cells (Levrand *et al.*, 2007) with studies rarely concentrating on heart cells. Furthermore, the small amount of research that has been published on heart cells involved cultured cells (Mishra *et al.*, 2009; Wald *et al.*, 2011). The few studies involving freshly isolated heart cells have been limited to the investigation of the effect of homocysteine on excitation. In particular, a study showed that exposure of isolated heart cells to 50 μM homocysteine significantly reduced maximum transient outward current measured at +60mV compared to controls, which may have implications for repolarisation during the action potential (Shontz *et al.*, 2001). The mechanisms underlying these cardiac electrophysiological effects and their relevance to holistic cardiac function during an oxidative insult are unclear. Therefore the aim of the second series of experiments was to investigate the effect of homocysteine on cardiomyocyte viability and morphology in the presence or absence of oxidative stress.

Chapter 2: Proof of concept: Effect of glycyl-glutamine on ischaemia reperfusion of the young adult rat heart.

2.1. Abstract

The amino acids, glycine and glutamine have been implicated in myocardial protection against ischaemic reperfusion. This study investigated whether such protection could be enhanced by simultaneously delivering these amino acids as the dipeptide, L-glycyl-L-glutamine (gly-gln). Hearts from 6-8 week-old male Wistar rats were perfused in the Langendorff mode. For measurements of functional performance and reperfusion damage the perfusion protocol comprised 20 minutes baseline perfusion, 40 minutes global normothermic ischaemia and 40 minutes reperfusion. Where used gly-gln was added at 0.5 mM, 2 mM and 5 mM to the perfusate 10 minutes into baseline perfusion and was present throughout ischaemia being washed out 10 minutes into reperfusion. Reperfusion damage was assessed using the release of lactate dehydrogenase into the coronary effluent during reperfusion as a biomarker. Functional performance, recovery and reperfusion damage were unaltered in hearts perfused with 0.5 mM gly-gln. By contrast the presence of 5 mM gly-gln significantly improved the recovery of the rate pressure product (5527 ± 957 mm Hg/beat/min in control vs. 9755 ± 765 mm

Hg/beat/min in 5mM gly-gln [$P < 0.05$ vs. control]), lengthened the time to ischaemic contracture (22.4 ± 1.3 minutes in control vs. 29 ± 1.7 minutes in 2 mM gly-gln ($P < 0.05$) vs. 29.3 ± 1.6 minutes in 5 mM gly-gln ($P < 0.01$) and significantly reduced reperfusion damage (LDH area under the curve in control 104 ± 12.3 vs. 50.8 ± 10.6 in 2 mM gly-gln ($P < 0.01$) vs. 46.2 ± 10.6 ($P < 0.01$) in 5 mM gly-gln). These findings provide proof of concept to support the hypothesis that gly-gln is a cardioprotective agent against ischaemia reperfusion.

2.2. Introduction

Reperfusion of the ischaemic myocardium produces harmful reactive oxygen species such as hydrogen peroxide, superoxide and hydroxyl radical which damage the organ and tissues (Andreadou *et al.*, 2009; King *et al.*, 2010). Studies have shown that the amino acids, glutamine and glycine are implicated in protective effects on the myocardium during ischaemia reperfusion (Jiang *et al.*, 2011; Khogali *et al.*, 1998; Nadtochiy *et al.*, 2009) of the perfused rat heart. Glycyl-glutamine is a dipeptide composed of glycine and glutamine which has higher solubility, and thermostability than either of the constituents glutamine and glycine. This dipeptide is transported by the proton-dependent transporter PEPT2 which has been found in different organs including the heart (Lin & King, 2007). This transporter has the capability to simultaneously transport glutamine and glycine as a dipeptide gly-gln; however, it is not clear whether simultaneous application of gly-gln will result in increased defence. Consequently the aim of this study was to examine the cardioprotective potential of gly-gln during ischaemia reperfusion in the isolated and perfused rat heart.

2.3. Materials and method

2.3.1. Animals

Six to eight week-old male Wistar rats were used in these experiments (Central Animal House, University of New England, NSW, Australia 2351). These experiments were performed with the approval of the Animals Ethics Committee (AEC09/152 and EC10/036). Animals were fed standard rat chow and water *ad libitum.*

All chemicals including enzymes, and amino acids were purchased from Sigma, Australia (14 Anella Ave, Castle Hill NSW 2154). Rats were killed by stunning followed by cervical dislocation. Subsequently, the hearts were removed and processed for cannulation using the Langendorff technique.

2.3.2. Preparation of solutions

For experiments utilizing the isolated and perfused heart solutions: Krebs± additives were prepared in the following manner. Two litre solutions: 120 mM of NaCl, 25 mM $NaHCO_3$, 11 mM Glucose, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 KH_2PO_4 made up to volume with double distilled water. On the day of the experiment, 2.4 ml of $CaCl_2$ from the 1M stock were added to the two litres of Krebs solution. Sufficient L-glycyl-L-glutamine was added to two litres of calcium containing Krebs to make final concentration of 0.5 mM, similarly 2 mM and 5 mM solutions were made up in the same manner. These solutions were stored in a refrigerator or cold room for no longer than four days prior to use.

2.3.3. Priming the equipment for ischaemia and ischaemic reperfusion

The temperature of the water bath was set to 38.5 ℃ so the final temperature when the solution reaches the heart was 37 ℃. The tubing system was connected through the pump flow from each chamber through the tubing system and controlled by a three-way valve in the tubing system, the direction in which the valve points was switched off. We isolated the valve below the chamber. The valve of the second chamber could then be pointed towards the first chamber to turn it on. A drainage tube runs beneath the

equipment and into a sink. The contents of the first chamber could be drained by pointing the valve towards the pump. The content of the second chamber could be drained by turning off the first chamber and turning the valve of the second chamber towards the first chamber. The beaker that was placed underneath the cannula was used to catch the solution as it passes through the equipment (Figure 4). A crocodile clip was used to fix the aorta to the cannula. This secured the heart which was attached to the cannula. Rats were sacrificed by cervical dislocation and their hearts were removed and prepared. On one side of the sink there was the Perspex plate which was used as a table for the rat cervical dislocation and on the other side of the sink; two sheets of paper were placed to facilitate disposal of the rat carcass after the process. Long forceps and scissors were also placed to be used in dissection and excision of the whole heart. Excised hearts were immediately placed in cooled Krebs solution. The first chamber contains Krebs solution with no additives while the second chamber contains Krebs solutions with added compounds (gly-gln). To ensure adequate oxygen supply to the heart, the carbogen supply (contains 95% oxygen and 5 % CO_2) was turned on so as to pass through both chambers ensuring that all pipes and both chambers are filled with oxygen so as not to damage the heart muscle through hypoxia. Perfusion of nutrients to the heart muscle

proceeds at the rate of 11 ml/min. controlled by a perfusion pump. To protect the heart muscle from air embolism, the system should be free of any air bubbles and this was done by bleeding the tubing system before starting the experiment. The Langendorff system (Figure 4) was primed and ready to receive the heart. Hearts from 6-8 week-old male Wistar rats were perfused in the Langendorff mode. For measurements of functional performance and reperfusion damage the perfusion protocol comprised 20 minutes baseline perfusion, 40 minutes global normothermic ischaemia and 40 minutes reperfusion were used. Where gly-gln was added at 0.5, 2 and 5 mM to the perfusate 10 minutes into baseline perfusion, was present throughout ischaemia, and was washed out 10 minutes into reperfusion. Reperfusion damage was assessed using the release of lactate dehydrogenase (LDH) into the coronary effluent during reperfusion as biomarker. At the end of the experiment the heart was dismounted from the equipment cut open and left to air dry in the warm lab. Subsequently the dry weight of the heart was measured and used to calculate lactate dehydrogenase release per mg dry weight. The release of lactate dehydrogenase was measured using a kit from Sigma and was performed according to the manufacturer's instructions (in *vitro* toxicology assay kit, lactate dehydrogenase based, catalogue no. TOX7).

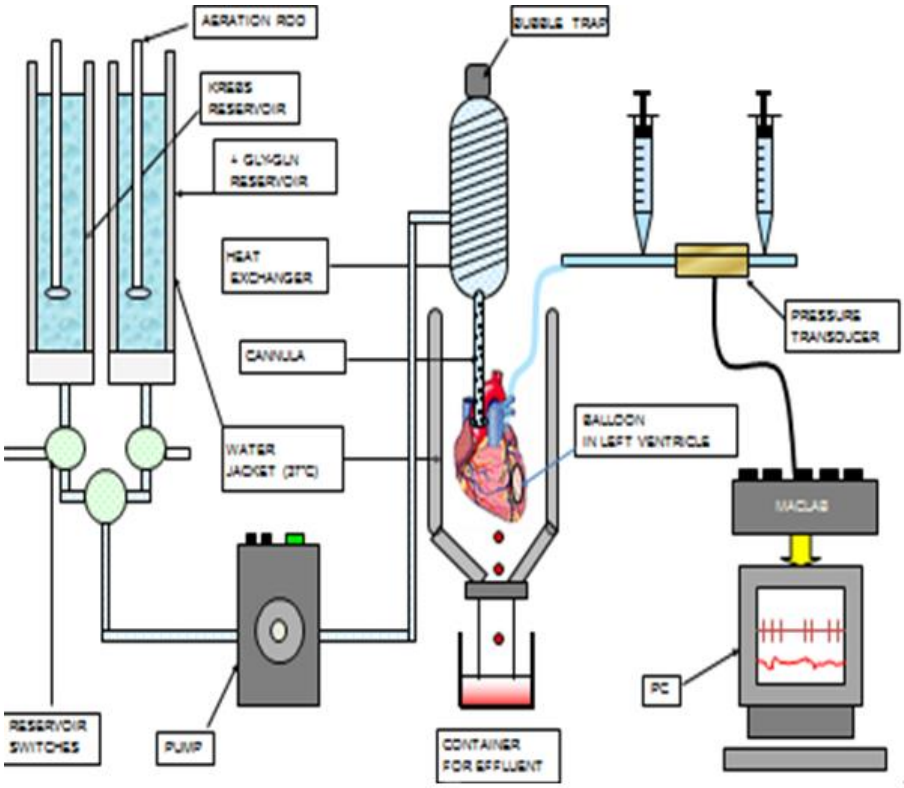


Figure 4: The Langendorff heart apparatus showing typical experimental set up.

A sample of the raw data obtained during a Langendorff experiment follows with some of the measures obtained. In this study measurement of the left ventricular developed pressure (LVDP), heart rate, rate pressure product (RPP) and time to ischaemic contracture were taken.

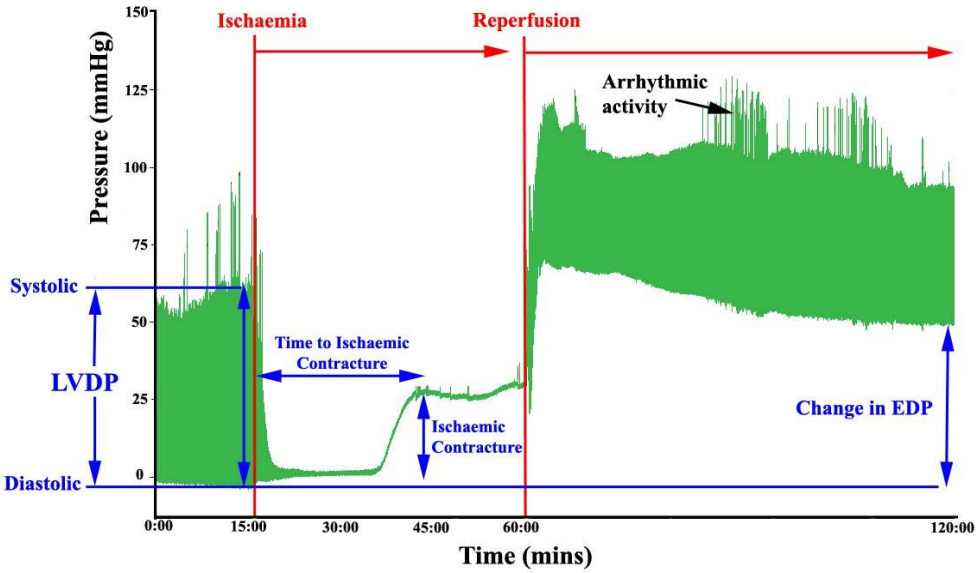


Figure 5: Representative left ventricular pressure trace from perfused heart.

Recordings were made at baseline (preischaemia), after 40 minute of regional ischaemia, and after 60 minute of reperfusion. Traces are compressed on the time (*X*) axis. The top and bottom boundaries of the green shaded area represent the systolic and diastolic pressures, respectively. The onset of ischaemia and reperfusion are indicated by arrows. Scale bars on each trace represent time. (*X*-axis) and mmHg (*y*-axis).

2.3.4. Cardiac functional parameters

Male Wistar rats ages 6-8 weeks were randomly assigned to one of 4 groups: 1) control, 2) + 0.5 mM gly-gln, 3) +2 mM gly-gln, or 4) + 5 mM gly-gln. The rats were humanely sacrificed, their heart quickly removed, cannulated and then perfused in the Langendorff mode (Figure 4) with oxygenated Krebs at 37 ºC as described previously. After 20 minutes equilibration, the rat hearts were exposed to 40 minutes global normothermic ischaemia followed by 30 minutes reperfusion. Where used, gly-gln was added to the perfusate 10 minutes before ischaemia and was then present until wash out after 10 minutes reperfusion.

Cardiac functional parameters (left ventricular developed pressure, LVDP; computer derived heart rate, HR; and rate pressure product, RPP (RPP=HR x LVDP) were measured using a water filled balloon inserted into the left ventricle and connected via a pressure transducer (mlt 844) to a PowerLab device (4/25). Reperfusion damage was evaluated using the release of lactate dehydrogenase (LDH) into timed collections of coronary effluent.

2.3.5. Data analysis

Data shown are means ± SE of n = 6-7. Statistical analysis was performed using ANOVA with a Tukey Post- test. using InStat Instant Biostatistics software version 3.0 (Graphpad software, La Jolla, CA, USA).

2.4. Results

2.4.1. Cardiac functional parameters

2.4.1.1. The effect of gly-gln on the left ventricular developed pressure.

Hearts from 6-8 week old rats were perfused in the Langendorff mode and exposed to global normothermic ischaemia and reperfusion in the presence of 0.5, 2 and 5 mM gly-gln. Figure 6 shows the left ventricular pressure measured at the end of baseline perfusion and at the end of reperfusion following ischaemia. No concentration of gly-gln affected the baseline LVDP compared to control. In contrast the presence of 5 mM gly-gln led to a significantly greater LVDP on reperfusion compared to control. Furthermore whilst ischaemia led to an approximately 50% reduction in LVDP in the controls, the percentage recovery of LVDP in the presence of 5 mM gly-gln was close to 100%.

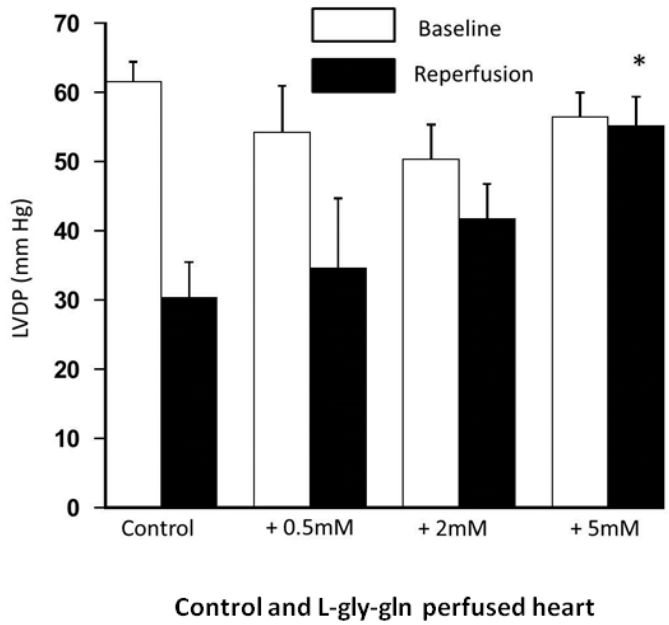


Figure 6 : Effect of gly-gln on the left ventricular developed pressure before and after ischaemia in young adult rat hearts perfused with or without (control) 0.5 mM, 2 mM or 5 mM gly-gln. * $P <$ 0.05 vs. reperfusion in control. Data shown are means ± SE of n=6-7.

2.4.1.2. The effect of gly-gln on heart rate

In addition to the left ventricular developed pressure, heart rate was measured in the same rat hearts at the end of baseline perfusion and the end of reperfusion. These results are shown in Figure 7. There were no significant differences between the baseline heart rate in controls versus the baseline heart rate in the presence of successive gly-gln concentrations. A similar picture was also apparent at the end of

reperfusion with no significant difference between the control and the successive gly-gln concentrations.

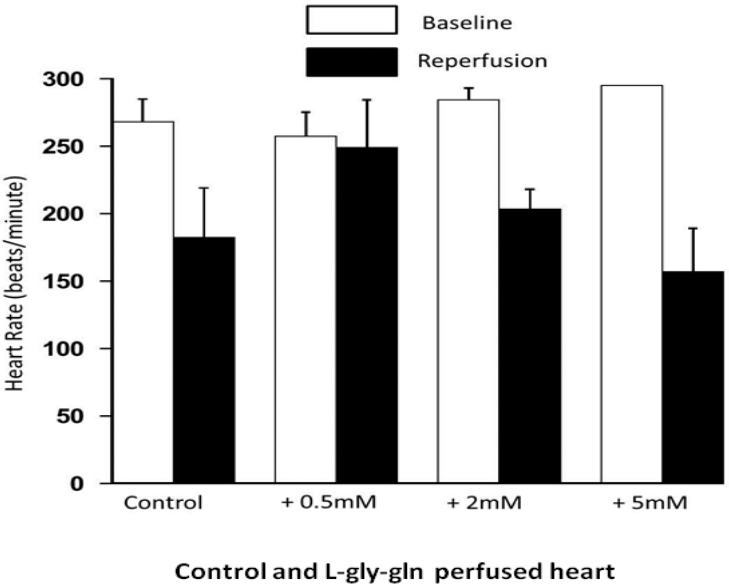


Figure 7: Effect of gly-gln on heart rate before and after ischaemia in young adult rat hearts perfused with or without (control) 0.5 mM, 2 mM or 5 mM gly-gln. Data shown are means ± SE of n=6-7. there were no significant differences

2.4.1.3. The effect of gly-gln on the rate pressure product

Figure 8 shows that the percentage recovery of the rate pressure product (RPP) during reperfusion was significantly better in the presence of 5 mM gly-gln as compared with control ($P < 0.05$). The presence of gly-gln did not significantly alter the pre-ischaemic rate pressure product.

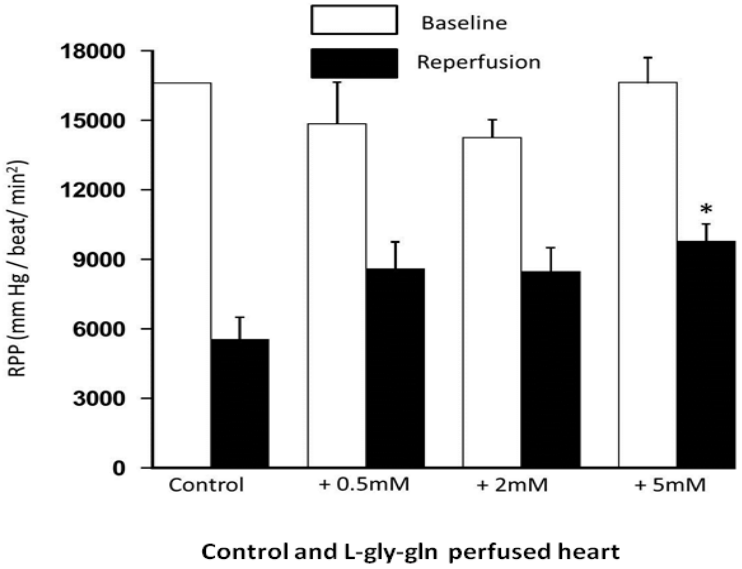


Figure 8: Effect on gly-gln on the rate pressure product (RPP) before and after ischaemia in young adult rat hearts perfused with or without (control) 0.5 mM, 2 mM or 5 mM gly-gln. * $P <$ 0.05 vs. reperfusion in control. Data shown are means ± SE of n=6-7.

2.4.1.4. The effect of gly-gln on reperfusion damage

Figure 9 shows the activity of lactate dehydrogenase (LDH) in samples of coronary effluent that were taken at intervals during reperfusion. The activity of LDH in each sample was plotted against time and the area under the curve calculated. The total LDH release indicates that myocardial damage was significantly less with either 2 mM or 5 mM gly-gln compared to control.

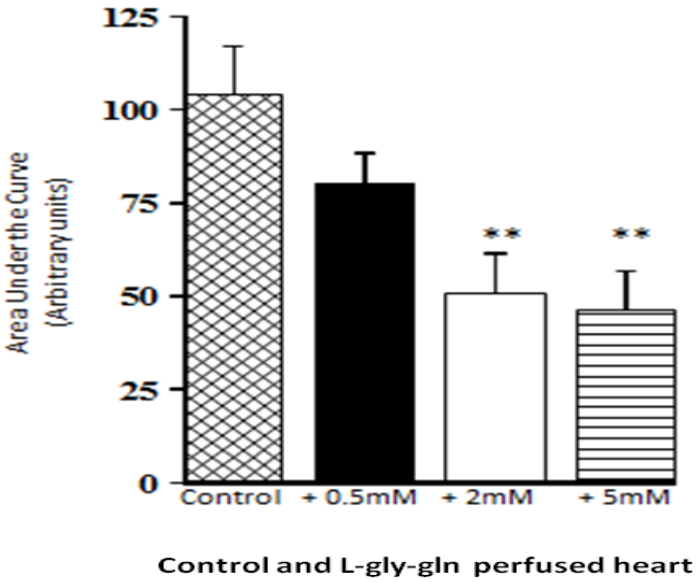


Figure 9: Effect on gly-gln on the extent of reperfusion damage. Graph of the mean area under the curve when the release of lactate dehydrogenase into the coronary effluent during reperfusion was measured. ** $P < 0.01$ vs. control. Data shown are means ± SE of n=6-7.

2.4.1.5. The effect of gly-gln on Ischaemic stress

Figure 10 show that the presence of 2 mM or 5 mM gly-gln significantly lengthened the amount of time taken to undergo ischaemic contracture by comparison with control.

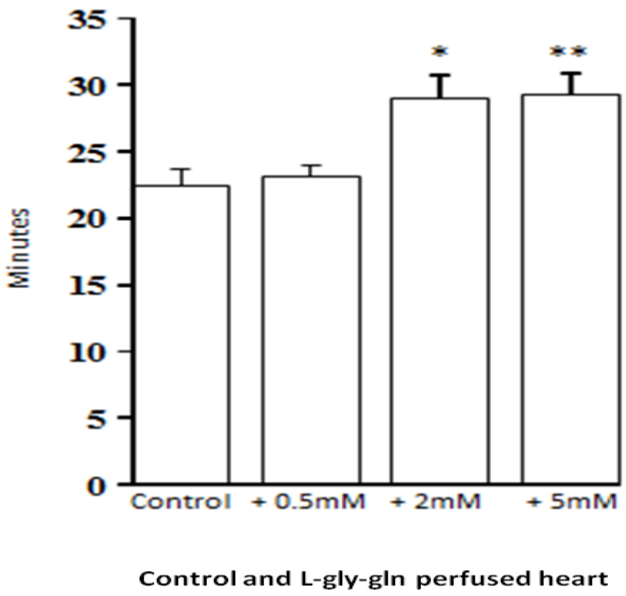


Figure 10: Effect on gly-gln on the time to ischaemic contracture during ischaemia in young adult rat hearts perfused with or without (control) 0.5 mM, 2 mM or 5 mM gly-gln. * $P < 0.05$ vs. control. ** $P < 0.01$ vs. control. Data shown are means ± SE of n=6-7.

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2.5. Discussion

In this study we used different concentrations of gly-gln to investigate possible cardioprotective effects by considering the effects on various physiological parameters following ischaemia in the Langendorff experiment. Both 2 mM gly-gln, and 5 mM gly-gln showed significant beneficial effects in preventing or attenuating functional compromise following ischaemia, while concentration of 0.5 mM gly-gln showed a protective trend that did not achieve significance (Almashhadany & King, 2013). In particular, the rate pressure product (RPP) was enhanced twofold in the presence of 5 mM gly-gln (Figure 8) whereas the time to ischaemic contracture was prolonged by 6-7 minutes (Figure 10). These improvements in cardiac function correlated with the dose dependant protective response seen in the treated animals as assessed by significant reduction in the release of marker enzyme LDH in the presence of 2 and 5 mM gly-gln dipeptide (Figure 9). This is a valuable biochemical biomarker and confirmation of the observed physiological responses. Gly-gln dipeptide is an efficient glutamine and glycine source (Jiang *et al.*, 2006). Individually, glycine and glutamine are highly compatible with cardiomyocyte use. Glutamine can be transported inside the cardiomyocytes via a high-capacity saturable stereospecific, sodium-dependent transporter in the cardiocyte membrane (Bolotin *et al.*,

2007; Khogali *et al.*, 2002; Rennie *et al.*, 1996). Glycyl-glutamine (gly-gln) is a dipeptide synthesised from glutamine and glycine which has higher solubility, and thermostability than either component amino acid. This dipeptide is transported by the proton-dependent transporter PEPT2 which has been found in the heart. This transporter has the ability to simultaneously transport glycine and glutamine as the dipeptide, gly-gln (Lin & King, 2007) and therefore glycyl-glutamine dipeptide use is more suitable. A study done by Qi Ren *et al* (2009) suggest that significant reduction in cardiac function can be caused by ischaemia-reperfusion injury and that 2.5 mM gly-gln can mitigate the effect of ischaemia-reperfusion injury by inhibiting myocardial cell membrane lipid peroxidation, reducing the generation of ROS and maintaining myocardial cell membrane permeability and stability (Qi Ren-bin, 2009). The results in this thesis are consistent with these earlier results but also show significant dose dependant reduction in lactate dehydrogenase and significant increase in time to ischaemic contracture. Lactate dehydrogenase is an important biomarker of cardiac damage, whilst time to ischaemic contracture is an important marker of energetic reserves during ischaemia. These results suggest that gly-gln shows promise as a combatant against ischaemia reperfusion injury. Gly-gln has

potential as a prophylactic treatment against ischaemic–reperfusion injury and may find a use in surgical procedures.

Chapter3 Supplementation with L-glycyl-L-glutamine Improves Functional Recovery Following Ischaemia Reperfusion in the Middle Aged Isolated Rat Heart

3.1. Abstract

The previous chapter demonstrated the proof of concept that gly-gln was a potential cardioprotective agent. These studies were however conducted in young and healthy adult rats. Cardioprotective techniques are known to have decreased effectiveness in the ageing population as the possibility of comorbidities increases with age. It was therefore considered important to investigate whether gly-gln was also a potential cardioprotective agent in an older age group. After 40 minutes ischaemia in hearts removed from 36 week old rats the percentage recovery of the left ventricular developed pressure was 18.5 ± 3.6% in control rats vs. 20 ± 4% in hearts perfused with 0.5 mM gly-gln; 34.2 ± 5.7% (P < 0.05 vs. control) in hearts perfused with 2 mM gly-gln and 40 ± 6% (P < 0.01 vs. control) for hearts perfused with 5 mM gly-gln. To investigate the protective mechanisms involved, measurements were made of TBARS and lactate. In separate experiments small samples of the left ventricle were collected at the beginning and end of ischaemia for lactate measurements and from the right ventricle at the end of ischaemia for

measurement of TBARS. Where used, control and different concentrations of gly-gln, 0.5 mM gly-gln, 2 mM gly-gln, and 5 mM gly-gln were added to the perfusate 10 minutes into baseline perfusion with wash out as before after 10 minutes reperfusion, 40 minutes global normothermic ischaemia.

Data presented are means ± SE of n=8 and compared using Student's unpaired t tests. The presence of 2 mM, 5 mM, gly-gln was accompanied by reduction in lactate and TBARS concentration ($P < 0.07$). There was also a trend towards a reduced lactate and TBARS concentrations in 0.5 mM gly-gln exposed samples, although this did not quite reach significance. These results suggest that gly-gln is an effective cardioprotective agent against ischaemia reperfusion in clinically relevant aged hearts.

3.2. Introduction

The main thrust of the myocardial protection studies presented in this thesis is to find a suitable method for protecting the older heart. Strategies targeting ischemia-reperfusion are designed to preserve intracellular metabolites and maintain ionic homeostasis. This presents a challenge in the older heart. A good example of this is ischaemic preconditioning, which is a powerful protector of the ischaemic heart, but is less effective in the post-infarcted-, failing- and/or aged- heart (Pantos *et al.*, 2007). Elderly patients are more likely to suffer from multiple comorbidities that increase the likelihood of myocardial damage (Jahangir *et al.*, 2007). Ischaemia- reperfusion can cause irreversible damage caused by accumulated Ca^{2+} via the Na^{+}/Ca^{2+} exchanger and generation of ROS (Suleiman *et al.*, 2008). Heart cells release free amino acids in response to cardiac insults (Schaffer *et al.*, 2002). The principal free amino acids glutamine, alanine, glutamate, taurine and aspartate also change in heart cells through cardiac insults throughout open heart surgery and in experimental models (Suleiman *et al.*, 2008). Amino acids play an important role in cardiomyocytes as they are involved in energy metabolism and therefore a role for supplementation is indicated.

3.3. Materials and methods

3.3.1. Animals

Thirty-six week-old male Wistar rats were used in these experiments (Central Animal House, University of New England, NSW, Australia 2351). These experiments were performed with the approval of the Animals Ethics Committee (AEC09/152 and EC10/036). Animals were fed standard rat chow and water *ad libitum.*

All chemicals including enzymes and amino acids were bought from Sigma, Australia (14 Anella Ave, Castle Hill NSW 2154). Rats were killed by stunning followed by cervical dislocation: Subsequently, the hearts were removed and processed for cannulation using the Langendorff technique.

3.3.2. Langendorff heart perfusion

The protocol used to perfuse isolate whole rat hearts and expose them to ischaemia reperfusion was identical to that described in chapter 2. The only difference was for these experiments the hearts were dissected from 36 week old rats.

3.3.3. Priming the equipment for ischaemia and ischaemic reperfusion

The protocol used to perfuse isolate whole rat hearts and expose them to ischaemia reperfusion was identical to that described in chapter 2 (Figure 4).

3.3.4. Preparation of heart tissue

Before sacrificing an animal, the equipment was checked for full readiness and particularly that there were no air bubbles in the coil. The container was filled with cold Krebs solution. The rat was sacrificed by stunning followed by cervical dislocation. The sacrificed rat was turned so that it is lying on its back, with the belly up. The still beating heart was exposed by cutting through the rib cage with sharp pointed scissors and the heart removed by cutting the aorta and leaving about 1 cm sufficient for attachment of the heart to the cannula. Excised hearts were immediately placed in cold Krebs solution and squeezed gently to remove blood from the aortic root. The excised heart was connected to the Langendorff equipment (Figure 11) by holding the aorta with the forceps and inserting in the cannula. A crocodile clip was used to secure the cannula in the heart. The aorta is the largest vessel emerging from the heart. Should there be difficulty to identify it, the heart is placed back into the container of the Krebs solution and

gently squeezed. This should open up the vessel and the blood may be ejected from the heart through the aorta. Pushing the heart too far up the cannula will block the opening of the coronary vessels and the heart will not perfuse properly. If the heart is cannulated too low this will let the heart slip off. Tying the heart using a thread was important to ensure perfusion at a rate of 11 ml/min and to avoid any leakage of the liquid. To ensure adequate oxygen supply to the heart when it was connected to the Langdendorff system, the carbogen supply (containing 95% oxygen and 5% CO_2) was turned on so as to ensure that a continuous flow of gas passed through all pipes and both chambers. Perfusion of nutrients to the heart muscle was delivered at a rate of 11 ml/min by perfusion pump. To protect the heart muscle from air embolism, care was taken to ensure that the Langendorff system was free of any air bubbles by letting Krebs solution run through the pipes before connecting the heart to the system. Once the heart was attached to the cannula via the aorta, the pump was turned on and the chamber is raised to maintain physiological temperatures (37 ℃). The heart was perfused for 10 minutes with Krebs solution from the first chamber, then for further 10 minutes using second chamber containing Krebs solution+ addition of gly-gln. For the control specimen perfusion proceeds for 20 minutes using Krebs solution only. After a total 18 minutes of

perfusion with empty chamber surrounding the heart, the chamber was then filled with the liquid collected from the coronary effluent for two minutes. At 20 minutes perfusion the pump was turned off which resulted in global normothermic ischaemia. After 20 minutes a small sliver of tissue from the left ventricle is taken and placed in pre-labelled Eppendorff tubes and then dropped into liquid nitrogen. The heart was submerged to the level of the tops of the ventricles in the coronary effluent, the temperature in the water bath was reduced to 34.5 ℃ and the rate of gas permeation slowed down. At the end of 40 minutes ischaemia, the solution was drained from the chamber that surrounds the heart and the chamber lowered down and the heart removed from the cannula for the sampling of the muscle tissue as before. After post-ischaemia sampling the rest of the heart was discarded. The samples (three from each heart) were removed from the liquid nitrogen after snap freezing and placed into the -80 ℃ freezer. Three samples were collected for 8 hearts in each experimental group (control, 0.5 mM gly-gln, 2 mM gly-gln, 5 mM gly-gln) at the beginning and end of ischaemia for lactate measurements in the left ventricle and the whole of the right ventricle at the end of ischaemia for TBARS.

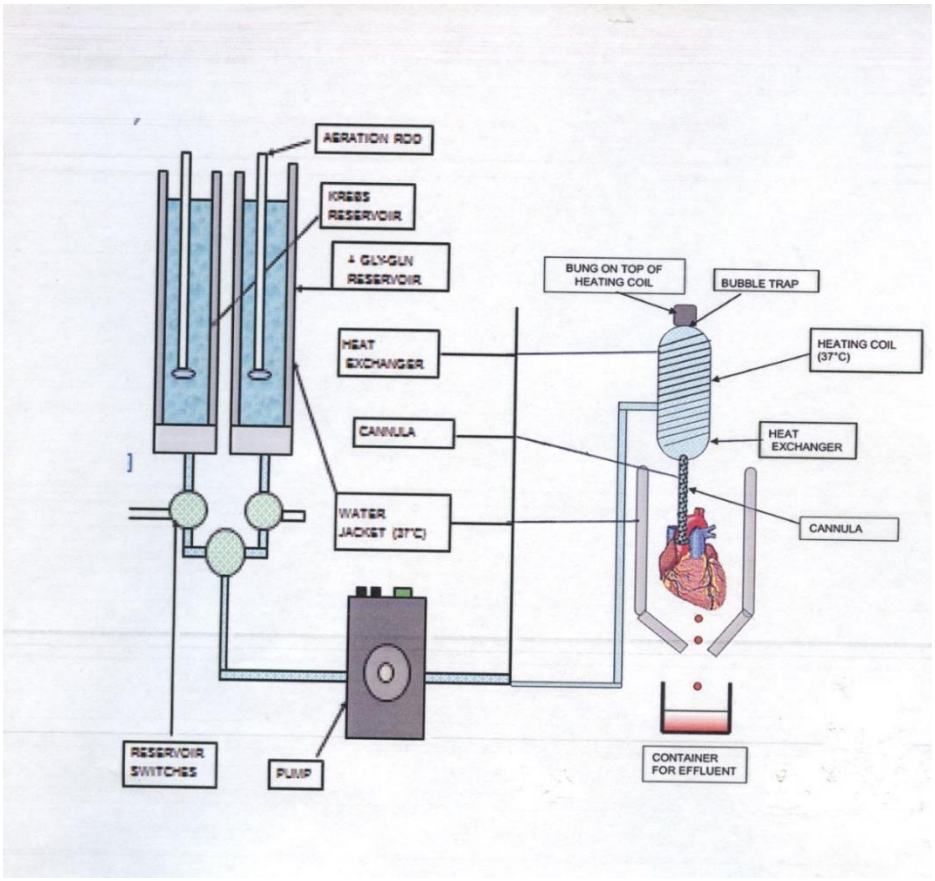

Figure 11: The Langendorff heart apparatus for heart perfusion.

3.3.5. Monitoring the response of the heart to the ischaemic challenge

3.3.5.1. Extraction of heart tissues in readiness for lactate measurement

A solution of 0.44 M K_2CO_3 was prepared. Glass tubes were labelled and placed on ice. Five hundred µl of 4.8% perchloric acid (PCA) was pipetted into each glass tube. The tubes were weighed and again placed on ice. Heart tissue samples were kept as cold as possible at all times to prevent sample degradation and prior to analysis were placed on ice. A little liquid nitrogen was placed into a mortar and pestle to cool it. Further liquid nitrogen was then added with heart tissue and the pestle was used to crush the frozen tissue. The crushed tissue was transferred to appropriately labelled glass tubes using a micro-spatula. Samples were briefly vortexed and centrifuged at 4000 rpm for 10 minutes at 4 ℃. The tubes were then reweighed. Three hundred µl of the supernatant was transferred into a further set of labelled pre-cooled glass tubes. The tubes containing the pellet were left in the ice box then stored at - 20 ℃. To the tubes containing the supernatant 300 µl of cooled K_2CO_3 was added. The mixed samples were vortexed for 10 seconds each, and then placed on ice for 2 minutes, thereafter remixed in vortex for 10 seconds, and then placed on ice for 2 minutes and vortexed for a final 10

seconds. The tubes were then centrifuged at 4000 rpm for 10 minutes at 4 ℃. The supernatant was pipetted into pre-labelled Eppendorff tubes and stored at -20 ℃. For determination of lactate in tissue extracts a solution of 20 ml containing 10 ml PCA and 10 ml K_2CO_3 was prepared. The water bath was equilibrated to 37 ℃. A lactate stock solution of concentration 10.96 mM was prepared. Lactate standard solutions with concentration 0, 0.05, 0.1, 0.2, 0.4 and 0.8 mM were prepared and labelled according to the table shown below:

Table 1: The reaction volume of lactate standards.

Tube	Lactate stock solution (µl)	PCA/K_2CO_3 (µl)	Final lactate concentration
1	0 (blank)	1000	0
2	4.6	995	0.05
3	9.2	991	1
4	18.4	982	2
5	36.8	963	4
6	73.6	926	8

Two ml glycine buffer, 4 ml H_2O and 0.1 ml lactate dehydrogenase solution were added to the β-NAD vial, the vial was then mixed very well by inverting it several times. 0.1 ml from each of the standard samples as well as from the heart tissue was added to 2.9 ml from the vial and mixed in the appropriately labelled tube. These tubes were incubated at 37 ℃ for 15 minutes. These samples were transferred to a quartz cuvette and the absorbance measured using a spectrophotometer at 340 nm. The readings of the standard samples were used to construct a curve from which the lactate concentrations in the heart samples could be extrapolated.

3.3.5.2. Estimation of lipid peroxidation measured as thiobarbituric acid reactive substances (TBARS)

The amount of lipid peroxidation in the various samples after ischaemic stress was determined as malondialdehyde (MDA) which reacts with the thiobarbituric acid reagent (TBA) to form a pinky-red coloured TBA-MDA complex (Buege & Aust, 1978). Samples in the presence of 50 mM AAPH (300 µl) were combined with 1ml of a solution containing (15% w/v) trichloroacetic acid and (0.375% w/v) thiobarbituric acid in 0.25 M HCl. The mixture was boiled for 15 minutes and allowed to cool on ice. Following

centrifugation at 10,000 rpm for 5 min, the absorbance of the supernatant was determined at 535 nm against a blank solution containing all the reagents except sample. The MDA concentration was calculated using a molar extinction coefficient of $1.56*10^5$ M^{-1} cm^{-1} (Wills, 1969). In the assay procedure for lipid peroxidation, right ventricle samples were homogenised for (30 second) in 500 µl double distilled water and then centrifuged at 4000 rpm for 10 minutes at 4 °C. Measurements were carried out by taking a mean of two readings. Two hundred fifty µl of each of the samples was pipetted into two separate labelled Eppendorff tubes. To each tube 250 µl of 50 mM (AAPH) was added and mixed well. Following this 1ml of a solution containing 15% (w/v) trichloroacetic acid (TCA) and 0.375% (w/v) TBA in 0.25 M HCl was added and placed into a boiling water bath for 15 minutes. The tubes were removed from the water bath and cooled on ice for a further 10 minutes. The tubes were then centrifuged at 10,000 rpm

for 5 minutes at 4 °C. The blank comprising to 150 µl AAPH mixed with 1ml of the TCA/TBA mixture was pipetted into a cuvette; the absorbance of the supernatant at 535 nm was read against the blank. The concentration of lipid peroxidation product in the samples was calculated using the Beer Lambert Law as described below:

$$Abs= \epsilon cl$$

Where ϵ is the molar extinction coefficient of MDA, which is $1.56*10^5$ M^{-1} cm^{-1} c is concentration (M) and l is the path length (1cm).

3.3.6. Measurement of protein concentration using Bradford's reagent

The Bradford assay is the most commonly used colourimetric assay for protein quantification. The assay was based on the shift of the Coomassie absorption maximum from 470 nm to 595 nm after protein binding at acidic pH. The protein concentration of an unknown sample can be determined by extrapolation from a calibration curve. The colourimetric reaction depends on the content of aromatic and basic amino acids. To create a calibration curve, bovine serum albumin (BSA) was used as standard. A one litre volumetric flask was covered with aluminium foil to protect it from light. One hundred mg Coomassie blue G was dissolved in 50 ml ethanol then the flask was put on ice for cooling. One hundred ml orthophosphoric acid (H_3P0_4) was added to the solution and made up to one litre with distilled water. This solution was then filtered into the aluminium foiled flask through two layers of Whatman no.1 filter paper. In the establishment of a protein concentration calibration curve using Bradford's Reagent, for the standard curve 0.02 g of bovine serum albumin (BSA) were dissolved in 10 ml of ultra-pure water to provide a BSA stock solution. This represents the BSA stock containing 2000 µg/ml BSA.

The initial BSA stock was divided into 250 µl aliquots and stored at -20 °C. The standards were prepared using the BSA stock solution and water according to the following table.

Table 2: The reaction volume of protein standards.

Tube	Volume of BSA stock (µ l)	Source	Volume of water (µ l)	Final protein concentration (µg/ml)
1	70	BSA stock	0	2000
2	75	BSA stock	25	1500
3	70	BSA stock	70	1000
4	35	Tube 2	35	750
5	70	Tube3	70	500
6	70	Tube5	70	250
7	70	Tube6	70	125
8	0		70	0 (blank)

Twenty µl of the standard or blank solutions were pipetted into the appropriately labelled test tubes. Twenty µl of the samples were also pipetted into test tubes. To each and every one of these tubes of both groups one ml of Bradford's Reagent was added and then incubation proceeded for 15 minutes at room temperature. The absorbance of the samples and standards were measured at 595 nm in the spectrophotometer. The standard curve of absorbance versus protein concentration for the standards manifests a straight line from which the unknown protein concentration may be extrapolated.

3.4. Results

3.4.1. Determination of lactate concentrations

Figure 12 shows the results for lactate. At the end of ischaemia the lactate concentration in the control was significantly greater compared to the value at the beginning of ischaemia in the control ($P < 0.05$). This was not the case in the presence of any of the different gly-gln concentrations where the value at the beginning and end of ischaemia were not significantly different from each other. The calculations of statistical significance between groups was determined by Student's unpaired t tests, data are mean± SD. (n=8-9).

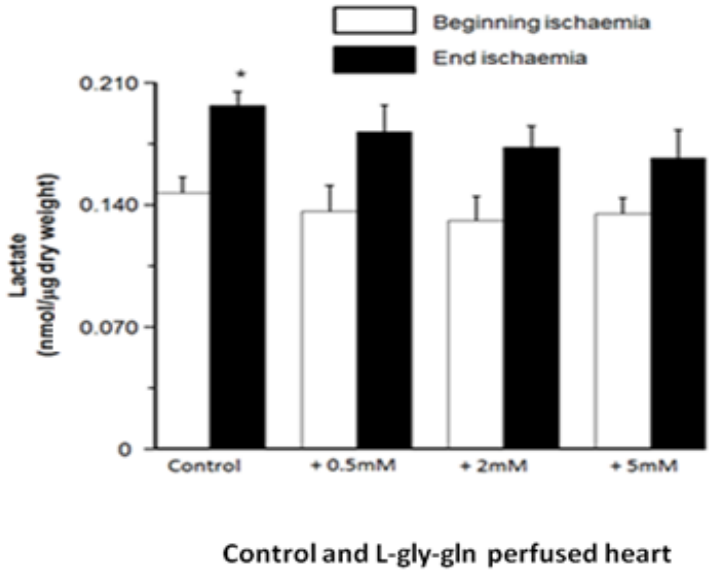

Figure 12: Effect of gly-gln on ischaemic stress in 36 week old rat hearts. Lactate concentrations measured at the beginning and end of 40 minutes global normothermic ischaemia. * $P < 0.05$ vs. control at the beginning of ischaemia. Data shown are means ± SE of n=8-9.

3.4.2. Thiobarbituric acid reactive substances (TBARS)

Figure 13 shows that although there was a strong trend towards a lower concentration of TBARS following treatment with 2 mM gly-gln and 5 mM gly-gln this did not reach significance ($P < 0.07$) due possibly to the large SE. There was also no significant differences between 0.5 mM gly-gln and control. This being said the apparent dose response is impressive.

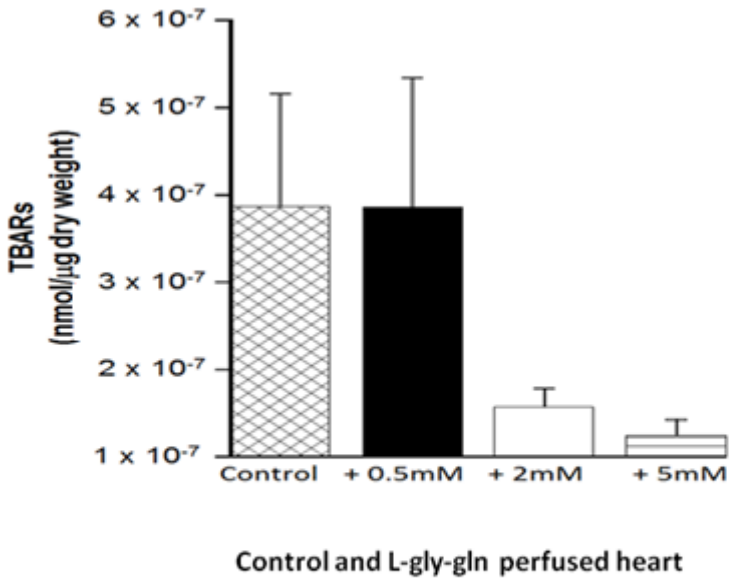

Figure 13: Effect of gly-gln on lipid peroxidation in samples of right ventricle from 36 week old rat hearts collected at the end of 40 minutes global normothermic ischaemia. TBARS: thiobarbituric acid reactive substances. Data shown are means ± SE of n=5-9. there were no significant differences

3.4.3. Cardiac functional parameters

3.4.3.1. The effect of gly-gln on the left ventricular developed pressure.

Figure 14 shows how hearts of young and older rats were affected by 40 minutes global normothermic ischaemia. For both ages the reperfusion left ventricular developed

pressure (LVDP) was worsened upon reperfusion, whilst the reperfusion LVDP of the 36-weeks-old was significantly lower than that of the 8-weeks-old.

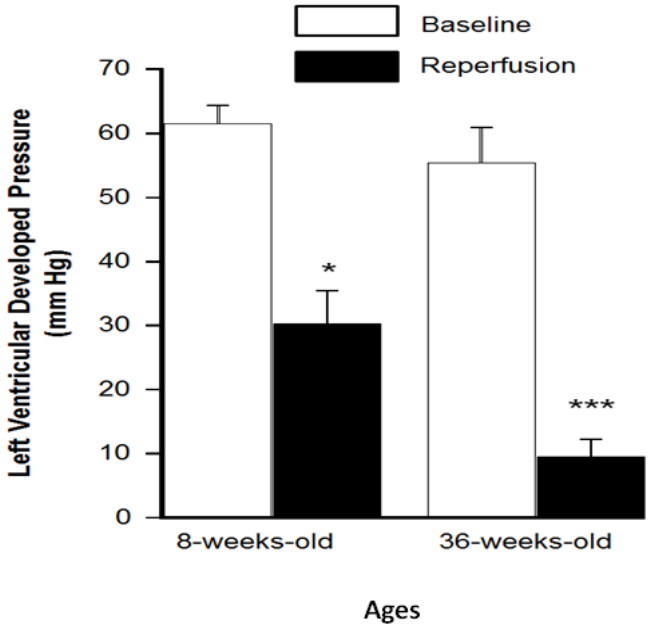

Figure 14: Effect of ageing on the functional performance of rat hearts during ischaemia reperfusion. Left ventricular developed pressure during baseline perfusion (open bars) and at the end of reperfusion following 40 minutes global normothermic ischaemia (solid bars).* $P < 0.01$ vs. baseline, ** $P < 0.05$ vs. reperfusion in 8-weeks-old. Data shown are the means ± SE of n=6-7.

3.4.3.2. The effect of gly-gln on recovery of the rate pressure product

Figure 15 shows the rate pressure product measured during baseline perfusion (before ischaemia) and at the end of reperfusion following 40 minutes global normothermic ischaemia. Three gly-gln concentrations were tested namely 0.5, 2 and 5 mM. The presence of 2 mM and 5 mM gly-gln led to a significant improvement in the post ischaemia rate perfusion product compared to control. Again the apparent dose response is impressive.

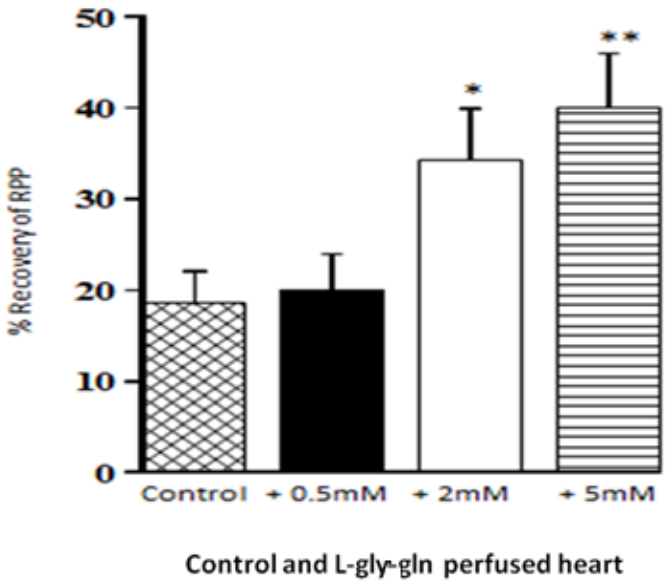

Figure 15: Effect on gly-gln on the recovery of the rate pressure product during reperfusion of middle aged rat hearts. ** $P < 0.01$ vs. control (ANOVA with a Tukey post-test). Data shown are means ± SE of n=6.

3.4.3.3. The effect of gly-gln on reperfusion damage

Figure 16 shows that the area under the curve indicative of LDH release was significantly less compared to control in the presence of either 2 or 5 mM gly-gln. In addition to measuring the functional performance of the rat hearts, the degree of reperfusion damage was also measured. This was assessed from the release of lactate dehydrogenase into the coronary effluent on reperfusion following the 40 minutes global normothermic ischaemia. Lactate dehydrogenase release vs. time was plotted and then the area under the curve calculated as an indication of the total time dependent release.

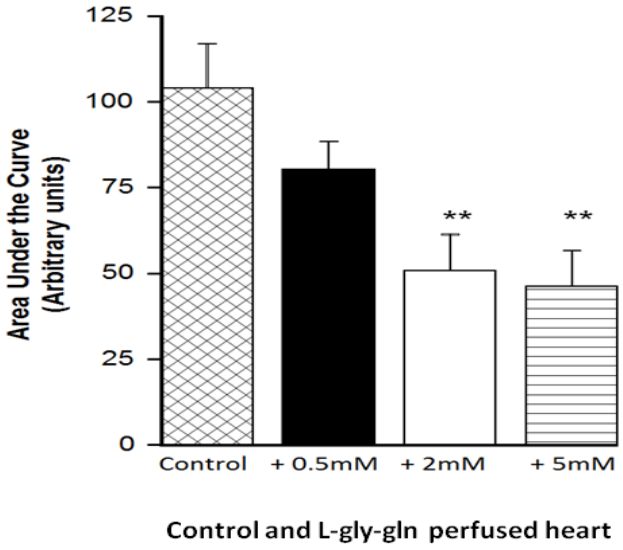

Figure 16: Effect on gly-gln on the extent of reperfusion damage. Graph of the mean area under the curve when the release of lactate dehydrogenase into the coronary effluent during reperfusion was measured. ** $P < 0.01$ vs. control. Data shown are means ± SE of n=6-7.

3.4.3.4. Time to ischaemic contracture

Figure 17 shows a similar pattern was seen when the time to ischaemic contracture was measured with the presence of 5 mM gly-gln significantly lengthening the time taken to undergo contracture compared to both controls and the +0.5 mM group.

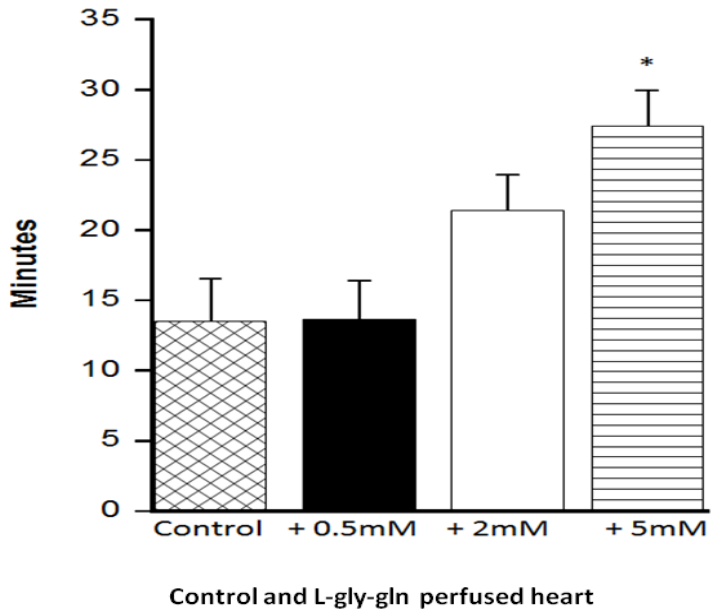

Figure 17: Effect on gly-gln on the time to ischaemic contracture during ischaemia in 36 week old rat hearts perfused with or without (control) 0.5 mM, 2 mM or 5 mM gly-gln. * $P < 0.05$ vs. control. ** $P < 0.01$ vs. control. Data shown are means ± SE of n=6.

3.5. Discussion

The previous chapter demonstrated the proof of concept that gly-gln was a potential cardioprotective reagent in the young and healthy rat. These experiments were performed on young adult rat hearts (8-week-old), where there is an abundance of literature investigating different modes of cardioprotection with which to compare the current findings. Figure 15 shows the rate pressure product measured during baseline perfusion (before ischaemia) and at the end of reperfusion following 40 minutes global normothermic ischaemia. Three gly-gln concentrations were tested namely 0.5, 2 and 5 mM. The presence of 5 mM gly-gln led to a significant improvement in the post ischaemia rate perfusion product compared to control. There was also a trend towards a higher rate pressure product in the presence of 0.5 and 2 mM gly-gln, however this did not reach significance. In addition to measuring the functional performance of the rat hearts, the degree of reperfusion damage was also measured. This was assessed from the release of lactate dehydrogenase into the coronary effluent on reperfusion following the 40 minutes global normothermic ischaemia. Lactate dehydrogenase release vs. time was plotted and then the area under the curve calculated as an indication of the total time dependent release. Figure 16 shows that the area under the curve was significantly

less compared to control in the presence of either 2 or 5 mM gly-gln. Taken together the findings in Figures 15 and 16 suggest gly-gln has potential as a cardioprotective agent supporting the proof of concept. An important aim of these experiments was to investigate the cardioprotective potential of gly-gln in older rats as being more representative of the age when humans are first diagnosed with coronary artery disease. In the first place a comparison was made of the vulnerability of 8-weeks-old and 36-weeks-old rat hearts to ischaemia reperfusion. Figure 14 shows how hearts of these ages were affected by 40 minutes global normothermic ischaemia. For both ages the reperfusion left ventricular developed pressure (LVDP) was worsened upon reperfusion, whilst the reperfusion LVDP of the 36-weeks-old was significantly lower than that of the 8-weeks-old. Figure 15 shows the effect of different gly-gln concentrations on the rate pressure product during reperfusion following 40 minutes global normothermic ischaemia in 36-weeks-old rat hearts. There was a significantly greater rate pressure product in the presence of 5 mM gly-gln compared both to control and to 0.5 mM gly-gln. A similar pattern was seen when the time to ischaemic contracture was measured (Figure 17) with the presence of 5 mM gly-gln significantly lengthening the time taken to undergo contracture compared to both controls and the +0.5 mM group. When it came to

reperfusion damage the area under the curve for lactate dehydrogenase release was significantly lower in the presence of both 2 mM and 5 mM compared to control (Figure16). In order to investigate the possible mechanisms underlying the protection afforded 36-weeks-old hearts by gly-gln lactate accumulation during ischaemia as an indicator of ischaemic stress and the accumulation of thiobarbituric acid reactive substances (TBARS) during ischaemia as an indication of lipid peroxidation were measured (Figure 13). Figure 12 shows the results for lactate. At the end of ischaemia the lactate concentration in the control was significantly greater compared to the value at the beginning of ischaemia in the control. This was not the case in the presence of any of the different gly-gln concentrations where the value at the beginning and end of ischaemia were not significantly different from each other. There were no significant differences in the TBARS data.

Though ischaemia-reperfusion can cause damage that affects any age, the elderly are at the highest risk, recent studies suggest that the response to ischaemia-reperfusion may vary as a function of age. The result of myocardial infarction in elderly patients is more severe (Grines & DeMaria, 1990; Lesnefsky *et al.*, 1994). In this chapter we considered it is important to investigate whether gly-gln was also a potential

cardioprotective agent in an older age group. Therefore the effects of 40 minutes ischaemia in hearts removed from 36 week old rats were investigated. We attempted to further correlate such putative effects with various biochemical biomarkers associated with damage to the heart. Studies have shown that glycine and glutamine individually are both useful in protecting cardiomyocytes in ischaemia-reperfusion injury as evidenced by increased RPP (Liu *et al.*, 2007; McGuinness *et al.*, 2009; Pan *et al.*, 2005; Støttrup *et al.*, 2006; Weinberg *et al.*, 1997). The amino acids glycine and glutamine improved myocardial protection against ischaemia reperfusion. An attractive alternative, which offers the potential for delivering a number of amino acids simultaneously is made possible through the existence of a proton-coupled oligopeptide transporter family, namely PEPT1, PEPT2, PEPT3 and PEPT4 (Daniel & Kottra, 2004). These transporters facilitate the proton and membrane potential dependent flux of all the possible 400 different di- and 8000 different tri- peptide combinations of the 20 proteinogenic amino acids as well as a broad range of peptidomimetic drugs, including several used in the fields of cardiac surgery and cardiology (e.g. ACE inhibitors (Daniel & Kottra, 2004). The functional expression of PEPT2 has been demonstrated in the guinea-pig and rat hearts (Lin & King, 2007). Present study extends these observations to biochemical biomarkers

associated with damage to the heart (LDH, lactate and TBARS) and shows dose dependant reduction in TBARS concentration and total LDH release indicator of myocardial damage was significantly less with either 2 mM or 5 mM gly-gln. Though the major sources of cardiomyocyte energy are fatty acid and glucose, studies done by Khogali *et al*., (2002), McGuinniss *et al*., (2009), Stottrup *et al*., (2006), Bolotin *et al*., (2007) and Zhang *et al*.,(2013) have shown that glutamine can increase energy synthesis in the pathological state. The result from the present study shows that gly-gln protects against the accumulation of lactate during ischaemia. This indicates that the dipeptide may be involved in energy production in the oxygen starved state and therefore, to an extent, mitigate against the pathological damaging anaerobic accumulation of lactate. The previously mentioned observation of dipeptide dependant delay in the time to ischaemia induced contracture (Figures 17) is entirely consistent with this result and has important implications for the post-crisis treatment of human infarcts. In addition, a dose dependant decrease in TBARS concentration in the gly-gln exposed samples (Figures 13) is consistent with an improved myocardial tissue energy metabolism and energy reserve in cardiac tissues after injury. These results may achieve statistical significance with further experiments; unfortunately time prevented the

completion of these additional experiments. These results suggest that gly-gln is an effective cardioprotective agent against ischaemia reperfusion in clinically relevant aged hearts.

Chapter4 : Utilising different methods to isolate fresh rat heart cells and to study the effect of homocysteine.

4.1. Abstract

Homocysteine has been implicated in augmented oxidative stress, and recognised as a risk factor for developing cardiovascular disease. However the effect on heart cells has been rarely studied. Every single rat heart cell potentially provides a powerful tool for heart research. Nevertheless the isolation of healthy cells is not easy and this imposes a significant impasse on their use in cellular studies of the heart. To overcome this problem we use the classic Langendorff technique that reproducibly isolates healthy heart cells, as well as facilitating easy handling and the rapid aortic cannulation of the small rat heart. There were two aims in this chapter a); to compare different methods of isolation and b); to investigate the effects of homocysteine with/without oxidative stress. Having isolated healthy rat heart cells a further aim was to investigate the impacts of exogenous homocysteine on the morphology and viability of isolated cardiomyocytes in the presence/absence of oxidative stress induced by H_2O_2 and to compare this in cardiomyocytes isolated under three different conditions. The three different conditions were isolation in Tyrode buffer , isolation in Tyrode plus taurine and isolation in Tyrode

with glutamate and carnitine added to the digest enzymes. After isolation the viability of cardiomyocytes isolated in Tyrode was 64.4% ± 1.8 (mean ± SE) which was significantly less than the 80.7% ± 2.4 (mean ± SE, $p < 0.001$ vs. Tyrode, ANOVA with a Tukey post - test) in Tyrode solution containing taurine and the 80.3% ± 1.9 (mean ± SE, $p < 0.001$ vs. Tyrode, ANOVA with a Tukey post-test) in Tyrode with added carnitine and glutamate to the digest enzymes. After 210 minutes incubation at 37°C there were more viable cells isolated in the presence of Tyrode with taurine compared to the other two isolation methods. In all groups the percentage viability and percentage of rod shaped cells decreased in the presence of H_2O_2, homocysteine and H_2O_2 plus homocysteine, however by far the greatest decline was seen with H_2O_2 plus homocysteine. These results suggest that homocysteine exposure during oxidative stress is damaging to myocardial structural homeostasis.

4.2. Introduction

Homocysteine is a thiol-containing, non –protein amino acid for which the normal plasma concentration; range from 5-15 μM. Hyperhomocysteinaemia (hHcy) arises when the plasma homocysteine concentration exceeds 15 μM (Levrand *et al.*, 2007) with an upper limit of over 400 μM such as occurs in the inherited condition, cystathionine β-synthase deficiency (Antoniades *et al.*, 2009). More modest increases in plasma homocysteine (15-100 μM) occur in approximately 5-7% of the general population and are recognized as a strong independent risk factor for coronary heart disease suggesting that elevated plasma homocysteine correlates with cardiovascular disease mortality, increased incidence of stroke, dementia, Alzheimer's disease, bone fracture, and a higher prevalence of chronic heart failure (Antoniades *et al.*, 2009; Levrand *et al.*, 2007). The pathogenesis of hyperhomocysteinaemia is thought to involve increased thrombogenicity, greater oxidative stress, over-activation of redox-sensitive inflammatory pathways, impaired endothelial function, atherogenesis, cardiac remodelling and contractile dysfunction (Levrand *et al.*, 2007). It is somewhat surprising, therefore that several large scale clinical trials targeting a reduction in plasma homocysteine concentration through vitamin supplementation have yielded unconvincing results,

although a recent meta-analysis suggests that the conflicting results may reflect differences between individual patients' anti-platelet therapies (Selhub, 2006). The majority of the evidence investigating the effects of homocysteine has been collected from vascular endothelial cells and smooth muscle cells (Levrand *et al.*, 2007) with studies rarely concentrating on heart cells. Additionally, the small amount of research that has been published on the heart cells largely involved cultured cells (Mishra *et al.*, 2009; Sipkens *et al.*, 2007; Wald *et al.*, 2011; Wang *et al.*, 2012; Zhao *et al.*, 2006). Culturing is known to stimulate the expression of one of the major enzymes involved in homocysteine metabolism, namely cystathionine β-synthase (Sipkens *et al.*, 2007) and to induce changes in the electrical activity of heart cells (Mitcheson *et al.*, 1998). Homocysteine has also been implicated in augmented oxidative stress, particularly leading to a reduction in nitric oxide availability in cultured heart cells (Sipkens *et al.*, 2007; Wald *et al.*, 2011) and in isolated hearts from different experimental models of hyperhomocysteinaemia (Becker *et al.*, 2005; Suematsu *et al.*, 2007). Nitric oxide is central in enabling the heart to relax and drugs such as amyl nitrite that increase nitric oxide availability are in the frontline of treatment for angina. Oxidative stress is known to play a role in the pathogenesis associated with a sudden ischaemia and subsequent

reperfusion of the heart's blood flow with ischaemia being the cause of the pain in angina. Ischaemia reperfusion is encountered during clinical procedures for cardiovascular disease including percutaneous coronary intervention, coronary artery bypass grafting, cardiac valve replacement and heart transplants, where the undesirable consequences can include damaging change to heart rate and rhythm, harmful alteration to blood flow and cell death (Suleiman *et al.*, 2011). In contrast homocysteine is structurally related to the cardioprotective agent, cysteine (King, 2010), raising the possibility of potential protective effects. The hypothesis here then can be stated thus: That modification in the plasma concentration of homocysteine affects the health and vulnerability of freshly isolated heart cells, which in turn will alter the risk of developing cardiovascular disease. Therefore the aim of this study was to specifically investigate 1); the effects of homocysteine on the functioning of freshly isolated heart cells, their response to oxidative stress and necrosis using isolated control cells and cells incubated in the presence of homocysteine, H_2O_2 and homocysteine+H_2O_2 and 2); comparison of the cellular morphology and viability during exposure to oxidative stress in the presence of 0.05 mM homocysteine and to compare this in cardiomyocytes isolated under three different conditions. The three different conditions were isolation in Tyrode buffer alone,

isolation in Tyrode plus taurine and isolation in Tyrode with glutamate and carnitine added to the digestive enzymes.

4.3. Materials and methods

4.3.1. Animals

Thirty six weeks old male Wistar rats were used in this experiment (Central Animal House, University of New England, NSW, Australia 2351). Animals were fed standard rat chow and water *ad libitum*. These experiments were performed with the approval of the Animals Ethics Committee (AEC13-053), University of New England, Armidale, NSW, Australia.

All chemicals including enzymes and amino acids were bought from Sigma Australia (14 Anella Ave, Castle Hill NSW 2154). Rats were killed by stunning followed by cervical dislocation: Subsequently, the hearts were removed and processed for cannulation using the Langendorff technique.

4.3.2. Preparation of solution for cardiomyocyte isolation.

Stock solutions (1M) of KCl, $MgCl_2$, $MgSO_4$, NaH_2PO_4, NaOH, $CaCl_2$, were prepared. These solutions were very stable when stored at 4 ℃. Stock containing 45 mM EGTA was prepared. The base solution for isolating heart cells is called Tyrode. Solution A was prepared by adding 137 mM of NaCl, 20 mM HEPES, 16 mM glucose, 5mM pyruvate, in 1500 millilitres of double distilled water and stirring until Dissolved then 10 millilitres of the 1M KCl stock (final concentration 5 mM), 3.6 millilitres of $MgCl_2$ stock (final concentration 1.8 mM), 2.4 millilitres of the 1M $MgSO_4$ (final concentration 1.2 mM), 2.4 millilitres of the 1M NaH_2PO_4 (final concentration 1.2 Mm), were added, with added 1M NaOH so the pH of the solution reached 7.25 then distilled water was added to a total volume of two litres. Solution A was used to prepare the followed solutions: Solution B (contains 0.75 mM $CaCl_2$); Solution C (contains 9 μm EGTA); Solution D (contains 1 mg /ml collagenase and 1 mg / 10ml protease); Solution E (contains 0.15 mM $CaCl_2$); Solution F (contains 0.5 mM $CaCl_2$); And solution G (contains 1mM $CaCl_2$).

4.3.3. Cardiomyocytes Isolation

The equipment set up for this experiment is shown in Figures 18 and 19. The circulating water bath was set at 37.5 ℃ and in a similar process to that described previously regarding preparation of the Langendorff system for perfusion and rat dissection to remove the heart for cannulation (Section 3.3.4, Preparation of heart tissue). Twenty ml of cold solution B were placed in a broad pot. Reservoir one was filled with solution B and the second reservoir filled with solution C. At this point the reservoir three was left empty. The oxygen supply was turned on so the solutions in reservoirs one and two were gently bubbled. The pump was set on 11 ml/minute. The isolated heart was placed into the pot containing solution B and then squeezed gently to remove blood from the aortic root. The heart was then cannulated. A clip was used to hold the heart on the cannula and a thread used to tie the heart onto the cannula to ensure good perfusion. The pump was turned on and the chamber was raised so the heart was kept warm. The heart was perfused for 4 minutes with solution B. Solution then began to drip from the heart. At first it was bloody showing that the heart was being correctly perfused. At times the heart was beating and this was also reassuring but is not an absolute requirement (calcium is important for contraction and the calcium concentration in the solution B is lower than *in*

vivo so the heart may not beat). Thus solution D was added to reservoir three. Solution D could become very frothy if it received too much oxygen. It must however be oxygenated so it was kept very gently bubbling. After complete perfusion with solution B the valve of second reservoir was turned on and the heart perfused with solution C for six minutes. By turning on solution C flow of solution B automatically stopped. During this time solution B voided out of reservoir one which was then filled with solution E. About five minutes into perfusion with solution C, the heart become slightly swollen and harder than when first cannulated. A complete removal of Ca^{2+} from the coronary artery was essential before the application of enzymes. If perfusion of EGTA is insufficient and the Ca^{2+} removal incomplete, the perfusion flow was stopped after a switch to the enzyme solution (D) and this led to poor tissue digestion and damage to the isolated cells. On the other hand if perfusion continued for too long the cells were paradoxically damaged by the Ca^{2+} free environment in EGTA and they had an impaired Ca^{2+} tolerance (Daly *et al.*, 1987; Wolska & Solaro, 1996). After six minutes the third reservoir was turned on and the heart began perfusion with solution (D). Perfusion continued for a further 1.3 minute and effluent collection was initiated. Whenever the solution in reservoir three becomes low it was topped up with recycled collected effluent. There was no set time to perfuse

with solution D. Nothing will happen for 5 minutes or so. Then lowered the chamber and feel the heart. When the heart had digested sufficiently it would feel soft but not completely sloppy and falling off the cannula (this is a signal of over digestion and the cells would be very fragile and soon die). If under-digested there will be very few isolated cells. If the solution D turnd red this means that the heart had blood clots in it, which have been digested in the presence of the enzymes. This was not necessarily a bad thing so one should not worry unduly about it. Once the heart was digested then the third reservoir was switched off and reservoir one turned on so the heart is perfused with solution E for five minutes. At this point there was no concern for air bubbles because once the heart had been digested air bubbles were not so damaging. The heart was collected into a weighing boat. The atria and vessels around the base of the heart were excised (properly digested these structures should be very soft but still quite distinct). The ventricles were cut into 4-5 pieces and the pieces placed into a small beaker. The weighing boat was washed with solution E (from reservoir one) and more added (about 10ml) into the beaker. The beaker was placed into a shaking water bath at 37 ℃ for six minutes with a moderate shake frequency. Whilst it was shaking, the solution E was drained out of reservoir one and replaced with solution F. A funnel with cotton lint was

placed into a beaker. Cotton lint was placed into the funnel (this is a material, usually white in colour that is loose weave). Then the first beaker was taken out of the water bath and digested material filtered through the cotton lint into the next beaker. The beaker was placed aside and left to stand at room temperature for 10 minutes. A clearly visible layer began forming at the bottom of the beaker. The supernatant was removed (solution over the top of the cell layer) without disturbing the sedimented cell layer. The cell layer was agitated gently. A few drops were taken and placed onto a microscope slide. Cells are viewed under the microscope at low power (×100). Ten ml of solution F was added to the cells and incubation proceeded for 10 minutes at room temperature. The solution F was drained out from reservoir one and replaced with solution G. The supernatant F was removed from the cells without disturbing the cell layer. After this ten ml of solution G was added to the cell layer. This experimental protocol was repeated more than four times with Tyrode, with added taurine, and with added carnitine + glutamic acid to the solution D (enzyme). At the end of this procedure >65% of the cells were rod shaped and able to exclude Trypan blue. All isolated cells were then divided into four groups. The cells in group one were incubated with 0.05 mM homocysteine, group two incubated with 0.2 mM H_2O_2, group three with 0.05 mM homocysteine + 0.2 mM H_2O_2, and group four

was the control. All cells were placed in a gently shaking water bath at 37 ℃ for 210 minutes.

At timed intervals an aliquot was taken from each group and placed on a microscope slide on the stage of a light microscope. A drop of Trypan Blue (0.4%) was added then a field was selected at random. In order to determine cell viability the numbers of cells able to exclude Trypan Blue (called viable cells in the results) vs those including Trypan Blue were counted. In order to determine cell morphology the number of cells showing rod shaped morphology vs. those displaying hypercontracted morphology were counted. Results are expressed a percentage of the total number of cells in the field.

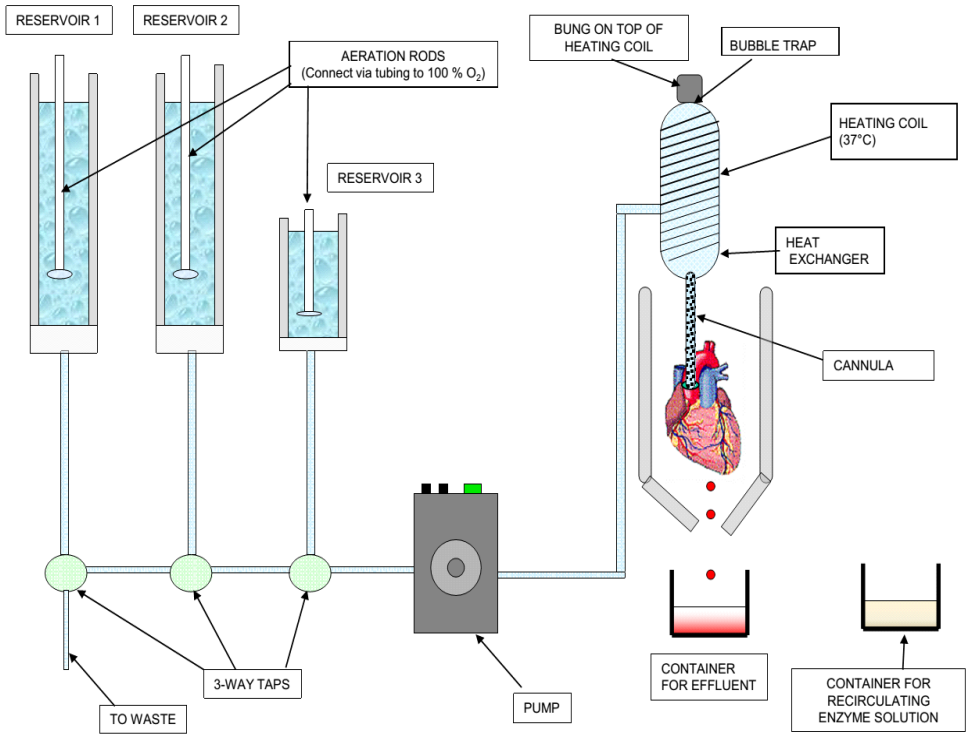

Figure 18: The schematic diagram of the Langendorff heart apparatus during an experiment showing the technique of heart cells isolation.

Figure 19: Photograph of heart cells isolation apparatus Langendorff.

4.3.4. Data analysis

Data values were expressed as Mean±SE (n=4-7).Comparison between data sets were made using ANOVA with a Tukey Post- test.

4.4. Results

4.4.1. The healthy appearance and shape of isolated cardiomyocytes.

Figure 20 is a photomicrograph of freshly isolated cardiomyocytes from the left and right ventricles of a Wistar rat. This photo shows that the majority of cells had the typical rod shaped morphology, and were quiescent at an extracellular Ca+2 concentration of 1 mM. A small number of hypercontracted cells excluding or including Trypan Blue can also be seen.

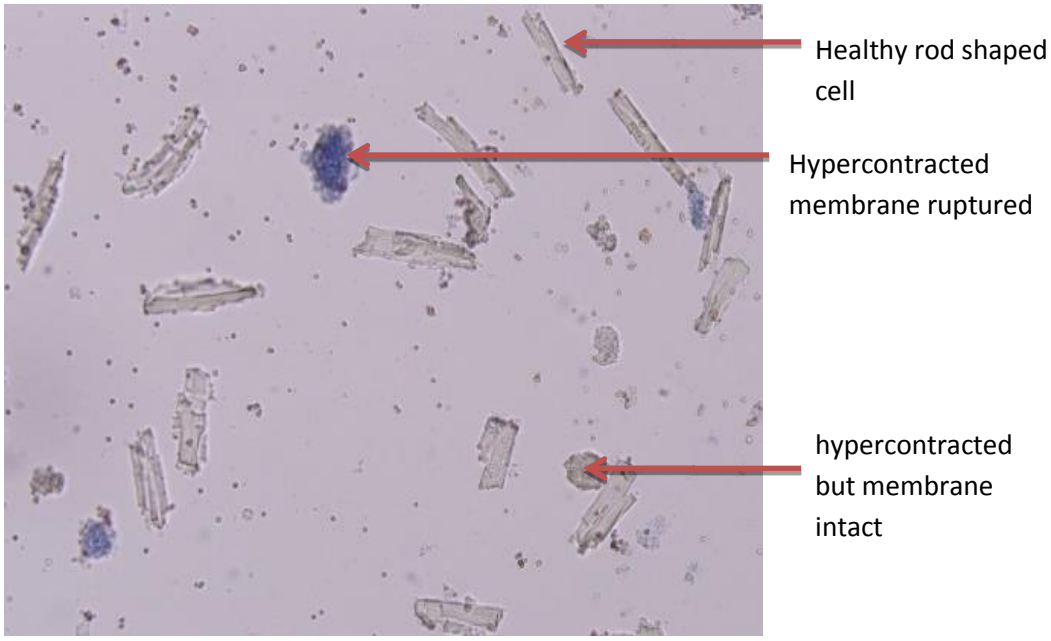

Figure 20: The appearance of cells at the time of isolation showing healthy rod shaped, hypercontracted membrane ruptured and hypercontracted but membrane intact.

4.4.2. Effect of homocysteine with/without oxidative stress on the viability of cells isolated in Tyrode.

Figure 21 shows there was significant reduction of cardiomyocyte viability over time in controls and in the presence of homocysteine. A larger decrease in cell viability over time was seen with oxidative stress and the biggest reduction observed in the presence of oxidative stress and homocysteine ($P < 0.01$). a b c d different letters represent different levels of significance.

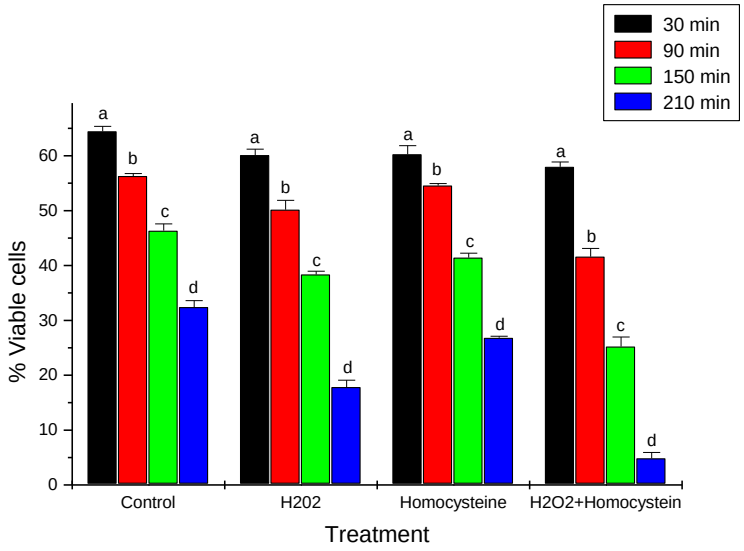

Figure 21: The effect of homocysteine on viability of isolated cardiomyocytes during exposure to oxidative stress over time using Tyrode solution, showing significant changes in the viability of isolated heart cells over time and during exposure to homocysteine and oxidative stress. Data are mean ± SE (n=4-7).

4.4.3. Effect of homocysteine with/without oxidative stress on the viability of cells isolated in Tyrode solution with added taurine.

Figure 22 shows a similar pattern to Figure 21 in that there is a progressive decrease in viability of the cells over time in both control and treated cells, ($P < 0.01$). Having said this it is clear that taurine offers some protection against oxidative insult. a b c d different letters represent different levels of significance.

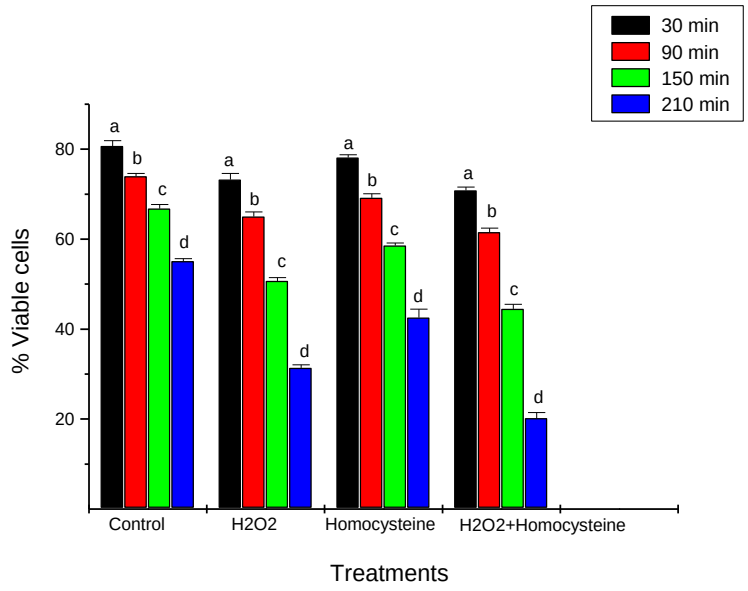

Figure 22: The effect of homocysteine on viability of isolated cardiomyocytes during exposure to oxidative stress over time using Tyrode solution with added taurine showing significant changes in the viability of isolated heart cells over time and during exposure to homocysteine and oxidative stress. Data are mean ± SE (n=4-7).

4.4.4. Effect of homocysteine with/without oxidative stress on the viability of cells isolated in Tyrode solution with added glutamic acid and carnitine to the collagenase and protease enzymes.

Figure 23 shows a similar pattern at different times to Figure 21 and 22. The control was significantly higher by comparison with oxidative stress and homocysteine treatments ($P < 0.01$). As expected at 30 minutes the viability of isolated cardiomyocytes was significantly higher ($P < 0.01$) by comparison with (90,150, and 210 minutes). a b c d different letters represent different levels of significance.

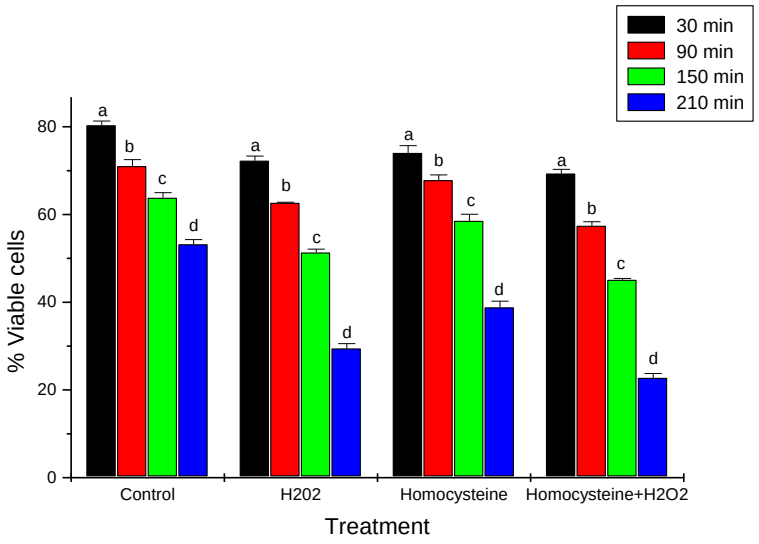

Figure 23: The effect of homocysteine on the viability of isolated cardiomyocytes during exposure to oxidative stress over time using Tyrode solution with added glutamic acid and carnitine to the digest enzymes, indicated above showing significant changes in the viability of isolated heart cells over time and during exposure to homocysteine and oxidative stress treatments. Data are mean ± SE (n=4-7).

4.4.5. Effect of homocysteine with/without oxidative stress on the viability of isolated heart cells in three different methods

Figure 24 shows a similar pattern at three different methods with same treatment and all after 210 minutes incubation there were significant changes in the viability of isolated heart cells ($P < 0.001$) in Tyrode solution with added taurine and Tyrode solution with added glutamic acid+carnitine to the digest enzyme in comparison with Tyrode alone, however there were no significant changes between the added two amino acids. There was significant reduction of cardiomyocyte viability over time in controls and in the presence of homocysteine. A larger decrease in cell viability over time was seen with oxidative stress with the biggest reduction observed in the presence of oxidative stress and homocysteine. It is clear that the amino acids provide some protection to cardiomyocytes. a b different letters represent different levels of significance.

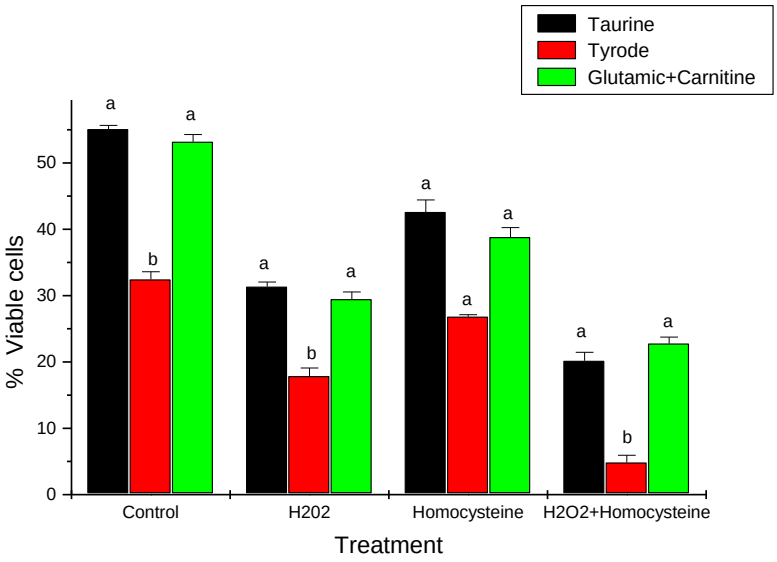

Figure 24: The effect of homocysteine on the viability of isolated cardiomyocytes during exposure to oxidative stress over 210 minutes time using Tyrode, Tyrode with added taurine, and Tyrode with added glutamic acid and carnitine to the digest enzymes, indicated above showing significant changes in the viability of isolated heart cells over time and during exposure to homocysteine and oxidative stress treatments. Data are mean ± SE (n=4-7).

4.4.6. Effect of homocysteine with/without oxidative stress on the morphology of cells isolated in Tyrode.

Figure 25 shows there was a significant reduction in the number of cardiomyocytes showing rod shaped morphology over time in the controls and in the presence of homocysteine ($p<0.05$). This decrease was enhanced in cardiomyocytes exposed to oxidative stress with the greatest reduction seen in cardiomyocytes exposed to oxidative stress and homocysteine ($P < 0.05$ and < 0.01). a b c d different letters represent different levels of significance.

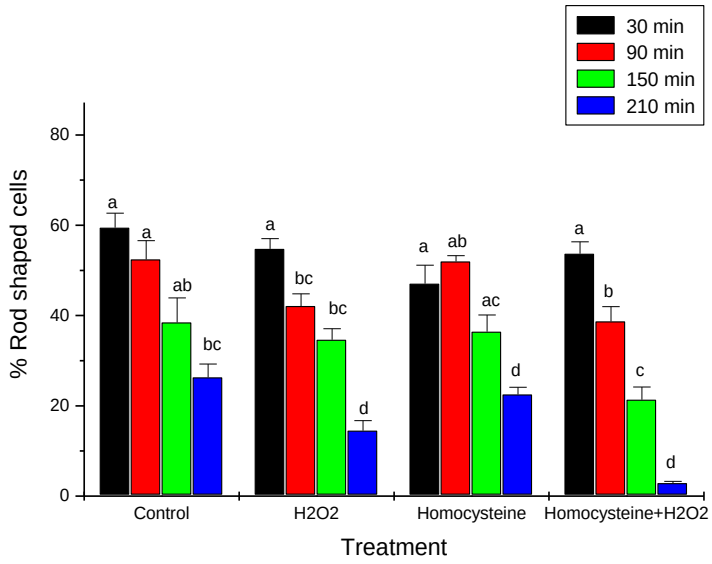

Figure 25: The effect of homocysteine on the number of rod shaped cells during exposure to oxidative stress over time using Tyrode solution, showing significant changes in the morphology of isolated heart cells over time and during exposure to homocysteine and oxidative stress. Data are mean ± SE (n=4-7).

4.4.7. Effect of homocysteine with/without oxidative stress on the morphology of cells isolated in Tyrode with added taurine.

Figure 26 shows there were significant reductions in rod shape cells at 90, 150, 210 minutes in control by comparison with 30 minutes control ($P < 0.01$) however, there were no significant changes over 90, 150, 210 minutes in control. Figure 26 shows a similar pattern in rod shape cells after insult with H_2O_2 and insult with homocysteine in that there is a progressive decrease in morphology of the cells over time in both control and treated cells ($P < 0.05$) and ($P < 0.01$) it is clear that normal morphology cells decreased during insult with oxidative over time more than control and homocysteine. Having said this it is clear that taurine offers some protection against oxidative insult. a b c d different letters represent different levels of significance.

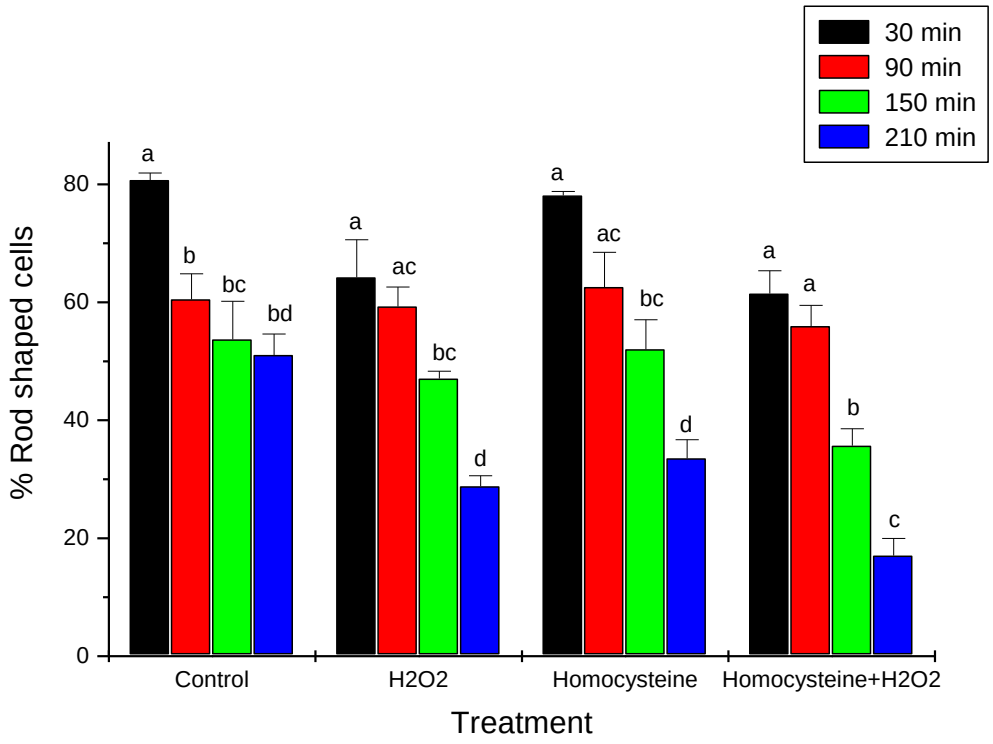

Figure 26: The effect of homocysteine on the number of rod shaped of isolated cardiomyocytes during exposure to oxidative stress over time using Tyrode solution with added taurine. Data are mean ± SE (n=4-7).

4.4.8. Effect of homocysteine with/without oxidative stress on the morphology of cells isolated in Tyrode solution with added glutamic acid and carnitine to the digest enzymes.

Figure 27 shows a similar pattern at different times to Figure 25 and 26. The control was significantly higher by comparison with oxidative stress and homocysteine treatments ($P < 0.01$). As expected at 30 minutes the rod shape cells number of isolated cardiomyocytes was significantly higher ($P < 0.05$) by comparison with 90,150, and 210 minutes ($P < 0.01$) and ($P < 0.05$). a b c d different letters represent different levels of significance.

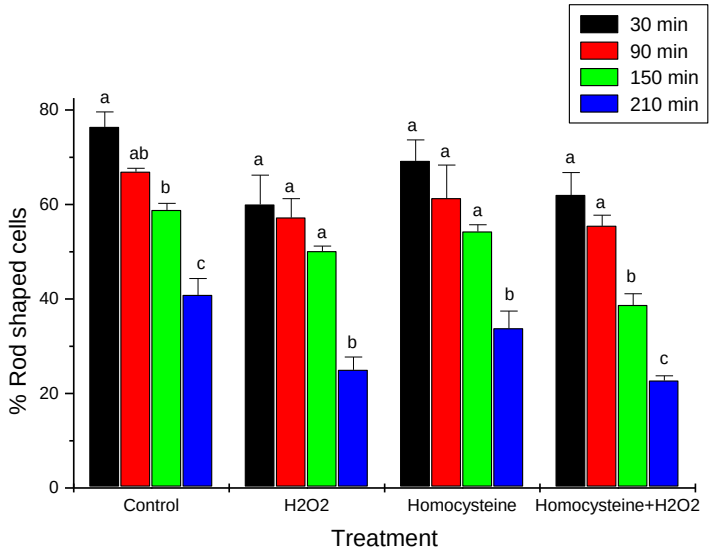

Figure 27: The effect of homocysteine on the number of normal rod shaped isolated cardiomyocytes during exposure to oxidative stress over time using Tyrode solution with added glutamic acid and carnitine to the digest enzymes. Data are mean ± SE (n=4-7).

4.5. Discussion

This study has described a classic technique (Langendorff) that was used to isolate heart cells from rat hearts perfused with digestive enzymes. This technique enables an easy and reproducible isolation of healthy cells from rat heart under physiological conditions. Collagenase digestion alone is usually insufficient therefore in this technique added proteases were used to facilitate tissue digestion and single cell dispersal, respectively.

An augmented plasma concentration of homocysteine has been recognised as an independent risk factor for development of cardiovascular disease (Antoniades *et al.*, 2009; Fryer *et al.*, 1993; gopinath, 2012). Recent studies investigated the possible mechanisms by which hyperhomocysteinaemia may impair vascular function (Antoniades *et al.*, 2009; Ross , 1986). However little work has been carried out investigating the effect of homocysteine on the functioning of freshly isolated heart cells and their response to oxidative stress, and susceptibility to apoptosis. Therefore the aim of this study was to test the hypothesis that homocysteine exposure, during stressful conditions in the heart cells would compromise cell performance. The findings of Levrand *et al.*,(2007) who found that 0.1 mM homocysteine induces cell death in cultured H9C2

cardiomyocytes (Levrand *et al.*, 2007) agrees with significant studies by Rohilla *et al.*, (2010) that hyperhomocysteinaemia increases oxidative stress during ischaemia reperfusion (Rohilla *et al.*, 2010). Therefore it was not surprising that the results in Figures (21, 22, and 23) suggested that homocysteine incubated cardiomyocytes were less able to withstand an oxidative insult compared to control. In conclusion exposure to 0.05 mM homocysteine of the freshly isolated heart cells during insult with oxidative stress reduces the number of viable cells possibly through a mechanism involving disrupted ionic homeostasis. These findings may contribute to an understanding of the greater vulnerability to cardiovascular disease shown by those suffering from genetic conditions causing deficiencies of cystathionine β-synthase, or in enzymes involved in methyl-B_{12} synthesis and homocysteine methylation (Pacher *et al.*, 1999; Refsum *et al.*, 1998; Selhub, 2006). Recent studies have shown that amino acids such as glutamine, taurine, glycine exhibit cytoprotective effects on the cardiomyocyte (Jiang *et al.*, 2011; King *et al.*, 2006; King *et al.*, 2003; Sahin *et al.*, 2011). However there are few studies demonstrating the protective effects of amino acids on freshly isolated heart cells. The results here showed that 40 mM taurine with Tyrode (Figures 22) protects cadiomyocytes by comparison with Tyrode alone and showed the response of freshly isolated heart cells

to oxidative stress with greater resistance compared with heart cells prepared from Tyrode (Figures 21). Similarly results showed that using Tyrode solution with added glutamic acid and carnitine to the digest enzymes (Figures 23) protects cardiomyocytes by comparison with Tyrode alone (Figures 21). Again it is clear that glutamic acid and carnitine itself provides significant protection against oxidative insult.

Chapter5：Conclusions overall

Compared to control, the addition of 2 mM or 5 mM L-glycyl-glutamine during ischaemia reperfusion of isolated and perfused rat hearts increased the time taken to ischaemic contracture; recovery of contractile function; decreased reperfusion damage; improved energy metabolism; reduced oxidative stress; and significantly improved the recovery of left ventricular developed pressure. At the end of ischaemia the lactate concentration in the control was significantly greater compared to the value at the beginning of ischaemia in the control. This was not the case in the presence of any of the different gly-gln concentrations where the value at the beginning and end of ischaemia were not significantly different from each other. Despite a strong dose related trend differences in the TBARs data did not quite reach significance.

These findings support a positive role for L-glycyl-glutamine in myocardial protection against ischaemia reperfusion.

Exposure to 0.05 mM homocysteine impairs the viability of isolated cardiomyocytes reduces the number of rod shape cells compared to control and compromises the contractility of freshly isolated cells during exposure to oxidative stress probably through

a mechanism involving disrupted ionic homeostasis. These findings may help to explain the greater vulnerability to cardiovascular disease display by those suffering from genetic conditions. Added taurine and glutamate exhibited protective effects on the cardiomyocyte by giving more resistance against insult by homocysteine and oxidative stress.

Chapter6：Future Research

The establishment of easy robust and reproducible heart cell isolation opens up the possibility of new frontiers in cardiovascular research. This includes further research investigating the long- term effects of hypertension contributing factor in the leading cause of death in Australia in 2007 (Health.A.S, 2010). With respect to homocysteine, future research is likely to focus on investigating homocysteine transport in heart cells. This is important because the rate and characteristics of homocysteine transport across the heart cell membrane is likely to be a rate limiting factor in controlling the intracellular homocysteine concentration .Other investigations will concentrate on the effects of homocysteine on signaling pathways in heart cells and the effects of homocysteine in the presence of the other cardiovascular risk factors such as hypertension (King *et al.*, 2012) and advanced age. Some of the best methods of protecting the heart during cardiac surgery become considerably less effective when applied to the ageing heart (Jahangir, 2007). More research is needed on the role of amino acids and antioxidant enzymes in protective mechanisms to cardiomyocytes in advanced age.

Chapter7 ：References

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