THE UNIVERSITY OF HULL

The Effects of Preconditioning Coronary Artery Disease Patients With Hyperbaric Oxygen Prior To Coronary Artery Bypass Graft Surgery & Cardiopulmonary Bypass

(PROPHYlACTiC)

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"It Always Seems Impossible

Until It is Done"

~Nelson Mandela

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Jeysen Z Yogaratnam

// Abstract

Introduction

Coronary artery bypass graft (CABG) is associated with periods of ischaemia and reperfusion, which may lead to myocardial dysfunction. In clinical studies, hyperbaric oxygen (HBO_2) treatment following an acute myocardial infarction (AMI), has been shown to limit myocardial injury and improve myocardial function. The primary efficacy objective of this study was to determine if systemically preconditioning coronary artery disease (CAD) patients with HBO_2, prior to first time elective on cardiopulmonary bypass (CPB) CABG surgery, leads to a remote preconditioning like effect that is capable of improving myocardial function following CABG. The main secondary objectives of this study were to assess the safety of HBO_2 preconditioning and, its effects on myocardial injury and post operative intensive care unit (ICU) length of stay. The exploratory secondary objectives were to assess the effects of HBO_2 preconditioning on surrogate serum biomarkers of endothelial and neutrophilic adhesiveness and, myocardial biomarkers of cardioprotection.

Methods

In this single centre, randomised control study, 81 patients, who were having first time elective on CPB CABG surgery, were recruited. 40 were randomised to the Control Group and 41 to the HBO_2 Group. Treatment with HBO_2 preconditioning was completed approximately 2 hours prior to CPB and consisted of two 30 minute sessions of 100% oxygen at 2.4 atmospheres (ATA) separated 5 minutes apart. Efficacy was measured by determining peri-operative haemodynamic measurements using a pulmonary artery (PA) catheter. Safety was measured by collecting peri-operative data on myocardial injury and adverse events (AEs) and, post operative days spent in ICU. Using collected peri-operative venous blood, myocardial injury was determined by measuring the concentration of serum Troponin-T. In these same venous blood samples, endothelial and neutrophilic adhesiveness was indirectly assessed by measuring the concentrations of sE-selectin, sP-Selectin and sICAM-1 and, sPSGL-1, respectively. Using intra-operative right atrial biopsies, the degree of cardioprotection provided by HBO_2 preconditioning was determined by measuring the quantity of myocardial eNOS and Hsp72. Analysis of the serum and myocardial biomarkers were done by ELISA.

Results

Compared to the Control Group, the HBO_2 Group demonstrated a significant improvement in left venticular stroke work (LVSW) 24 hours post CPB (p=0.005). While there were no significant safety findings, there were fewer cardiovascular, pulmonary, renal and neurological AEs in the HBO_2 Group. This group also had a significantly shorter post operative ICU length of stay. 1 hour post HBO_2 preconditioning, the concentration of sPSGL-1 increased significantly in the HBO_2 Group. At all time points, the peri-oprative concentration of sPSGL-1 was higher in the HBO_2 Group but none of the changes were significant. The latter was also the case for the peri-operative concentration of sP-Selectin, apart from following the period of ischaemic and reperfusion, when it was lower in the HBO_2 Group. Intra-operatively, the concentration of sE-Selectin increased significantly in the HBO_2 Group and was higher in this group throughout the peri-operative period. During this intra-operative period also, the concentration of sICAM-1 was higher in the HBO_2 Group and the increase was particularly significant following the period of ischaemia and reperfusion. 24 hours post CPB, the concentrations of all the serum soluble adhesion molecules were higher in the HBO_2 Group. No significant differences were observed between the groups with respect to the concentrations of serum Troponin-T and, the quantity of myocardial eNOS and Hsp72. However, in the HBO_2 Group, the peri-operative concentrations of serum Tropinin-T, eNOS and Hsp72 were lower. Furthermore, while there was a pre-CPB reduction of both eNOS and Hsp72, following ischaemia and reperfusion, the quantity of both these myocardial biomarkers were increased.

Conclusion

From this study, it can be concluded that HBO_2 preconditioning of patients with CAD prior to on CPB CABG, is capable of improving myocardial function 24 hours post CABG. Additionally, the data suggest that this may also be a safe modality of treatment as it did not lead to significant post operative AEs, limited peri-operative myocardial injury and reduced post operative ICU length of stay. It also led to increased post operative concentrations of the measured surrogate biomarkers of endothelial and neutrophilic adhesiveness, with a number of significant peri-operative changes. Finally, while HBO_2 treatment did not lead to significant changes in the myocardial biomarkers of cardioprotection, the quantities of these increased in the HBO_2 Group following ischaemia and reperfusion, suggesting that it may be capable of inducing endogenous cardioprotection following ischaemia and reperfusion.

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   Jeysen Zivan Yogaratnam, Gerard Laden, Leigh Anthony Madden, Levant Guvendik, Mike Cowen, Anne-Marie Seymour, John Greenman, Alex Cale, Steve Griffin
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   Jeysen Zivan Yogaratnam, Gerard Laden, Leigh Anthony Madden, Levant Guvendik, Mike Cowen, Anne-Marie Seymour, John Greenman, Alex Cale, Steve Griffin
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5. **Hyperoxic Hyperbaric Preconditioning Induces Endogenous Myocardial Protection Against Ischaemic Reperfusion Injury"**
   J.Z. Yogaratnam, G. Laden, S.C. Griffin, A.R.J.Cale, M. Cowen, L.Guvendik
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   Jeysen Zivan Yogaratnam
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3. **Hyperbaric Oxygen & Cardiovascular Preconditioning**
   Jeysen Zivan Yogaratnam
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4. **Hyperbaric Oxygen Induced Myocardial Protection & Revascularisation Via Reactive Oxygen Species**
   Jeysen Zivan Yogaratnam
   University of Karolinska Postgraduate Course in Hyperbaric Medicine, Stockholm, Sweden (April 2006)

5. **Hyperbaric Medicine, Ischaemic Reperfusion Injury, & Organ Protection: Molecular Mechanisms"**
   Jeysen Zivan Yogaratnam
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Abbreviations

- ADP: Adenosine DiPhosphate
- AE: Adverse Event
- AMI: Acute Myocardial Infarction
- ATA: Atmosphere Absolute
- ATP: Adenosine TriPhosphate
- AF: Atrial Fibrillation
- BMI: Body Mass Index
- BIPAP: Biphasic Positive Airway Pressure
- °C: degree Celsius
- CABG: Coronary Artery Bypass Graft
- CAD: Coronary Artery Disease
- CI: Cardiac Index
- CK: Creatine Kinase
- CK-MB: Creatine Kinase-MB
- CO: Cardiac Output
- CPAP: Continuous Positive Airway Pressure
- CPB: Cardiopulmonary Bypass
- CVVH: Continuous Venovenous Haemofiltration
- cDNA: complementary DeoxyriboNucleic Acid
- DNA: DeoxyriboNucleic Acid
- dNTP: deoxyriboNucleotide TriPhosphate
- dATP: deoxyAdenine TriPhosphate
- dGTP: deoxyGuanosine TriPhospnate
- dCTP: deoxyCytosine TriPhosphate
- dTTP: deoxyThymine TriPhosphate
- DVT: Deep Vein Thrombosis
- EF: Ejection Fraction
- EDTA: Ethylene Diamine Tetraacetate
- ECG : Electrocardiogram
- GTP: Guanosine Triphosphate
- HBO_2: Hyperbaric Oxygen
- Hct: Haematocrit
- HOT-MI: Hyperbaric Oxygen Therapy in Myocardial Infarction
- HOT-PI: Hyperbaric Oxygen Therapy in Percutaneous Interventions
- HR: Heart Rate
- HRP: Horseradish Peroxidase
- HSF: Heat Shock Factor
- Hsp: Heat Shock Protein
- ICAM-1- Intercellular Cell Adhesion Molecule-1
- IL: Interleukin
- IPC: Ischaemic Preconditioning
- IRI: Ischaemic Reperfusion Injury
- IABP: Intra-Aortic Balloon Pump
- LAD: Left Anterior Descending

- L-NAME: L-Nitro-Arginine Methyl Ester
- LPS: Lipopolysaccharide
- LVDP: Left Ventricular Developed Pressure
- LVEDP: Left Ventricular End Diastolic Pressure
- LVEF: Left Ventricular Ejection Fraction
- LVSP: Left Ventricular Systolic Pressure
- LVSW: Left Ventricular Stroke Work
- LVSWI: Left Ventricular Stroke Work Index
- MAP: Mean Arterial Pressure
- MAPK: Mitogen Activated Protein Kinase
- MI: Myocardial Infarction
- mmHg: millimetres of Mercury
- M-MLV RT: Moloney Murine Leukemia Virus Reverse Transcriptase
- MPAP: Mean Pulmonary Artery Pressure
- mRNA: messenger Ribonucleic Acid
- NFκB: Nuclear Factor Kappa B
- NO: Nitric Oxide
- O_2: Oxygen
- ·OH: Hydroxyl Radical
- Oligo-dt: Oligo dinucleotide
- PAI-1: Plasminogen Activator Inhibitor Type 1
- PCI: Percutaneous Coronary Interventions
- PCR: Polymerase Chain Reaction
- PCWP: Pulmonary Capillary Wedge Pressure
- PE: Pulmonary Embolus
- PKC: Protein Kinase C
- PVR: Pulmonary Vascular Resistance
- PVRI: Pulmonary Vascular Resistance Index
- RNA: Ribonucleic Acid
- ROS: Reactive Oxygen Species
- RPM: Revolutions Per Second
- rTPA: recombinant Tissue Plasminogen Activator
- RT-PCR: Reverse Transcriptase Polymerase Chain Reaction
- RVSW: Right Ventricular Stroke Work
- RVSWI: Right Ventricular Stroke Work Index
- STK-streptokinase
- SV: Stroke Volume
- SVR: Systemic Vascular Resistance
- SVRI: Systemic Vacular Resistance Index
- TBE: Tris-borate-EDTA
- TIA: Transient Ischaemic Attack
- TNF-α: Tumour Necrosis Factor Alpha
- t-PA: tissue Plasminogen Activator
- SPECT: Single Photon Emission Computer Tomography
- u-PA: urokinase Plasminogen Activator
- WP: Weibel Palade

1. Introduction

Coronary artery bypass graft (CABG) surgery has been shown to be a life saving procedure (Guyton, 2006) which leads to longer survival (Eagle et al., 2004) and better quality of life (Loponen et al., 2007) when compared with medical therapy or percutaneous intervention, in specific groups of patients suffering from coronary artery disease (CAD) (Eagle et al., 2004). This surgical procedure, when performed during cardiopulmonary bypass (CPB), requires a relatively bloodless operating field without any myocardial contractions. This is achieved by clamping the aorta with a cross-clamp, which induces a controlled global myocardial ischaemia due to the reduced coronary perfusion. As a result of this myocardial ischaemia, the heart becomes relatively motionless heart and this facilitates the anastamosis of the bypass conduit to the coronary artery (distal anastamosis). In order to protect the myocardium during this period of ischaemia, one of two very different myocardial protective strategies maybe utilised. One strategy involves the use of a potassium enriched solution known as St. Thomas Cardioplegia solution (Hearse et al., 1976) during the global ischaemic period. This hyperkalaemic solution causes a cardiac arrest and stand still, while at the same time providing the myocardium with substrates necessary to keep it metabolically viable (Rosenkranz, 1995). The other strategy, known as hypothermic (28°C-32 ^{o}C) intermittent ischaemic fibrillatory arrest (Lucas et al., 1980), involves the use of short, repeated intervals of controlled global ischaemia, by clamping the aorta, together with a controlled electrically

induced ventricular fibrillation. This leads to a relatively motionless heart while also protecting the myocardium (Abd-Elfattah et al., 1995).

While on CPB CABG using either of these myocardial protective strategies has been shown to be a safe and effective means of providing myocardial protection (Alex et al., 2005, Alhan et al., 1996, Anderson et al., 1994, Liu et al., 1998, Scarci et al., 2009), the need for the application of an aortic cross-clamp to achieve a bloodless operating field without myocardial contractions, followed by its removal, also means that during CABG surgery, the myocardium is exposed to durations of ischaemia followed by durations of reperfusion. This ischaemia and reperfusion leads to an injury known as ischaemic reperfusion injury (IRI) (Venugopal et al., 2009) which can cause mortality, myocardial infarction (MI), unstable angina, ventricular failure, life-threatening arrhythmia, renal insufficiency and stroke (Alexander et al., 2005, Desai et al., 2004, Eagle et al., 2004, Edwards et al., 1994, Ferguson et al., 2002, Manning and Hearse, 1984). Despite efforts to improve the post operative outcomes, post CABG adverse events (AEs) remain common and add substantially to hospital costs for this procedure (Brown et al., 2008). It has been reported that over half of in-hospital deaths after CABG occur in patients with normal baseline left ventricular function and, are related to IRI and low cardiac output (CO) in post operative period (O'Connor et al., 1998). Furthermore, 25% to 45% of patients dying soon after CABG have histological evidence of IRI at autopsy (Bulkely and Hutchins, 1977, Moore and Hutchins, 1981, Weman et al., 2000).

1.1 Myocardial Ischaemic Reperfusion Injury

The common denominator of myocardial IRI involves the acute interruption of coronary blood flow, due to obstruction, followed by the return of coronary flow when that obstruction is removed or bypassed (Piper et al., 1998, Prasad et al., 2009). During the period of no flow, cells are metabolically compromised by hypoxic conditions that cause cellular dysfunction and eventually lead to cell death. When the coronary blood flow is restored, this return of myocardial perfusion, paradoxically, despite restoring the ischaemic myocardium with oxygen (O_2) and metabolic substrates, leads to another form of myocardial damage termed ‘reperfusion injury’ (Ferrari et al., 1993, Prasad et al., 2009). This is defined as the death of myocytes, alive at the time of reperfusion, as a direct result of one or more events initiated by reperfusion (Braunwald and Kloner, 1985). One of the causes for reperfusion injury is a second period of decreased flow that occurs during reperfusion. This is known as the no-reflow phenomenon (Ito, 2006, Kloner et al., 1974, Krug et al., 1966). This phenomenon is associated with an increased incidence of acute myocardial infarction (AMI), myocardial rupture and death (Abbo et al., 1995). The mechanism underlying this reperfusion injury is attributed to plugging of the vasculature with neutrophils (Engler et al., 1983) and platelets (Michaels et al., 2000), capillary endothelial damage and intraluminal

swelling (Engler et al., 1983) and, cellular oedema compressing the capillaries (Manciet et al., 1994).

The cellular damage that results from myocardial ischaemia and reperfusion can be reversible or irreversible and is related to the duration of the preceding ischaemic insult (Jennings and Reimer, 1983). When myocardial ischaemia is limited to periods of less than 20 minutes, reperfusion leads to recovery following transient changes in cellular structure, function and metabolism. These changes manifest clinically as depressed myocardial contractility, which may persist for a variable period of time. This condition is not associated with myocardial necrosis and is termed ‘myocardial stunning’. This is defined as the mechanical dysfunction that persists after reperfusion of previously ischaemic tissue in the absence of irreversible damage including myocardial necrosis (Hess and Kukreja, 1995). However, when myocardial blood flow is restored after an ischaemic period of greater than 20 minutes, the myocardium sustains a reperfusion injury that results in myocardial necrosis and the extent is directly proportional to the duration of the ischaemic insult (Jennings and Reimer, 1983).

1.2 The Inflammatory Response of Ischaemic Reperfusion Injury

Myocardial ischaemia elicits an acute inflammatory response that is greatly augmented by reperfusion (Hearse and Bolli, 1992, Steffens et al., 2009, Suleiman et al., 2008). In the human heart, this acute inflammatory response is associated with the production of reactive oxygen species (ROS) and lipid peroxidation products and, is accompanied by intra-coronary release of pro-inflammatory cytokines and vasoactive substances (Vaage and Valen, 1993). Part of this ischaemic-reperfusion induced inflammatory damage is mediated by neutrophils which accumulate in myocardium under the influence of chemo-attractants (Entman et al., 1991, Mehta et al., 1988, Reimer et al., 1989, Vinten-Johansen, 2004). In addition to physically plugging capillaries (Engler et al., 1983), neutrophils also bind to the endothelial surface and cause endothelial cell dysfunction and tissue destruction through a variety of cytotoxic mechanisms that involve ROS, cytotoxic enzymes and cytokines (Inauen et al., 1990).

1.2.1 Neutrophil & Endothelial Adhesion Molecules

Neutrophil mediated endothelial injury is dependent on the interaction of adhesion glycoproteins [L-selectin, Sialyl Lewis^x, P-selectin glycoprotein ligand-1 (PSGL-1), and CD11/CD18] expressed on the surface of circulating neutrophils, with adhesion molecules expressed on the surface of the endothelium [P-selectin, E-selectin, and intracellular adhesion molecule-1 (ICAM-1)].

1.2.1.1 The Selectin Family Of Adhesion Molecules

The selectin family of adhesion molecules mediates the initial capture of neutrophils from the blood stream, before their firm adhesion and diapedesis at the sites of tissue injury and inflammation (Ebnet and Vestweber, 1999). L-selectin is expressed constitutively on neutrophils (Bevilacqua and Nelson, 1993) and is rapidly shed after neutrophil activation (Kishimoto et al., 1989). P-selectin, is constitutively stored in the Weibel-Palade bodies of endothelial cells and in the α granules of platelets and, is rapidly mobilised to the cell surface in response to various inflammatory stimuli like thrombin, histamine, and ROS (Lorant et al., 1991, Patel et al., 1991). E-selectin expression is largely restricted to endothelial cells and is activated by stimuli such as endotoxins, the pro-inflammatory cytokine interleukin (IL)-1 and tumour necrosis factor (TNF) (Bevilacqua et al., 1989). Unlike P-selectin, E-selectin expression takes 4-8 hours to occur following stimulation (Lasky,

1995) as E-selectin expression requires *de novo* messenger mRNA and protein synthesis. It usually returns to baseline levels 24 hours after the stimulating event.

In the first 20 minutes after tissue injury, neutrophil rolling on the vascular endothelium is mainly mediated by P-selectin, with minimal L-selectin contribution (Griffin et al., 1990). Subsequently, the role of P-selectin diminishes due to internal degradation, and L-selectin becomes the principal mediator of neutrophil rolling. There is little appreciable role for E-selectin in the early response to injury. Rather, initial interaction between the neutrophil and the vascular endothelium, mediated via P-and L-selectins, is later followed by a stronger interaction involving E-selectin and subsequently integrins, prior to extravasation of the neutrophil, via the blood vessel wall, into lymphoid tissues and to the sites of inflammation (Lasky, 1995). To date, the best characterised cell adhesion ligand for P- and E-selectins is PSGL-1 (Cummings, 1999), which is found on the surface of neutrophils. It is a stronger ligand for P-selectin than for E-selectin (Moore et al., 1992).

1.2.1.2 ICAM-1

ICAM-1, is expressed on the surface of endothelial cells, epithelial cells and fibroblast (Rothlein et al., 1986). Its expression is induced by cytokines IL-1, IL-6 and TNF-α (Frangogiannis et al., 2002, Frangogiannis et al., 1998). Once stimulated, its expression is significantly increased within 4 hours of induction and cell surface levels may remain elevated for days (Lindsberg et al., 1996). ICAM-1 is one of the primary ligands for the neutrophilic adhesion molecule, CD-18 (Albelda et al., 1994).

1.2.1.3 Ischaemic Reperfusion Injury & Adhesion Molecules

In experimental mouse models, ischaemia followed by brief (Palazzo et al., 1998a) and prolonged (Jones et al., 2000) periods of reperfusion have been shown to upregulate P-Selectin expression in the coronary circulation and enhance neutrophil accumulation. However, in mice that were made genetically deficient for P-Selectin, neutrophil accumulation and myocardial injury were attenuated following IRI (Jones et al., 2000, Palazzo et al., 1998a). Similarly, it also been demonstrated that E-Selectin was upregulated in the mouse myocardium following ischaemia and reperfusion (Jones et al., 2000), and that genetic deficiency of E-Selectin conferred myocardial protection by limiting myocardial infarct size owing to the attenuation of neutrophil accumulation (Sligh et al., 1993). It has also been demonstrated that mice that were made genetically deficient of ICAM-1, showed a significant reduction in myocardial necrosis in association with an

attenuation of myocardial neutrophil infiltration following both brief (Palazzo et al., 1998b) and extended (Jones et al., 2000) periods of reperfusion.

Due the deleterious effects of neutrophil-endothelial interactions via the selectin group of adhesion molecules, many selectin inhibitory therapies have been developed. These therapies involve the use of monoclonal antibodies (Nigam and Kopecky, 2002), Sialyl Lewis^x analogues, PSGL-1 analogues (Ley, 2003), nitric oxide (NO) enhancing agents (Li et al., 2009) and small-molecule selectin antagonist (Onai et al., 2003). More recently, there has also been evidence to suggest a role for hyperbaric oxygen (HBO_2) in limiting neutrophil-endothelial interactions (Buras and Reenstra, 2007).

1.3 Ischaemic Reperfusion Injury & Reactive Oxygen Species

While the lack of oxygen during ischaemia is deleterious to myocardial survival, the restoration of oxygen during reperfusion of the previously ischaemic myocardium, paradoxically, has also been demonstrated to lead to a myocardial injury which is worse than the injury induced by ischaemia alone (Hearse et al., 1973). It has now been established that the presence of oxygen during the early moments of reperfusion of the ischaemic myocardium leads to an oxidative stress via a burst of ROS (Kevin et al., 2003, Park and Lucchesi, 1999). These ROS arise from a variety of sources, such as the xanthine oxidase system, activated neutrophils, the electron transport chain of mitochondria, and the arachidonic acid pathway (Kloner et al., 1989, Kukreja and Hess, 1992).

ROS have been suggested to be responsible for the post ischaemic myocardial dysfunction characterized by myocardial stunning and post ischaemic arrhythmia (Kevin et al., 2005, Kloner et al., 1989, Kukreja and Hess, 1992). At present, controversy exist as to as to whether ROS leads to MI as there have been some experimental studies which support the use of anti-oxidant (Ambrosio et al., 1986, Chi et al., 1989, Kilgore et al., 1994, Naslund et al., 1986) and others (Downey et al., 1991, Nejima et al., 1989, Ooiwa et al., 1991, Uraizee et al., 1987, Vanhaecke et al., 1991) which do not support the use of anti-oxidants to limit the possibility of ROS induced MI.

In an experimental study, it was demonstrated that when the myocardium was exposed to ischaemia, the generation of ROS rapidly increased, but this increase did not occur at the same rate during subsequent episodes of ischaemia and reperfusion (Das et al., 1999b). It was suggested that the initial ischaemia and reperfusion led to the development of an oxidative stress that adapted the myocardium to the subsequent oxidative stresses of ischaemia and reperfusion and this, resulted in the observed ROS mediated reduction in ischaemia-reperfusion induced injury. The myocardial protective abilities of the ROS adapted myocardium were completely abolished when the myocardium was pre-perfused with N-acetyl cysteine to scavenge ROS, suggesting that redox signaling may play a crucial role in generating survival signals during myocardial adaptation to ischaemia (Maulik et al., 1998). In keeping with this, ROS have been demonstrated to function as signaling molecules (Herrlich and Bohmer, 2000, Rosette and Karin, 1996). By initiating genetic expression and, the consequent synthesis of a variety of functional and structural proteins, ROS signaling may allow for the adaptation and survival of the cells (Das and Maulik, 2003, Das and Maulik, 2004) or, depending on the intensity and duration of the signal, activate the processes responsible for the cell damage or death (Buttke and Sandstrom, 1994, Das, 2001). Thus, it is possible that an oxidative attack may induce either a loss or a gain of a function or lead to a switch to a different function.

1.4 Ischaemic Reperfusion Injury, Nitric Oxide & Nitric Oxide Synthase

The administration of NO donors prior to ischaemia have been demonstrated to reduce myocardial infarct size and endothelial dysfunction as a result of IRI (Bolli, 2001). Furthermore, pre-treatment with drugs that enhance NO release such as statins (Lefer et al., 1999), calcium channel antagonist (Asanuma et al., 2001), ACE inhibitors (Hartman et al., 1994) and dexamethasone (Hafezi-Moghadam et al., 2002), have also been shown to protect the myocardium against IRI. While these provide evidence to support the role for exogenous administration of NO to initiate protection against IRI, there is also evidence, based on models of ischaemic preconditioning (IPC), demonstrating a role for endogenously induced NO in mediating early (Han et al., 2008b) and delayed (Bolli, 2000) myocardial protection against IRI.

1.4.1 Nitric Oxide & Nitric Oxide Synthase

NO, first characterized as endothelium-derived relaxation factor, is a ubiquitous signalling messenger molecule that is involved in neurotransmission, inflammatory and immune responses and, vascular homeostasis (Gong et al., 2004, Moncada et al., 1988). It is formed by the oxidation of the guanidine moiety of L-arginine resulting in the products NO and L-citrulline (Schulz et al., 2004). This reaction is catalysed by nitric oxide synthase (NOS). Once formed, it diffuses freely and has a half-life of only 3-5

seconds (Tuteja et al., 2004) before being rapidly oxidized to the stable inactive end products of nitrite and nitrate (NO^-_2 and NO^-_3) (Geller and Billiar, 1998).

At present, there are three known isoforms of NOS, neuronal NOS (also known as nNOS or NOS 1), inducible NOS (also known as iNOS or NOS 2) and endothelial NOS (also known as eNOS or NOS 3). All three isoforms have been shown to be present in the myocardium (Forstermann et al., 1994, Massion et al., 2005, Xuan et al., 2000) and all have been found to play a role in myocardial NO production (Jugdutt, 2002). nNOS and eNOS are continuously present and are thereby termed constitutive NOS (cNOS). In contrast, iNOS, which is not typically expressed in resting cells, must first be induced by a stress such as ischaemia (Bolli et al., 1997), hypoxia (Jung et al., 2000), lipopolysaccharides (Yin et al., 2007) and cytokines (Balligand et al., 1994).

1.4.2 Nitric Oxide & Its Mechanisms for Myocardial Protection

NO has a negative inotropic and chronotropic (Finkel et al., 1992) effect on the myocardium. The capacity for NO to reduce myocardial contractility (Brady et al., 1993) serves to reduce oxygen consumption and demand. This NO function involves cyclic guanosine monophosphate (cGMP), a secondary messenger produced by the action of NO on soluble guanylate cyclase (Mittal CK, 1982). Guanylate cyclase catalyses the conversion of guanosine triphosphate (GTP) to cGMP. cGMP exerts it protective effects by reducing the influx of calcium through L-type calcium channels (Han et al., 1996, Mery et al., 1993). This, prevents calcium overload which is one of the critical features of mitochondrial dysfunction during IRI (Jennings and Reimer, 1991).

NO has also been shown to maintain coronary vasodilatory tone (McGowan et al., 1994), reduce post ischaemic hyperpermeability (Kubes and Granger, 1992, Noel et al., 1995), decrease platelet adhesion and aggregation (Radomski et al., 1987), reduce neutrophil adherence, migration and associated injury (Conger and Weil, 1995, Jordan et al., 1999, Ronson et al., 1999) and, impair mast cell activation (Johnson et al., 1990). It also inhibits neutrophilic generation of the ROS superoxide (Kubes et al., 1991, Sato et al., 1996). The neutrophil anti-adhesive effects mediated by NO occurs via NO's ability to inhibit the vascular endothelial expression of adhesion molecules, in

particular P-selectin, E-selectin and ICAM-1 (De Caterina et al., 1995, Ohashi et al., 1997).

1.5 Ischaemic Reperfusion Injury & Heat Shock Proteins

Studies in the early 1990's demonstrated that increased expression of Heat Shock Protein (Hsp) may protect the myocardium from a stressful stimulus such as ischaemia and reperfusion (Donnelly et al., 1992, Heads et al., 1996, Radford et al., 1996, Yellon et al., 1992). Amongst the many Hsp, the major inducible form of the Hsp70 stress protein, Hsp72, has been shown to directly protect against myocardial ischaemic damage, improve metabolic and functional recovery and, reduce myocardial infarct size (Hutter et al., 1996, Marber et al., 1995, Radford et al., 1996).

Experimental studies have demonstrated that short durations of global or regional myocardial ischaemia lead to the induction of myocardial Hsp70 (Currie, 1987, Dillmann et al., 1986, Marber et al., 1993) and that the increase in Hsp70 was associated with the protection of the myocardium against a subsequent ischaemic episode (Currie et al., 1988). This increase in Hsp70 was also observed following repeated, brief episodes of coronary artery occlusion prior to prolonged ischaemia and was associated with reductions in myocardial infarct size (Marber et al., 1993). The latter findings also suggests that the degree of cardioprotection correlated directly with the quantity of Hsp70 produced by the preceding stress event (Marber et al., 1994).

1.5.1 Heat Shock Protein 70 & Its Mechanism For Myocardial Protection

During myocardial IRI, there is an oxidative stress that is associated with the generation of ROS (Misra et al., 2009). When the production of ROS exceeds the capacity of endogenous detoxification mechanisms, myocardial cells are damaged, either by ROS directly or by the ROS-dependent triggering of a cascade of pro-inflammatory events. Hsp may favourably interfere with the ROS-induced injuries because of its role as biological molecular chaperones in ensuring quality control of protein folding in major subcellular compartments (Feder and Hofmann, 1999, Li et al., 2002, Santoro, 2000). Hsp70 in particular has a number of cytoprotective functions such as ensuring the appropriate folding of proteins (Bukau and Horwich, 1998), the maintenance of structural proteins (Palleros et al., 1991), the refolding of misfolded proteins (Palleros et al., 1991), the translocation of proteins across membranes and into various cellular compartments (Bukau and Horwich, 1998, Chirico et al., 1988, Deshaies et al., 1988), the prevention of protein aggregation (Bukau and Horwich, 1998) and, the degradation of unstable proteins (Bukau and Horwich, 1998). Additionally, there are also studies that have shown that levels of Hsp closely parallel the activity of antioxidant enzymes such as catalase. This indirectly supports the suggestion that Hsp may also be involved in pathways which are activated to counteract ROS-dependent cellular and tissue damage (Currie et al., 1988, Karmazyn et al., 1990, Mocanu et al., 1993).

Hsp may also function as immunodominant signalling molecules that is capable of downregulating the production of pro-inflammatory cytokines, such as TNF-α, interferon-γ, IL-1α, IL-1β, and IL-6 (Pockley, 2002) and also improving cellular tolerance to inflammatory cytokines such as TNF-α and IL-1 (Jaattela and Wissing, 1993, Muller et al., 1993). In a myocardial model of ischaemia and reperfusion, it has been demonstrated that the upregulation of Hsp70 is associated with the suppression of inflammatory cytokines in (Grunenfelder et al., 2001). It has also been proposed that Hsp could interfere with the signaling pathway of the transcription factor NFκB. This pathway is involved in the transcription of several pro-inflammatory genes. It has been shown that the heat shock response inhibits the activation of the NFκB pathway, resulting in suppression of the cellular inflammatory response (Malhotra and Wong, 2002). The down regulation of cytokine production by Hsp may account for the Hsp-dependent protection of the myocardium during experimental ischaemia and reperfusion.

The myocardial protection provided by Hsp may also be linked to mechanisms involving NO and NOS. Although the exact mechanism involved in the interaction between NO and Hsp is unknown, evidence suggests that NO could trigger Hsp70 synthesis and expression, and this seems to ultimately protect the myocardial cells against the cytotoxic effects of TNF-α (Latchman, 2001). Additionally, it has also been demonstrated in an experimental model of myocardial ischaemia that increased activity of iNOS was associated with reduced myocardial injury and that this protective effect

was paralleled by an elevated expression of Hsp70, with reduced activity of NFκB (Zingarelli et al., 2002).

Hsp may also play a role in cellular protection during periods of energy depletion as cellular ATP depletion has been shown to induce Hsp expression (Benjamin et al., 1992). It has been demonstrated that treatments leading to the accumulation of cellular Hsp70 can reduce protein aggregation resulting from ATP loss (Kabakov et al., 2002). Furthermore, it has also been shown that heat shock not only results in Hsp70 induction but also protection from energy deprivation (Gabai and Kabakov, 1993). This suggest that Hsp accumulation may confer an 'ATP-sparing' effect (Gabai and Kabakov, 1993, Kabakov, 1997).

In addition to an anti-apoptotic function (Garrido et al., 2001, Li et al., 1996), Hsp70 has also been reported to improve the process of fibrotic repair after myocardial injury by enhancing the synthesis of collagen and repair of ion channels (Latchman, 2001). It has been shown that the opening of ATP-sensitive potassium channels, especially in the mitochondria, is a crucial step for ATP synthesis in myocardial cells (Eells et al., 2000). By repairing these ion channels under stressful conditions, Hsp70 upregulation may protect the mitochondrial energy metabolism as well as cellular function in the injured myocardium (Sammut et al., 2001).

1.6 Myocardial Preconditioning

The concept of myocardial preconditioning began with the understanding from experimental studies in late 1970's which demonstrated that while 40 minutes of sustained ischaemia was associated with severe ATP depletion and cell death (Jennings et al., 1978, Reimer and Jennings, 1979), four 10 minute periods of coronary artery occlusion produced no more ATP depletion than a single 40 minute occlusion and did not cause necrosis (Reimer et al., 1986). Its was observed that intermittent brief periods of ischaemia prevented the cumulative effects of a prolonged period of ischaemia by reducing harmful catabolites such as lactate and hydrogen ions which were washed out with each reperfusion (Reimer et al., 1986). These findings led to the hypothesise that multiple brief ischaemic episodes may actually protect the myocardium during a subsequent sustained ischemic insult. This hypothesis was later proven (Murry et al., 1986) and marked the beginning for the era of myocardial preconditioning. In that 1986 study by Murry, anaesthetised open-chest dogs were exposed to four cycles of 5 minutes of coronary artery occlusion followed by 5 minutes reperfusion before being exposed to 40 minutes of coronary occlusion and 4 days of reperfusion. The protocol of four short cycles of ischaemia followed by reperfusion was termed ischaemic preconditioning (IPC). That study demonstrated that dogs exposed to IPC developed significantly smaller infarct sizes than the control animals.

1.6.1 Ischaemic Preconditioning

Since the discovery of IPC, its myocardial protective capacity has been widely studied (Bolli et al., 2007, Das and Das, 2008, Downey et al., 2007, Hausenloy et al., 2005) and now, it is clear that IPC is cardioprotective against myocardial IRI (Bolli, 2001, Bolli et al., 2007). A recent meta-analysis concluded that IPC reduces post operative ventricular arrhythmias, inotrope use and shortens post operative intensive care unit (ICU) stay (Walsh et al., 2008).

IPC induces two distinct windows for myocardial protection (Kuzuya et al., 1993, Marber et al., 1993). The first window of myocardial protection, known as the 'early phase' or 'Classical Preconditioning' (Pagliaro et al., 2001) develops within minutes and lasts 1-2 hours after IPC while the second window, known as the 'late phase' or Delayed Preconditioning' (Bolli et al., 2007), develops within 24 hours following the initial IPC and lasts for 48-72 hours. The early phase of IPC has been shown to result from the rapid post-translational modifications of existing myocardial proteins whereas the late phase of IPC is mediated by cardioprotective gene expression and the synthesis of new proteins in the myocardium (Bolli et al., 2007). Furthermore, the early phase of IPC has been shown to protect against MI but fails to limit the degree of myocardial contractile dysfunction or stunning while, the late phase of IPC protects against both myocardial cell death and preserves post ischaemic left ventricular function (Bolli et al., 2007).

The initial triggers for IPC, which are released within minutes of ischaemia, include adenosine, opioids, bradykinins and prostaglandin. In the early phase of IPC, these molecules lead to the activation of G-protein-coupled receptors and mitochondrial K_{ATP} channels, ROS generation and, the stimulation of a series of protein kinases which include protein kinase C (PKC), tyrosine kinase and members of the Mitogen Activated Protein Kinase (MAPK) family (Das and Das, 2008, Penna et al., 2009). Following the initial trigger, the effects of the late phase of IPC are mediated mainly via newly synthesised proteins such as NOS and maybe even Hsp (Das and Das, 2008, Heusch et al., 2008).

In the first clinical application of IPC in 1993, which involved aortic clamping, it was reported that a protocol of IPC during surgery led to preservations of ATP levels in the myocardium of patients undergoing on CPB CABG (Yellon et al., 1993). It was subsequently reported that in patients undergoing on CPB CABG, a protocol of IPC was also capable of reducing myocardial injury (Jenkins et al., 1997). In addition to ATP preservation (Lu et al., 1997) and attenuating myocardial injury (Ji et al., 2007, Szmagala et al., 1998, Teoh et al., 2002a, Teoh et al., 2002b), IPC during cardiac surgery has also demonstrated the ability to improve ventricular function (Lu et al., 1997, Wu et al., 2002, Wu et al., 2001), limit arrthymias (Wu et al., 2002), heart rate variability (Wu et al., 2005) and inotrope use (Wu et al., 2002). However, the risks associated with repeated clamping and unclamping of the aorta, as required for an IPC protocol, in addition to the routine clamping and unclamping that has to occur to perform on CPB CABG surgery, has meant

that the routine clinical use of IPC has been limited. Due to this, the concept of myocardial preconditioning has been further investigated using remote IPC (RIPC), which does not require an invasive intra-operative myocardial IPC protocol, and, pharmacological preconditioning using adenosine, bradykinin, opioids and inhalation anaesthetics (Granfeldt et al., 2009, Hausenloy and Yellon, 2009, Venugopal et al., 2009).

1.6.2 Remote Ischaemic Preconditioning

Myocardial RIPC involves applying the IPC protocol to an organ or tissue which is distant from the heart (Hausenloy and Yellon, 2008). This method of myocardial preconditioning was first discovered in 1993, when it was observed that a period of brief ischaemia and reperfusion in the left circumflex coronary artery territory of dog hearts, prior to a prolonged period of acute coronary occlusion in the left anterior descending (LAD) artery territory, later led to a substantial reduction in myocardial infarct size (Przyklenk et al., 1993). Subsequently, the cardioprotective effects of RIPC has also been demonstrated using RIPC protocols to the kidney (Pell et al., 1998), intestine (Gho et al., 1996) and limb (Birnbaum et al., 1997).

The mechanism for RIPC is unclear but it appears to closely resemble that for IPC (Granfeldt et al., 2009). At present, there are three general theories regarding the mechanism for RIPC (Hausenloy and Yellon, 2008, Walsh et al., 2009). The first theory is the neural hypothesis which suggests that ischaemic remote organs release endogenous substances, such as adenosine and bradykinin, which activate local afferent neural pathways to

trigger end-organ protection. The second theory is the humoral theory which suggests that the remote organs release humoral mediators, which again maybe adenosine and bradykinin, into the bloodstream and these substances are transported to distant organs where they directly trigger intracellular survival pathways. The third theory is the inflammatory suppression theory which suggests that the transient remote organ ischaemia produces a systemic inflammatory response which is capable of protecting distant organs from a subsequent IRI. While it is unclear which of these theories are accurate, it is also possible that all are true and that they maybe an interaction between all mechanisms of all three theories.

In the clinical setting, in 2005, a study was conducted where the non-dominant arm was rendered ischaemic by inflation of a blood pressure cuff for 5 minutes followed by 5 minutes of reperfusion for 3 cycles (Loukogeorgakis et al., 2005). Following this, the contralateral arm was subjected to 20 minutes of sustained ischaemia via cuff inflation followed by critical assessment of conduit vessel vasoreactivity. In that study, it was clearly demonstrated that both the early and late phases of IPC were present in RIPC and that the effects were mediated via neuronal activity as treatment with an autonomic blocker, trimetaphan, abolished the preservation of flow-mediated vasodilatation in the subjects that were treated with RIPC protocol.

The clinical effects of RIPC in cardiac surgery were first published in 2006. In that study, 37 children undergoing repair of congenital heart defects were randomised to either a RIPC group or a control group (Cheung et al., 2006). RIPC was induced by four cycles of 5 minutes of

lower limb ischaemia followed by 5 minutes of reperfusion using a blood pressure cuff. It was found that post operative serum Troponin-I and inotrope use were significantly lower in the RIPC group. Additionally, the RIPC group had a significantly lower post operative airway resistance. In the setting of on CPB CABG, a randomised control study had been conducted where 57 patients undergoing elective surgery were randomly allocated to receive RIPC or not (Hausenloy et al., 2007). The RIPC group was subjected to 3 cycles of 5 minutes of upper arm ischaemia that was induced by an automated blood pressure cuff-inflation followed by 5 minutes of reperfusion just prior to the start of CABG. The total Troponin-T released from the heart during the 72 hour study period was significantly reduced by 43% in the RIPC group compared to the control group. However, as that was a small study, and a previous small under-powered clinical study involving RIPC and CABG found no cardioprotective effects (Gunaydin et al., 2000), larger randomised control studies are required to conclusively assess the effects of RIPC in patients having CABG.

1.6.3 Pharmacological Preconditioning

Despite the cardioprotective effects of IPC and RIPC, both these techniques are still not part of the routine myocardial protective strategies used during cardiac surgery. This is perhaps because the protocols for each of these procedures adds further time to an already long operation and, particularly with regards to IPC, there is the added concern of unnecessarily cross-clamping an aorta which may already be atherosclerotic. Following the understanding of some of the basic mechanisms involved in inducing the beneficial effects of IPC and RIPC, it became clear that the cardioprotective effects of IPC and RIPC prior to sustained myocardial ischaemia can be mimicked with the use of pharmacological agents (Andreadou et al., 2008, Huffmyer and Raphael, 2009).

As pharmacological agents are more readily available and easily applicable to clinical practice than IPC or RIPC, it may be of more use in attempts to induce myocardial protection. At present there are a large number of pharmacological agents which have been shown to induce myocardial preconditioning and cardioprotection. This includes NO related agents such as NO donors (Takano et al., 1998) and sodium nitrite (Shiva et al., 2007), phosphodiaesterase inhibitors (Das et al., 2005), adenosine (Toombs et al., 1992), bradykinin (Wall et al., 1994), opiod agonist (Das and Das, 2008, Downey et al., 2007, Schultz and Gross, 2001), muscarinic agents (Przyklenk and Kloner, 1995), angiotensin AT_1 agonist (Fryer et al., 2002) and endothelin (Erikson and Velasco, 1996). In addition to this, a number of

noxious stimuli such as heat stress (Joyeux-Faure et al., 2003), ROS (Penna et al., 2009), cytokines (Wang and Yin, 2006) and endotoxins (Harder et al., 2005, Wang et al., 2002), when applied sparingly, have also been shown to trigger myocardial preconditioning.

In clinical studies involving CABG and pharmacological preconditioning, adenosine preconditioning has been shown to reduce peri-operative myocardial enzyme release and improve post operative myocardial function (Lee et al., 1995, Mentzer et al., 1997, Zarro et al., 1998). While the Acadesine 1024 Trial (Mangano et al., 2006) which investigated the use of acadesine, a purine analoge of adenosine which acts on adenosine receopts to increase adenosine levels, failed to show a statistically significant difference in post procedure MI, a meta-analysis of acadesine showed that it use led to a 27% decrease in MI and a 26% decrease in the combined outcome of stroke, MI, or cardiac death (Mangano, 1997). Pharmacological preconditioning with bradykinin on the other hand, has only demonstrated a weak anti-inflammatory cardioprotective effect but at the expense of significant haemodynamic compromise (Wang et al., 2009b, Wei et al., 2004). Studies have also shown that the use of the mitochondrial K_{ATP} channel opener, diazoxide, as a pharmacological preconditioner prior to CABG, had an anti-inflammatory effect and was associated with improved post operative myocardial functional recovery but did not reduce myocardial injury (Wang et al., 2003, Wang et al., 2004). Where the use of the pharmacological inhibitor of the Na^+-H^+ exchanger (NHE), which prevents intra-cellular calcium overload, is concerned, the GURDIAN clinical trial (Boyce et al., 2003) reported that

preconditioning with the NHE inhibitor cariporide, reduced all-cause mortality and myocardial infarction at 36 days and 6 months post CBAG. Subsequently, the EXPIDITION clinical trial (Mentzer et al., 2008) confirmed the early cardioprotective benefits of cariporide, but not the 6 month outcomes. However, that study also demonstrated that cariporide was associated with increased mortality from cerebrovascular events. In a recent pilot study, preconditioning with the calcium sensitizer, levosimendan, was also found to reduce myocardial injury in the setting of CABG (Tritapepe et al., 2006). The anti-C5a antibody, pexelizumab, which inhibits complement immune activation, was in vestigated in the PRIMO-CABG study (Verrier et al., 2004) and showed a strong trend twards reduced mortality or MI but again, these results were not confirmed in the larger follow-up study PRIMO-CABG II (Testa et al., 2008). The pyridoxine metabolite and purinergic receptor antagonist, pyridoxal-5'-phosphate, which is capable of reducing intra-cellular calcium over load and limiting IRI (Kandzari et al., 2005) was initially shown in the MEND-CABG-I clinical study to be capable of possibly lowering post CABG MI size (Tardif et al., 2007). However, this finding was not reproduced in the larger MEND-CABG II clinical study which instead demonstrated a small increase in mortality (Alexander et al., 2008).

Clinical studies investigating the pharmacological preconditioning effects of volatile anaesthetic agents have led to inconsistent results with respect to the reduction in myocardial enzyme release (Piriou et al., 2004, Tomai et al., 1999, Wang et al., 2004), although a few studies (De Hert et al., 2003, Landoni et al., 2008) have demonstrated an anti-stunning

effect with higher post operative cardiac index, reduced post operative low-output states necessitating inotrope use and shorter post operative ICU length of stay. A recent meta-analysis however, determined that volatile anaesthetics are capable of reducing myocardial injury but had no beneficial effect on observed clinical outcomes (Yu and Beattie, 2006). As with RIPC, a large multi-centre randomised control study is required to definitively determine if preconditioning with volatile anaesthetics can positively impact on clinical outcomes post cardiac surgery.

1.6.4 Hyperoxic Preconditioning

The concept of inducing myocardial protection by preconditioning with oxygen was first investigated in 2001. It was hypothesised that pre-treatment with high doses of oxygen, hyperoxia, to induce a low-grade oxidative stress prior to ischaemia and reperfusion, may evoke myocardial protection by mimicking both an early and late IPC like effects (Tahep ld et al., 2001). In that experimental study, rats were exposed to >95% oxygen for 60 or 180 minutes. Isolated rat hearts were then Langendorff-perfused immediately (early) or 24 hours (delayed) after hyperoxia before being subjected to 25 minutes of global ischaemia followed by 60 minutes of reperfusion. It was determined that both 60 and 180 minutes of hyperoxia induced a low grade systemic oxidative stress. In the early model, only exposure to >95% oxygen for 180 minutes, but not 60 minutes, was associated with significantly reduced reperfusion arrhythmias. However, in the delayed model, 24 hours after exposure, both 60 and 180 minutes of exposure to >95% oxygen were associated with significantly reduced reperfusion arrhythmias. In both the early and delayed model, exposure to >95% oxygen for both 60 and 180 minutes led to significant reductions in left ventricular end diastolic pressure (LVEDP). However, where left ventricular developed pressure (LVDP) [LVDP = left ventricular systolic pressure (LVSP) –LVEDP] was concerned, only the early model showed significant improvement post IRI following both 60 and 180 minutes of exposure to >95% oxygen. The delayed model only showed improvement following 60 minutes of >95% oxygen and not after 180 minutes. This was also the case for coronary flow. With respect

to myocardial infarct size, only the early model that was exposed to >95% oxygen for 60 minutes showed a reduction in infarct size post IRI.

Subsequently, the same group conducted another study to determine the dose of oxygen and verify the length of time of hyperoxic preconditioning which would induce myocardial protection. In that later study, it was shown that preconditioning rats for 60 minutes with ≥95%, 80% or 60%, but not 40% oxygen, improved post IRI myocardial function and coronary flow (Tahepold et al., 2002). Exposure for 60 minutes to either ≥95% or 80%, but not 60% or 40% oxygen, reduced LVEDP and the myocardial infarct size after IRI. LVDP and coronary flow were also significantly better in those groups. Furthermore, it was also demonstrated that exposure to ≥95% oxygen for 60 and 180 minutes in rats, led to significant reductions in LVEDP compared to the control group post IRI. Moreover, exposure to ≥95% oxygen for 60 and 180 minutes also led to smaller reductions in LVDP and improved coronary flow post IRI. Where myocardial infarct size was concern, exposure to ≥95% oxygen for 60 minutes in rats, led to reductions in post IRI infarct sizes. When the delayed effects of hyperoxic preconditioning were examined, it was also found that 24 hours after treatment with ≥95% oxygen for 60 and 180 minutes, prior to IRI, led to significant inhibition the increase in LVEDP and an attenuation in post ischaemic depression of LVDP. The delayed effects of 60 minutes of hyperoxia prior to IRI was also associated with a significantly reduced attenuation of coronary flow.

These early studies (Tahepold et al., 2002, Tahepold et al., 2001) suggest that preconditioning with ≥95% oxygen is capable of inducing

both early and delayed cardiprotective effects following IRI, in a way that mimics the cardioprotective effects of IPC and RIPC. Shorter durations (60 minutes) of hyperoxic preconditioning , in the early model, is capable reducing in post IRI myocardial infarct size This is not seen with longer durations (180 minutes) of hypeoxia in both the early and delayed model of hyperoxia and neither is it seen in the delayed models hyperoxia prior to IRI which use a shorter duration (60 minutes) of hyperoxia. In the both the early and delayed models of hyperoxia prior to IRI, both the shorter (60 minutes) and the longer (180 minutes) durations of hyperoxia are capabale of improving myocardial function. Where coronary flow is concerned, while in the early model of hyperoxia prior to IRI, both the shorter (60 minutes) and the longer (180 minutes) durations of hyperoxia are capable of improving flow, this only occurs with the shorter (60 minutes) duration hyperoxia in the delayed models of hyperoxia prior to IRI. With respect to reperfusion arrhythmias, in the early model of hyperoxia prior to IRI, a longer duration (180 minutes) of hyperoxia is required to limit the appearence of these while in the delayed model both the shorter (60 minutes) and the longer duration (180 minutes) of hyperoxia have the capacity to attenuate arrhythmias.

More recent studies however, on the duration of hyperoxia in relation to the early (Colantuono et al., 2008, Kaljusto et al., 2008) and late (Baharvand et al., 2009, Esmaili Dehaj et al., 2009) cardioprotective effects of hyperoxic preconditioning prior to IRI have clearly demonstrated that higher doses of oxygen (>95% oxygen), for durations of as little as 60 minutes and, for as long as 180 minutes, are able to reduce myocardial infarct

size and arrhythmias following both a short and a long duration between hyperoxia and a subsequent event of IRI. Furthermore, it has also been recently demonstrated that the myocardial protective effects of the delayed phase of hyperoxic preconditioning prior to IRI may last for longer than 48 hours and that this phase may be prolonged to up to 72 hours by repeated, intermittent exposure prior to the insult of IRI (Baharvand et al., 2009).

While hyperoxia of ≥95% for 60 minutes immediately prior to IRI is capable of reducing myocardial infarct size and reduce the increase in LVEDP in mouse hearts, durations of hyperoxia for as short a period of as 30 minutes, 24 hours prior to IRI, has also been shown to reduce myocardial infarct size and limit the increase in LVEDP in an experimental model (Tahepold et al., 2002). In another recent experimental study, it was also demonstrated that 100% oxygen for 30 minutes, prior to IRI, is also capable of improving coronary flow, LVDP and LVEDP and, reducing myocardial infarct size as early as 30 minutes after ischaemia (Colantuono et al., 2008).

As the administration of 80% oxygen for less than 24 hours is considered clinically safe (Kabon and Kurz, 2006), it would indeed be interesting to determine the effects of a short duration of preconditioning with 100% oxygen, prior to IRI, in a clinical setting. While the clinical effects of preconditioning with this dose has yet to be investigated, in a recent clinical study (Karu et al., 2007), 40 CABG patients were randomly exposed to either 40% oxygen or >96% oxygen for 120 minutes prior to IRI. In this study, it was observed that post operatively, Troponin-I, CK-MB and lactate levels did not differ between the groups. Neither were there any differences seen in the post

operative haemodynamic parameters. There was however a transient significant reduction in the pro-inflammatory cytokine, IL-6 in the first 20 minutes after reperfusion in the group that was exposed to >96% oxygen.

Under normal physiological conditions, 1% to 4% of available body oxygen is converted to ROS. This process is greatly accelerated in conditions which enable the breathing of hyperoxic air, thus leading to oxygen tensions higher than normal (Brueckl et al., 2006). While it is unclear how high ROS production has to be before it triggers preconditioning effects, what is clear is that hyperoxia increases ROS production, reaching the threshold level when oxygen is adminstered for approximately 120 minutes at a saturation of between 80% to 95% (Esmaili Dehaj et al., 2009). It has been demonstrated that hyperoxia increases ROS generation in a time and dose dependent manner (Brueckl et al., 2006). Several studies using infusions of low concentrations of ROS (Tritto et al., 1997, Vanden Hoek et al., 1998, Zhai et al., 1996) and administration of anti-oxidants (Das et al., 1999b, Tanaka et al., 2002, Toufektsian et al., 2003) have demonstrated that ROS acts as the trigger for the preconditioning process. It is currently the thinking that the cardiprotective effect of hyperoxic preconditioning, and the ROS it generates, prior to IRI, may have to do with the activation of NFκB and the dual role in may play in the adapted myocardium (Choi et al., 2006, Tahepold et al., 2003). While the activation of NFκB is know to lead to pro-inflammatory effects (Blackwell and Christman, 1997), its activation by hyperoxia prior to myocardial IRI, has been shown to lead to the upregulation of the rapidly inducible IFκB, which is capable of reducing the inflammation during a

subsequent sustained ischaemia by inhibiting NFκB activation (Tahepold et al., 2003).

1.7 Hyperbaric Oxygen

In hyperbaric oxygen (HBO_2) therapy, the patient intermittently breathes pure oxygen (100%) at a pressure greater than atmospheric pressure, usually between 1.5 absolute atmosphere (ATA) to 3 ATA, at room temperature, for a duration not longer than 2 hours, (Kim et al., 2001) while in a steel or polymer chamber. This mode of oxygen therapy is capable of increasing arterial and tissue oxygen tension up to 2000mmHg and 400mmHg, respectively (Choi et al., 2006).

HBO_2 is known mainly for its use as the treatment of choice in carbon monoxide poisoning, gas embolism and decompression sickness (Kindwall EP, 2002). In these conditions, generally only one or two HBO_2 therapy sessions are required. The experience of HBO_2 medicine specialists and, to a certain extent, the scientific literature, also support the use of HBO_2 as an adjuvant treatment for a number of other clinical conditions such as complex, refractory wounds (Morykwas and Argenta, 1997), intracranial abscess, radiation tissue injury, crush injuries, compartment syndrome, acute traumatic peripheral ischaemia, burns and IRI (Kindwall EP, 2002). However, such use requires numerous visits, with up to as many as sixty sessions lasting between 30 to 90 minutes a session, depending on the clinical condition. At present, there are no standard protocols for these clinical conditions. The therapeutic protocols vary according to the severity of the clinical condition and the treatment centre.

1.7.1 Hyperbaric Oxygen In Ischaemic Reperfusion Injury

Two phenomena may be involved to give HBO_2 therapy an anti-ischaemic effect:

a) The first phenomena is related to Henry's Law which states that *"the amount of any given gas that will dissolve in a liquid at a given temperature is a function of the partial pressure of the gas in contact with the liquid and the solubility coefficient of the gas in the liquid"*. As HBO_2 involves the administration of oxygen at pressures above atmospheric pressure at room temperature, this enables higher partial pressures of oxygen to be achieved in the blood and this has been clearly demonstrated in a recent clinical study (Weaver et al., 2009).

b) The second phenomena involves the ability for HBO_2 to improve the elasticity and therefore the deformability of red blood cells, which enables them to reach ischaemic tissues (Mathieu D, 1984).

These two phenomenon are capable of improving tissue oxygenation and thus increasing local metabolism. These potential benefits, have led to research into the use of HBO_2 for attenuating myocardial ischaemia and infarction (Shandling et al., 1997, Thomas et al., 1990) and, improving myocardial function (Dekleva et al., 2004).

A number of experimental studies have investigated the effects of HBO_2 during ischaemia and reperfusion. It appears that HBO_2 treatment prior to (Chen et al., 1998), during (Yamada et al., 1995) or after ischaemia (Nylander et al., 1987) or, immediately upon reperfusion (Yamada et al., 1995) is capable of preserving cellular ATP. Furthermore, it has also been observed that HBO_2 treatment prior to the insult of ischaemia and reperfusion (Chen et al., 1998), is capable of leading to an increase in ATP concentration that is associated with an attenuation of adherent neutrophils and lipid peroxidation.

In an experimental model of IRI, HBO_2 has also been shown to enhance endothelial cell-derived fibrinolysis and blood flow (Tjarnstrom et al., 2001). In that study, cultured endothelial cells were subjected to simulated ischaemic reperfusion by anoxia, followed by reperfusion with either HBO_2 or normobaric air for 1.5 hours. The HBO_2 treated cells exhibited significant increases in all the measured fibrinolytic factors [tissue plasminogen activator (t-PA), urokinase plasminogen activator (uPA) and plasminogen activator inhibitor type 1 (PAI-1)], suggesting that HBO_2 treatment following ischaemia (during reperfusion) has the potential to also limit vascular thrombosis.

1.7.2 Hyperbaric Oxygen & Myocardial Ischaemic Reperfusion Injury

In 1976, an experimental study was conducted to investigate the effects of HBO_2 on the myocardium (Kawamura et al., 1976). In that study, HBO_2 treatment consisting of 100% oxygen at 2 ATA was administered to one group of dogs before temporary occlusion of the left coronary artery and to another group immediately after the temporary occlusion of the left coronary artery. The duration of coronary occlusion ranged from 30 minutes to 2 hours. A control group of dogs also had durations of temporary left coronary artery occlusion in a similar range but did not receive HBO_2 treatment. Following sacrifice, which occured 4 hours after HBO_2 treatment in the group treated with HBO_2 prior to coronary artery oclusion and 5 days after coronary occlusion in the group that was treated with HBO_2 following coronary artery oclusion, it was discovered that, compared to the control animals, the myocardial ischaemic area in dogs that were treated with HBO_2, either before or after coronary oclusion, was markedly reduced and the myocardial muscles around the arterioles and sinusoids were viable. That study also found that serious arrhythmia, especially ventricular fibrillation, was suppressed in the treated dogs and they were haemodynamically more stable during the operative period. That study was the first to demonstrate, both macroscopically and histologically, that treatment with HBO_2 either before or after an ischaemic reperfusion event, was capable of limiting myocardial infarct size.

The myocardial infarct sparing property of HBO_2 was later further demonstrated in another experimental study. In that study involving an open-chest rabbit model, the left coronary artery was occluded for 30 minutes, to induce ischaemia, followed by 3 hours of reperfusion (Sterling et al., 1993). During this ischaemia and/or reperfusion, the controls group was ventilated with 100% oxygen at 1 ATA while the 'treated' group was exposed to HBO_2 at 2.5 ATA. The animals exposed to HBO_2 during ischaemia only, reperfusion only, or ischaemia and reperfusion, had significantly smaller infarcts compared to the control animals. When HBO_2 was administered 30 minutes after the onset of reperfusion, no myocardial protection was seen. This study suggests that treatment with HBO_2 is only capable of inducing myocardial protection during periods of ischaemia and reperfusion but not if administered shortly after reperfusion has begun.

The concept of using HBO_2 to limit myocardial IRI has also been investigated by using HBO_2 enriched solution. In an attempt to determine if perfusion with a solution of HBO_2 through the anterior interventricular vein (AIV) would reduce myocardial infarct size and neutrophil activation, using three groups, animals were exposed to IRI by 60 minutes of balloon occlusion of the LAD to induce ischaemia followed by 120 minutes of reperfusion (Johnson et al., 2004). Following IRI, the first control group was not treated with any further intervention during reperfusion while the second control group received arterial blood, drawn from the femoral artery, perfused into the AIV for the first 90 minutes of reperfusion. In the third experimental

group, a solution of HBO_2 enriched arterial blood was perfused through the AIV either immediately after reperfusion or 30 minutes after reperfusion, for a duration of 90 minutes. The oxygen level of blood delivered into the AIV in experimental group was between 1000 and 1400mmHg. In that study, compared to the control groups and the experimental group treated with HBO_2 30 minutes after reperfusion, in the experimental group that was treated with with HBO_2 immediately upon reperfusion, the endocardial blood flow was significantly higher in at the risk regions of the myocardium. Furthermore, the myocardial infarct size was also significantly smaller in this group compared to the other groups. It was also determined that in the HBO_2 group the smaller infarct size was associated with reduced reperfusion association neutrophil activation. This study supports the premise that increasing myocardial tissue oxygenation early during reperfusion is cardioprotective but delayed oxygenation, during reperfusion, is of limited cardioprotective value.

1.7.2.1 Hyperbaric Oxygen Preconditioning

While the the concept of treatment with HBO_2, prior to ischaemia and reperfusion to mitigate myocardial IRI, was first investigated in 1976 by Kawamura et al. (Kawamura et al., 1976), it was not until 2001 when the biology behind this mode of treatment was further investigated to understand how it may prophylactically induce myocardial protection prior to IRI. In a study by Kim et al (Kim et al., 2001), rats were intermittently exposed to 100% oxygen at 3 ATA for 1 hour daily for 5 days and then sacrificed after 24 hours of recovery in room air. The isolated rat hearts were

subjected to 40 minutes of ischaemia and 90 minutes of reperfusion. It was found that HBO_2 pre-treatment enhanced enzymatic activity and gene expression of the anti-oxidant enzyme catalase and, this was associated with a significant reduction in myocardial infarct size. A catalase inhibitor, 3-amino-1,2,4-triazole, completely abolished the infarct-limiting effect of HBO_2 pre-treatment. This concept of inducing cellular protection, via HBO_2, prior to cellular injury is now known as HBO_2 preconditioning (Kim et al., 2001) and its appears that its myocardial infarct limiting capacity is similar to that observed during IPC (Murry et al., 1986).

Further evidence of the MI sparing capacity of HBO_2 preconditioning comes from another recent experimental study (Han et al., 2008a). In that study 108 rats were randomly divided into four groups: normoxia + sham surgery (CS), normoxia + permanent occlusion of the LAD coronary artery (CMI), HBO_2 preconditioning + sham surgery (HS) and HBO_2 preconditioning + permanent LAD occlusion (HMI). Rats receiving HBO_2 preconditioning were exposed to 100% oxygen at 2.5 ATA for 60 minutes, twice daily for 2 days prior to myocardial ischaemia induced by LAD ligation. There was a 12 hour interval between HBO_2 preconditioning sessions and, a 12 hour interval between the last HBO_2 preconditioning session and the LAD ligation. Rats in the normoxia group were time-matched with the HBO_2 group and maintained under normoxic conditions prior to LAD occlusion. At day 3 after LAD ligation, the infarct size of the HMI group was found to be significantly smaller than that of the CMI group. The LVSP was significantly

improved in the HMI group compared to the CMI group at 3 and 7 days after LAD occlusion. That study also found that the capillary density was significantly increased in the ischaemic myocardium that was preconditioned with HBO_2. The findings from this randomised control experimental study clearly demonstrates that HBO_2 preconditioning is capable of leading to cardioprotection by improving myocardial myocardial function and blood flow as a result of increased myocardial capillary density and, as such, leading to a reduction in myocardial infarct size following ischaemia.

The concept of utilising HBO_2 preconditioning to induce myocardial protection has also been investigated in terms of determining the effects of HBO_2 on a common cause of myocardial ischaemia, coronary atherosclerosis. In a study involving rabbits fed an atherogenic diet, the animals were exposed to HBO_2 consisting of 100% at 2.5 ATA for 90 minutes, 5 days a week for 10 weeks (Kudchodkar et al., 2000). The control rabbits had the same diet but were not treated with HBO_2. In that study it was determined that repeated short periods of HBO_2 exposure dramatically reduced the development of atherosclerosis by reducing the accumulation of cholesterol in the aortic wall. Treatment with HBO_2 also substantially reduced the accumulation oxidised LDL and HDL cholesterol in the plasma, liver and aortic tissue which according to the investigators, may explain the slower progression of atherosclerosis in the HBO_2 treated animals. Furthermore, HBO_2 treatment also prevented the decrease in the plasma anti-oxidant activity of paraoxonase and caused a significant acceleration in the regression of aortic

atherosclerotic lesions. The investigators of that study suggests that HBO_2 possibly inhibits the initial development of atherosclerosis by suppressing the recruitment and proliferation of macrophages and the thus the formation of foam cells via paraoxonase anti-oxidation mediated reduction in the formation of lipid-derived oxidative products. This is a reasonable suggestion as others have also shown that HBO_2 has the capacity to enhance the activity of the serum anti-oxidant paraoxonase and this is known to cause a reduction in the oxidation of tissue and plasma lipids (Aviram et al., 1998). In a later study, the same group also found that HBO_2 treatment resulted in significant reduction in aortic cholesterol content and decreased fatty streak formation (Kudchodkar et al., 2007). In examining the possible biochemical mechanisms for these findings, this group found that a 10 week treatment with HBO_2 led to significant reduction in auto-antibodies against oxidatively modified LDL [levels of auto-antibodies to oxidised LDL have been shown to reflect levels of oxidised LDL in the circulation and correlate positively with the progression and regression of experimental atherosclerosis in mouse models (Tsimikas et al., 2001, Zhou et al., 1998)]. There were also profound changes in the redox state of aortic tissues with significant increases in aortic glutathione, oxidized gluththione reductase activity, glutathione S-transferase activity and catalase thus, suggesting that HBO_2 treatment had induced an environment that inhibits oxidation. They concluded that this anti-oxidant response may be the key to the anti-atherogenic effect of HBO_2 treatment.

1.7.3 Hyperbaric Oxygen Induced Neutrophil Attenuation & Adhesion Molecule Expression

One of the possible means for HBO_2 induced attenuation of IRI may be via the interference of neutrophil infiltration (Atochin et al., 2000, Hong et al., 2003). Findings in various experimental models have shown that neutrophil recruitment as a result of ischaemia and reperfusion was significantly reduced by peri-IRI HBO_2 treatment in IRI models of the liver (Chen et al., 1998, Kihara et al., 2005), intestine (Tjarnstrom et al., 1999, Yamada et al., 1995), gracilis muscle (Zamboni et al., 1996), brain (Atochin et al., 2000, Miljkovic-Lolic et al., 2003) and testis (Kolski et al., 1998). Real time videomicroscopy of HBO_2-treated muscle flaps following ischaemia and reperfusion have demonstrated a decrease in the adhesion of neutrophils to the vascular endothelium and a greater microvascular diameter (Zamboni et al., 1993, Zamboni et al., 1996). Studies by others suggest HBO_2 limits neutrophil-endothelial adhesion by inhibiting the polarization of the adhesion molecule CD18 on the surface of neutrophils (Khiabani et al., 2008), but not its expression (Hong et al., 2003, Larson et al., 2000), and inhibits the expression of the cellular adhesion molecule, ICAM-1 (Buras et al., 2000, Hong et al., 2003).

In a recent clinical study (Alex et al., 2005), 64 CAD patients were randomised to either a group preconditioned with hyperbaric air (n=31), or HBO_2 consisting of two 30 minute periods of 100% oxygen at 2.4 ATA separated 5 minutes apart (n=33). Preconditioning with air or HBO_2 took place for 90 minutes, 24 hours, 12 hours and 4 hours, respectively, prior to on CPB CABG. In that study it was observed that patients who were preconditioned with three sessions of HBO_2 (HBO_2 preconditioning) prior to the insult of IRI during on CPB CABG, in contrast to the control patients, did not experience any significant increase in post operative (2 hours and 24 hours post CPB) plasma CD18 and this was not accompanied by any significant neurocognitive decline 4 months post CABG. This suggests that perhaps cerebral vascular flow may have been better preserved in patients preconditioned with HBO_2 as a result of the attenuation of ischaemia reperfusion induced neutrophil-endothelial interaction and perhaps a reduction in the ensuing endothelial injury.

In the clinical study by Alex *et.al*, it was also observed that in addition to attenuating the rise in plasma CD18, three sessions of HBO_2 prior to the insult of IRI also reduced the post operative expression of serum soluble E-selectin (sE-Selectin) following CABG (Alex et al., 2005). However, in that study, HBO_2 preconditioning was also found to increase the post operative expression of serum soluble ICAM-1 (sICAM-1) and soluble P-selectin (sP-Selectin). In experimental models of IRI, it has been demonstrated that HBO_2 treatment during ischaemia (Hong et al., 2003) or following IRI (Buras et al.,

2000, Hong et al., 2003), decreased the expression of ICAM-1 in a muscular flap (Hong et al., 2003) and in the endothelium (Buras et al., 2000). These studies suggest that HBO_2 prior to IRI i.e. HBO_2 preconditioning (Alex et al., 2005), is capable of limiting the rise in plasma CD18 and, while HBO_2 during ischaemia and HBO_2 post IRI (Hong et al., 2003) does not alter neutrophilic CD18 expression, it does inhibit neutrophilic CD-18 polarization and thus neutrophil-endothelium adhesion (Khiabani et al., 2008). It remains unclear as to whether the attenuation in the rise of plasma CD-18 is linked to the inhibition in the distribution of CD-18 on the surface of neutrophils. Is it possible that the oxidative stress of HBO_2 redistributes the neutrophilic CD18 thus limiting the cleavage of CD18 from the surface of neutrophils into the plasma? Additionally, while HBO_2 during ischaemia (Hong et al., 2003) and HBO_2 following IRI (Buras et al., 2000, Hong et al., 2003) is capable of downregulating endothelial ICAM-1 expression, it also appears that HBO_2 prior to IRI was capable of increasing the presence of serum sICAM-1 (Alex et al., 2005). Is there a possible link between HBO_2 induced increase in sICAM-1 and the downregulation in the expression of endothelial ICAM-1?. Could it be that the lack of endothelial ICAM-1 following HBO_2 treatment is due to these adhesion molecules being shed into the circulation, as a result of the oxidative stress of HBO2, thus leading to increased circulating sICAM-1?.

1.7.4 Mechanisms of Action of Hyperbaric Oxygen in Ischaemic Reperfusion Injury

HBO_2 has been demonstrated to increase ROS generation (Benedetti et al., 2004, Conconi et al., 2003, Gregorevic et al., 2001) and has also been shown to be protective to the myocardium (Kim et al., 2001, Kudchodkar et al., 2000, Shandling et al., 1997, Sharifi et al., 2004, Sterling et al., 1993, Swift et al., 1992). It is therefore plausible to suggest that there is a therapeutic association between HBO_2 generated ROS and myocardial protection. There maybe six possible complex and intertwining mechanisms by which HBO_2 generated ROS may lead to reduced endothelial and myocardial injury *(Figure 1.1)*.

Mechanism 1: eNOS & NO

ROS, such as superoxide or hydrogen peroxide, are capable of modulating the expression of eNOS (Drummond et al., 2000, North et al., 1996) and NO production (Lebuffe et al., 2003). This may occur by ROS functioning as signaling molecules (Hool, 2006, Lebuffe et al., 2003) to activate NOS (Drummond et al., 2000, Lopez-Ongil et al., 1998) and produce NO (Mathy-Hartert et al., 2002, Whorton et al., 1997). Via direct (Elayan et al., 2000) and indirect (Thom et al., 2003) measurements of ROS, HBO_2 induced ROS has been shown to activate NOS (Elayan et al., 2000, Thom et al., 2003) and produce NO (Elayan et al., 2000, Thom et al., 2003).

Mechanism 1a: Peroxynitrite

HBO_2 generated ROS, in particular, superoxide, may play a therapeutic role in the attenuation of neutrophil adhesion via peroxynitrite production *(Figure 1.1: Mechanism 1a)*. It is possible that by combining with NO to form peroxynitrite (Beckman and Crow, 1993, Beckman and Koppenol, 1996, Liu et al., 1994), NO may act as a sink to reduce the contribution of IRI generated superoxide towards excessive free radical chain reaction propagation (Pryor and Squadrito, 1995, Rubanyi et al., 1991). Given the suggestions in favor of the use of HBO_2 as a therapeutic modality against IRI (Alex et al., 2005, Bertoletto et al., 2008, Chen et al., 1998, Henninger et al., 2006, Nie et al., 2006, Nylander et al., 1985, Nylander et al., 1987, Sterling et al., 1993, Tomur et al., 2005, Yamada et al., 1995, Yin and Zhang, 2005, Yu et al., 2005), it is unlikely that HBO_2 generated ROS leads to irreversible cellular injury. Paradoxically, it is more likely that, in balanced quantities, the HBO_2 generated ROS, such as superoxide, play a pharmacological role in the attenuation of neutrophil adhesion possibly via peroxynitrite production from the NO. Peroxynitrite in intermediate concentrations (2-5μM) has been shown to reduce neutrophil adhesion by inhibiting P-selectin expression on endothelial cell (Nossuli et al., 1998) thus limiting endothelial injury via neutrophil-endothelial adhesion.

In addition to P-Selectin attenuation, in an experimental model of IRI, it was demonstrated that peroxynitrite administration was also capable of inhibiting the expression of ICAM-1 mRNA (Liu et al., 2000). This

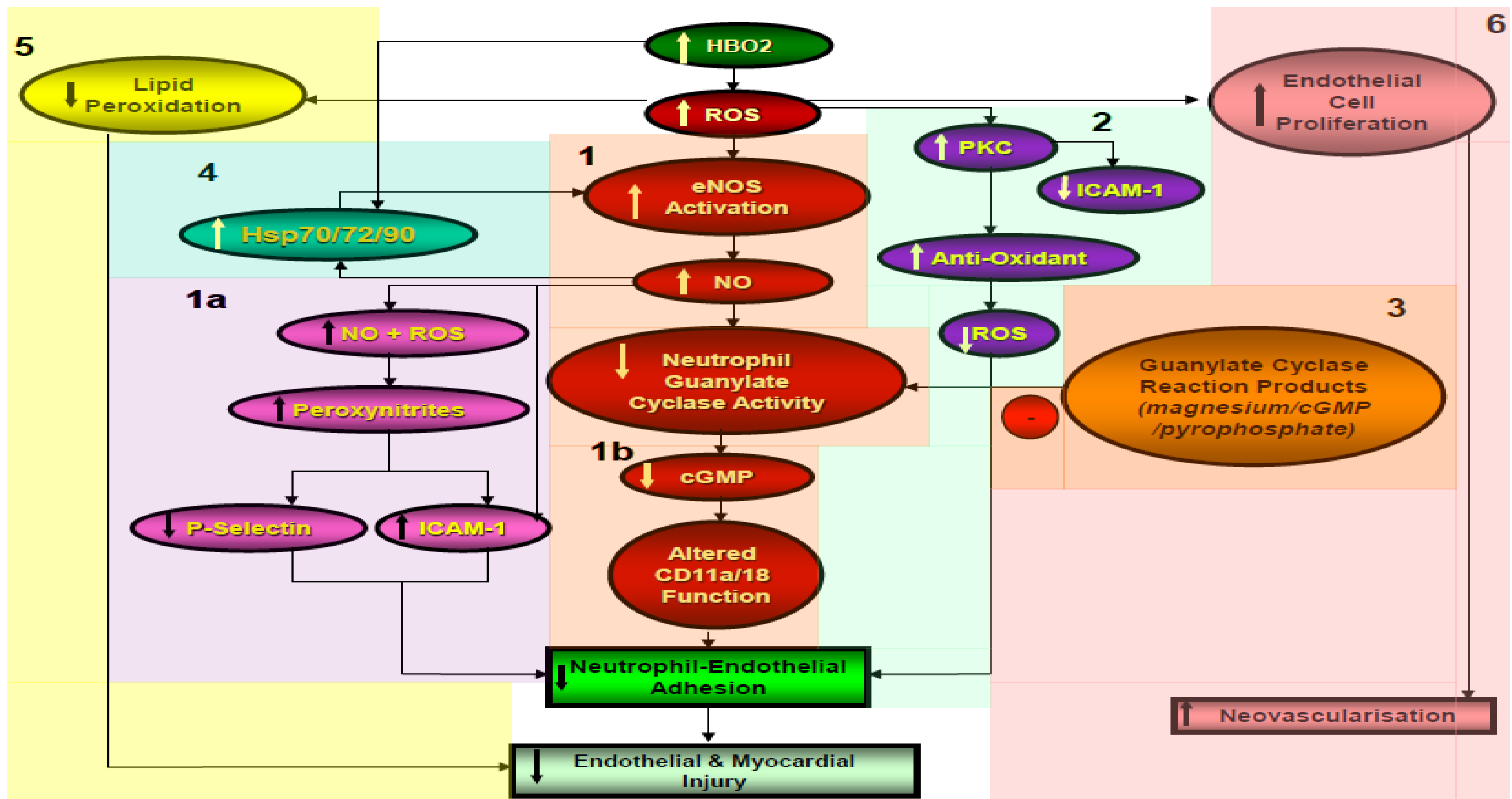
suggests that peroxynitrite is also capable of impairing neutrophil adhesion to the vascular endothelium by inhibiting the synthesis of ICAM-1 *(Figure 1.1: Mechanism 1b)*. This in turn will limit the neutrophil-endothelial adhesion that is known to occur as part of the 'no-reflow' phenomenon of IRI (Engler et al., 1986). As HBO_2 generated ROS could react with HBO_2 induced NO to produce peroxynitrite, and HBO_2 has been shown to lead to a down regulation of ICAM-1 expression (Buras et al., 2000), this mechanism could account for the observed HBO_2 associated preservation of capillary flow (Chen et al., 1998, Sirsjo et al., 1993, Zamboni et al., 1993) and hence the myocardial protection in models of IRI (Cabigas et al., 2006a, Sterling et al., 1993).

Mechanism 1b: CD-18

Studies have shown that HBO_2 exposure inhibits the extracellular binding domain of neutrophil guanylate cyclase (Thom et al., 1997) and impairs the synthesis of cGMP (Chen et al., 1996). This leads to an alteration in the function of the neutrophil specific adhesion molecule CD18 (Thom et al., 1997) and its distribution on the neutrophilic surface (Khiabani et al., 2008). CD18, as part of the heterodimeric protein CD11/18, binds to the ligand ICAM-1 that is located on the endothelial cell (Malik and Lo, 1996). This alteration in neutrophilc CD18 function and distribution, may limit the ability of neutrophils to adhere to endothelial cells via the interaction of CD18 with ICAM-1. In keeping with this, HBO_2 has also been shown to be capable of reducing neutrophil adhesion to the vascular wall (Khiabani et al., 2008, Zamboni et al., 1993). It is possible that the

mechanism underlying this effect is related to the ability of HBO_2 to stimulate eNOS (Cabigas et al., 2006b) and produce NO (Thom et al., 2003) which in turn inhibits neutrophil membrane-bound guanylate cyclase (Banick et al., 1997) thus limiting cGMP synthesis and altering the function of CD18 *(Figure 1.1: Mechanism 1b)*. Studies have shown that inhibition of eNOS promotes the adherence of neutrophils to the microvasculature while increased concentrations of eNOS has an anti-adherent effect (Kubes et al., 1991, Lefer and Lefer, 1996).

Figure 1.1: HBO_2 Possible Mechanisms of Action



Mechanism 1: HBO_2 generated ROS attenuating of endothelial injury via eNOS & NO. **Mechanism 2:** HBO_2 generated ROS activation of PKC inducing anti-oxidation and attenuation of ICAM-1. **Mechanism 3:** HBO_2 induced impairment of guanylate cyclase reaction products. **Mechanism 4:** HBO_2 generated ROS attenuating endothelial injury via Hsp. **Mechanism 5:** HBO_2 induced ROS attenuating lipid peroxidation. **Mechanism 6:** HBO_2 generated ROS enhancing endothelial cell proliferation.

Mechanism 2: PKC

Exposure to HBO_2 (Buras et al., 2000, Shinomiya et al., 1998) has been demonstrated to downregulate ICAM-1 expression. In studies involving myocardial IPC, it appears that the ROS, superoxide, is involved in the induction of increased ischemic tolerance (Baines et al., 1997, Mori et al., 2000, Osada et al., 1994, Tanaka et al., 1994, Tritto et al., 1997). IPC has also been shown to induce an endothelial protective effect by suppressing the expression of ICAM-1, a process which is mediated by production of NO, ROS, and PKC (Beauchamp et al., 1999). Furthermore, there is also evidence to suggest that both superoxide and hydrogen peroxide can stimulate PKC activity (Ogata et al., 2000). Hence, it may be possible that superoxide and/or hydrogen peroxide are able to participate in the regulation of ICAM-1 expression, through PKC (Beauchamp et al., 1999, Niwa et al., 1996, Suzuki et al., 1997a). This activation of PKC in turn, may lead to decreased amounts of free radical production during the actual insult of ischaemia and reperfusion possibly via PKC mediated anti-oxidant defences (Niwa et al., 1996). This mechanism may also partially explain the ability for HBO_2 generated ROS to limit neutrophil adhesion *(Figure 1.1: Mechanism 2).*

Mechanism 3: Neutrophil Guanylate Cyclase Reaction Products

Research (Russwurm and Koesling, 2004) suggest that when NO encounters guanylate cyclase, it binds to the heame moiety of guanylate cyclase to become active and produce cGMP. As such, how is it that in the presence of HBO_2 induced NO, there is an inhibition of guanylate cyclase, as has been previously demonstrated (Banick et al., 1997, Chen et al., 1996), which in turn leads to the impairment of cGMP synthesis and alteration in the function of CD18. Here in with may lie an another possible mechanism of action for HBO_2 induced NO *(Figure 1.1: Mechanism 3)*. It is now known that for guanylate cyclase to be activated by NO, it requires the presence of its reaction products (magnesium/cGMP/pyrophosphate) and sufficient NO (substrate) (Russwurm and Koesling, 2004) in order for it to transit, in a reversible manner, via a dinitrosyl state, to form an active five-coordinated NO-guanylate cyclase species. Based on the available evidence from the observed actions of HBO_2 on guanylate cyclase, perhaps it would be possible to suggest that the lack of activation of guanylate cyclase following HBO_2 could be due to the following two possible scenarios. First, and perhaps more easily appreciated, is that some how, HBO_2 limits the reaction products of guanylate cyclase. HBO_2 has certainly been shown to limit cGMP production (Chen et al., 1996, Thom et al., 1997) together with an attenuation of guanylate cyclase function (Thom et al., 1997). Second, and assuming that HBO_2 has no effect on guanylate cyclase's reaction products, while HBO_2 may generate NO, it also generates ROS such as superoxide. The inactivating

reaction between NO and superoxide to form peroxynitrite, may limit the available NO that binds to guanylate cyclase. While some NO may bind to guanylate cyclase, the lack of sufficient free NO to reversibly bind to it and form an active five coordinated species via a dinitosyl state may lock the NO-bound guanylate cyclase in an inactive five coordinated species or in the inactive six coordinated dinitrosyl state. Either of these two scenarios may limit guanylate cyclase's cGMP production. cGMP is not only one of the reaction products of guanylate cyclase but also a second messenger needed for the appropriate functioning of neutrophil CD18.

Mechanism 4: Hsp

It has been shown that the synthesis of Hsp70 is significantly induced in the lymphocytes of human subjects after just a single treatment with HBO_2 (Dennog et al., 1999). Furthermore, it has also been demonstrated that HBO_2 administration results in a time and dose dependent increase in Hsp70 expression at both mRNA and protein levels (Shyu et al., 2004). This suggests that Hsp70 may play a part in the mechanism for HBO_2 induced cellular protection *(Figure 1.1: Mechanism 4).*

It is also possible that this HBO_2 induction of Hsp synthesis occurs via the expression of NO. Using a rat heart model of IRI at various concentrations of oxygen and pressure, it was demonstrated that there was a 4 fold increase in association of Hsp90 with eNOS following preconditioning with HBO_2 (Cabigas et al., 2006b). This was in addition to an increase in

eNOS and nitrite plus nitrate (breakdown products of NO) content in the myocardium of rats preconditioning with HBO_2. Furthermore, it was also demonstrated that the myocardial protective effects of HBO_2 preconditioning were reversed by L-Nitro-Arginine Methyl Ester (L-NAME) (an NO inhibitor). These findings were also associated with the observation that preconditioning with HBO_2 resulted in a decrease in myocardial infarct size and increased recovery of left ventricular diastolic pressure. While that study did not show a significant increase in total Hsp90 content following HBO_2 preconditioning, the investigators felt that the increased association of Hsp90 with eNOS supported the notion that Hsp90 helps eNOS to produce NO and that this was responsible for the observed myocardial protection against IRI following HBO_2 preconditioning.

Mechanism 5: Lipid Peroxidation

Superoxide has been hypothesised to be involved in reactions antagonizing lipid peroxidation (Thom and Elbuken, 1991) and, in certain doses, may function as a terminator of lipid peroxidation (Nelson et al., 1994). HBO_2 given before ischaemia-reperfusion (Chen et al., 1998, Gurer et al., 2006) and during ischaemia (Bosco et al., 2007), has also been demonstrated to reduce lipid peroxidation. The reduction in ischaemia reperfusion mediated lipid peroxidation has also been observed following post IRI treatment with HBO_2 (Rubinstein et al., 2009). In a model of myocardial IPC, which also involves the generation of ROS, it was demonstrated that the beneficial effects of IPC was associated with both a reduction in neutrophil adhesion and lipid

peroxidation (Zhao et al., 2003). It is therefore also plausible to suggest that, HBO_2, via its ROS generation, is also capable of mediating the attenuation of lipid peroxidation and this leads to the associated reduction in neutrophil adhesion *(Figure 1.1: Mechanism 5)*.

Mechanism 6: Endothelial Cell Proliferation

HBO_2 has been shown to stimulate neovascularization in flaps, wounds, irradiated tissue and grafts (Bayati et al., 1998, Manson PN, 1980, Marx et al., 1990, Meltzer and Myers, 1986, Nemiroff, 1987, Zhang et al., 1998) and, angiogenesis (Godman et al., 2009, Han et al., 2008a, Khan et al., 2009, Ren et al., 2008, Sheikh et al., 2005). ROS have been shown to be able to function as signaling molecules (Suzuki et al., 1997b) that are a able to stimulate endothelial cell proliferation (Heinloth et al., 2000) and neovascularization (Monte et al., 1994). As HBO_2 is a modality of treatment which is capable of generating ROS, it is also reasonable to suggest that via its ROS signaling properties, HBO_2 may be capable of improving blood flow through endothelial cell proliferation and neovascularisation thus, alleviating tissue ischaemia *(Figure 1.1: Mechanism 6)*.

1.8 Hypothesis

The hypothesis of this study is that systemically preconditioning CAD patients with 100% oxygen, at a pressure above that of atmospheric pressure i.e. HBO_2, prior to first time elective on CPB CABG, leads to a remote preconditioning like effect that is capable of leading to post operative improvements in cardiovascular function.

1.9 Objective

1.9.1 Primary Objective

The primary cardiovascular efficacy objective of this study was to determine if systemically preconditioning CAD patients with HBO_2 prior to first time elective on CPB CABG, remotely precontioned the myocardium by leading to a better post operative improvement in myocardial left ventricular stroke work (LVSW) compared to CAD patients who were not preconditioned with HBO_2.

1.9.2 Secondary Objectives

Main Secondary Objectives:

The main secondary objectives of this study were to assess, in comparison to the Control Group, the effects of HBO_2 preconditioning on:

a) other peri-operative cardiovascular efficacy parameters.

b) patient outcomes with respect to the safety.

Exploratory Secondary Objectives:

The exploratory secondary objectives for this study were to assess, in comparison to the Control Group, the effects of HBO_2 preconditioning on:

a) surrogate biomarkers of endothelial and neutrophilic adhesiveness.

b) myocardial biomarkers of cardioprotection.

Additionally, a *post-hoc* analysis was also done to determine the cost effectiveness of HBO_2 preconditioning .

1.10 End Points

1.10.1 Primary Endpoint Measure

The primary cardiovascular efficacy endpoint measure of this study was the mean (or median) measurement of myocardial LVSW 24 hours post CPB in both groups.

1.10.2 Secondary Endpoint Measures

Main Secondary Endpoint Measures:

The secondary endpoint measures of this study were the mean (or median or incidence or proportion) measurements, in both groups, with respect to the following:

a) at all the pre-specified time points, the peri-operative cardiovascular haemodynamic parameters, as described in *Table 2.4*.
b) post operative adverse events (AEs) described in *Table 2.3*.

In this study, an AE was defined as any new untoward medical occurrence or worsening of a pre-existing medical condition in a patient treated with HBO_2 and that does not necessarily have a causal relationship with this treatment. An AE can therefore be any unfavorable and unintended sign (including an abnormal laboratory finding or prolonged admission), symptom, or disease temporally associated with the use of HBO_2, whether or not considered related to the HBO_2.

Exploratory Secondary Endpoints:

The exploratory secondary endpoint measures of this study were the mean (or median) measurements, in both groups, at all of the pre-specified time points, with respect to the following:

a) concentration of serum soluble adhesion molecules which included:

    i. soluble E-Selectin
    ii. soluble P-Selectin
    iii. soluble ICAM-1
    iv. soluble PSGL-1

b) quantity of intra-operative myocardial cardioprotective proteins which included:

    c) eNOS
    d) Hsp72

The *post-hoc* cost effectiveness analysis of HBO_2 preconditioning was done by estimating the cost for HBO_2 treatment and the daily post operative cost for the use of an intensive care bed in both groups.

2. Methods

2.1 Ethical & Hospital Approvals

Prior to commencing this study, ethical and hospital approval was obtained from the Hull & East Riding Local Research Ethics Committee (approval number: 04/Q1104/26) and the Hull & East Yorkshire NHS Trust (approval number: R0047), respectively. This clinical study conforms with the Declaration of Helsinki, the principals of Good Clinical Practice (GCP) and the principals of the International Conference of Harmonisation.

2.2 Study Population

The study population was defined as all patients with coronary artery atherosclerosis who were admitted to the Hull & East Yorkshire NHS Trust for elective first time CABG surgery using CPB and hypothermic intermittent ischaemic fibrillatory arrest. From January 2005 to July 2006, this study population consisted of 474 patients who had been admitted under the care of the two cardiac surgeons who had agreed to be part of this study.

2.2.1 Inclusion Criteria

The inclusion criteria was:

1. patients undergoing elective first time CABG using CPB and hypothermic intermittent ischaemic fibrillatory arrest.

2.2.2 Exclusion Criteria

The exclusion criteria were:

1. Patients with a high surgical risk which included:
   a) **Age < 20 and > 85 years**.
   b) **Ejection fraction < 30%**.
   c) **Unstable angina**.
   d) **< 1 month post MI**.
   e) **Cardiac disease other than CAD**.
   f) **Organ failure**.
   g) **History of chronic obstructive pulmonary disease (COPD), pneumothorax, pulmonary bullae, convulsions, myopia or intraocular lens.** Patients with a history of any these clinical conditions were excluded as exposure to the pressure changes during HBO_2 preconditioning may lead to complications.

2. **Current use of $K^{+}_{(ATP)}$ channel openers, oral hypoglycaemic, opioid analgesics or catecholamines.** Patients on any form of these agents were excluded as these agents have demonstrated some capacity to provide myocardial protection.

2.3 Statistical Plan

The statistical plan and analysis for this study was done in collaboration with the appointed study statistician, Dr.E.Gardiner.

2.3.1 Sample Size

Based on the haemodynamic observations from another study involving pharmacological preconditioning and myocardial protection (Wang et al., 2003), the sample size calculations for this study was based on the assumption that compared to the Control Group, treatment with HBO_2 preconditioning in the HBO_2 Group, would lead to a 7.5% improvement in LVSW, 24 hours post CPB. Furthermore, based on a previous clinical study (Alex et al., 2005) involving HBO_2 preconditioning and CBAG, a within-group standard deviation of 6.25% was assumed. A two-sided 5% significance level and a 90% power were pre-specified for this study.

Allowing for the detection of a 7.5% difference in the primary endpoint measure between the two treatment groups and, allowing for increased variance of interaction and estimates relative to the main effect (Piantadosi, 1997), it was estimated that a minimum of 60 patients would be

required to show statistically significant difference between the groups, with respect to LVSW at the time point '24 hours post CPB'. Based on the previous clinical study by Alex *et.al* (2005) involving HBO_2 and CABG, a drop-out rate of 35% was estimated for this study. Taking this into account, it was estimated that a minimum of 80 patients would need to be randomised to detect the a difference of 7.5% between the groups with respect to the primary endpoint measure.

However, as the detection of a 7.5% difference between the groups is small, it was expected that there would also be the possibility that this sample size may not be large enough to detect any difference between the groups and a Type 2 error (false negatives) made be made. Additionally, as this study has multiple secondary endpoints, which would involve multiple statistical testing, this may also increase the risk of a Type 1 error (false positives). To avoid making conclusions that were based on Type 1 and Type 2 errors, it was planned that definitive conclusions, based on statistical analysis, can only be drawn for the comparison of the primary endpoint between the groups, and not the secondary endpoints.

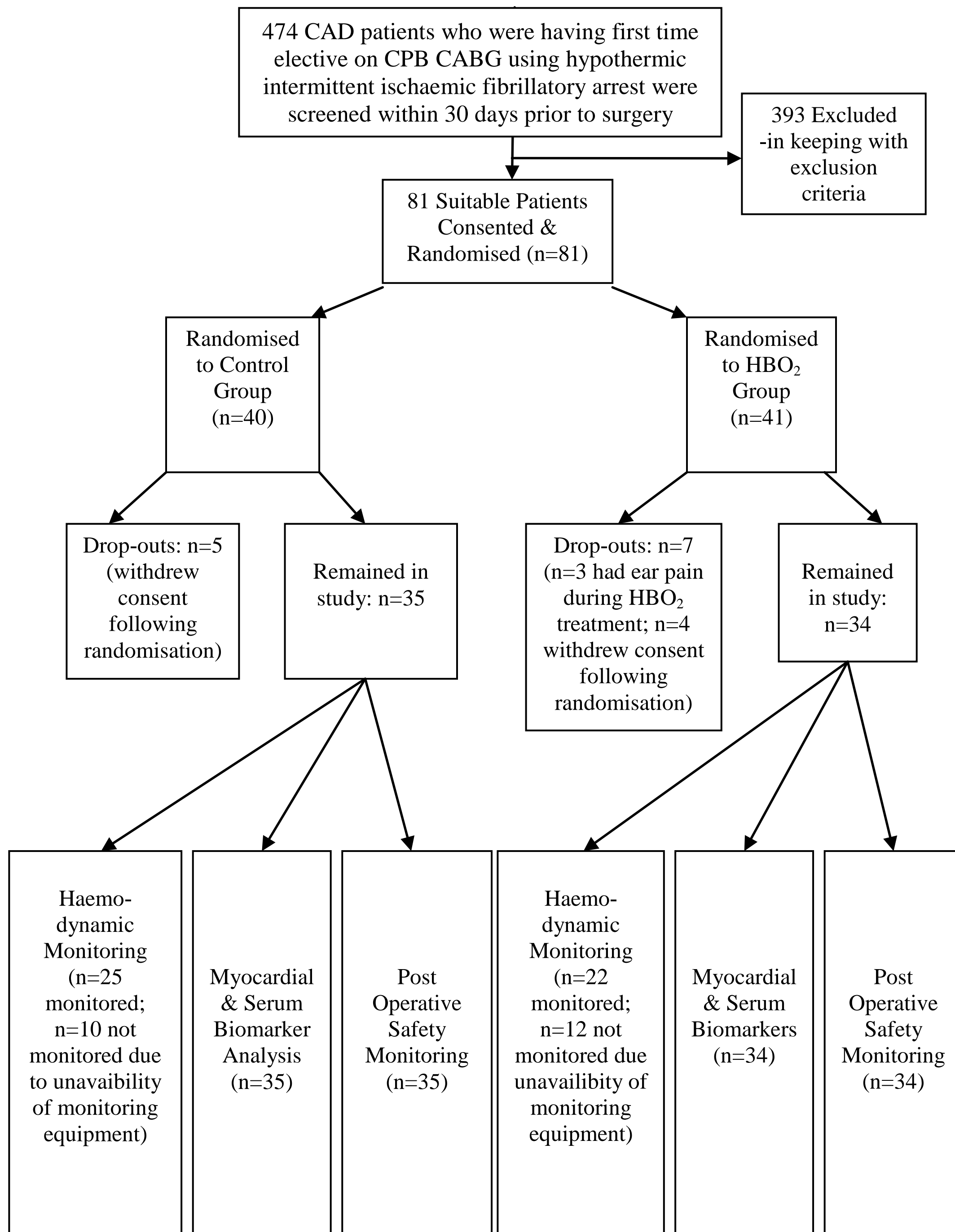
In this study, it was also planned that the main analysis for comparing the secondary endpoints measures between the groups would be a descriptive analysis of the means (or medians or proportions), with the statistical analysis only providing a sensitivity measure to support estimates of the descriptive differences that are reported.

2.3.2 Sample Population

Screening for suitable patients from the study population took place during the pre-assessment clinical visits. During these visits, which occurred up to 30 days prior to surgery, patients had routine investigations conducted as part of their work-up prior to surgery. From the study population of 474 patients, 81 matched the study criteria and were recruited following informed consent. 40 patients were randomised to the Control Group and 41 patients were randomised to the HBO_2 Group. The patients were aware of their randomisation group. None of the surgeons, junior doctors, anaesthetists, perfusionists or nursing team were aware of the study group allocations of the patients.

Following randomisation, there were 5 drop-outs from the Control Group and 7 from the HBO_2 Group. The total drop-out rate for this study was 13.6%, which was less than was expected. The drop-out rate for the Control Group was 13% while for the HBO_2 group it was 17%. The reason for the 5 drop-outs from the Control Group was a withdrawal of consent following randomisation. Of the 7 drop-outs from the HBO_2 Group, 4 were due to withdrawal of consent following randomisation and 3 were due to ear pain during the initial pressurisation protocol of the HBO_2 preconditioning treatment. *Figure 2.1* shows the CONSORT Flow Diagram of patients recruited to this study.

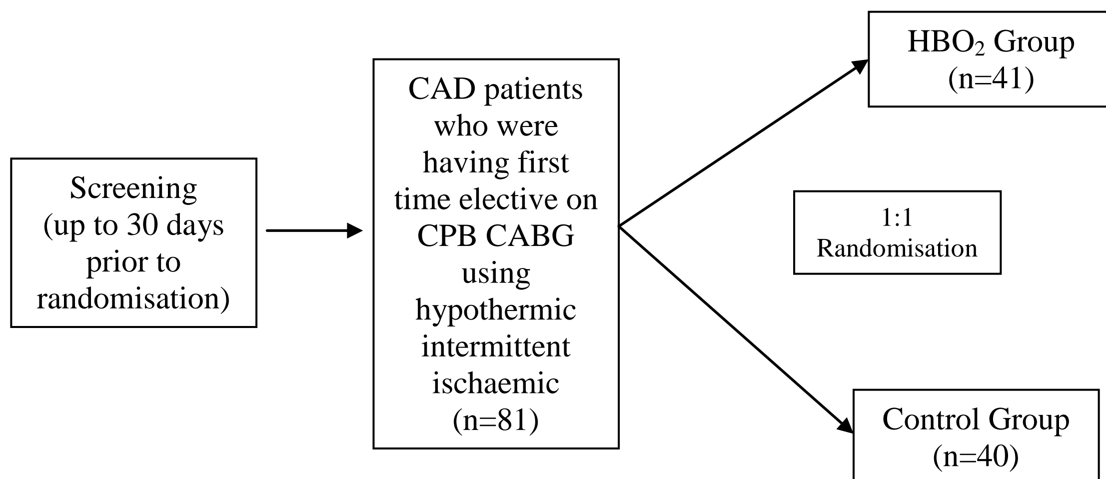
Figure 2. 1: Consort Flow Diagram



2.3.3 Study Design

This study was designed as a pilot proof of concept, phase 2, prospective, single centre randomised control study that was based on a previous clinical study involving HBO_2 and CABG (Alex et al., 2005). In this study, following screening during the pre-admission clinic visit, suitable consenting patients were randomised to either the Control Group or to the HBO_2 Group. Randomisation was done in a 1:1 manner *(Figure 2.2)*. Randomisation was carried out by pulling out sealed envelopes in sequence from a box. The random treatment allocations were contained within the envelope. The random treatment allocation list was prepared by the study statistician.

Figure 2. 2: Study Design



2.3.4 Statistical Methods

Repeated measures of ANOVA was used to analyse the data involving haemodynamic and safety measurements and, the concentrations of serum Troponin-T, soluble adhesion molecules and, myocardial eNOS and Hsp72. Wilcoxon Signed Rank Test was used to analyse pre and post HBO_2 data involving serum soluble adhesion molecules concentrations. Independent sample *t*-test was used to analyse post operative length of stay in ICU, intra and post operative blood loss and, post operative blood transfusion. The Chi-Square test was used to analyse categorical differences between the groups.

Data were analysed in accordance with the principals of intention to treat basis (ITT) (Molenberghs, 2007). Where data were missing, no imputation was undertaken. As there was no imputation of data, where large amounts of data were missing, only an on-treatment analysis was performed. To determine if the data were normally distributed or skewed, both the means and the medians were calculated. A large disparity between these would suggest that the data were skewed. Where the data were skewed, natural logarithmic (ln) transformation of the data were performed to normalise the data and enable parametric statistical analysis. Once parametric statistical analysis was performed, the data from logged results were transformed back into original estimates to provide the geometric mean which represents the ratio of the HBO_2 Group to Control Group values (Altman, 1991).

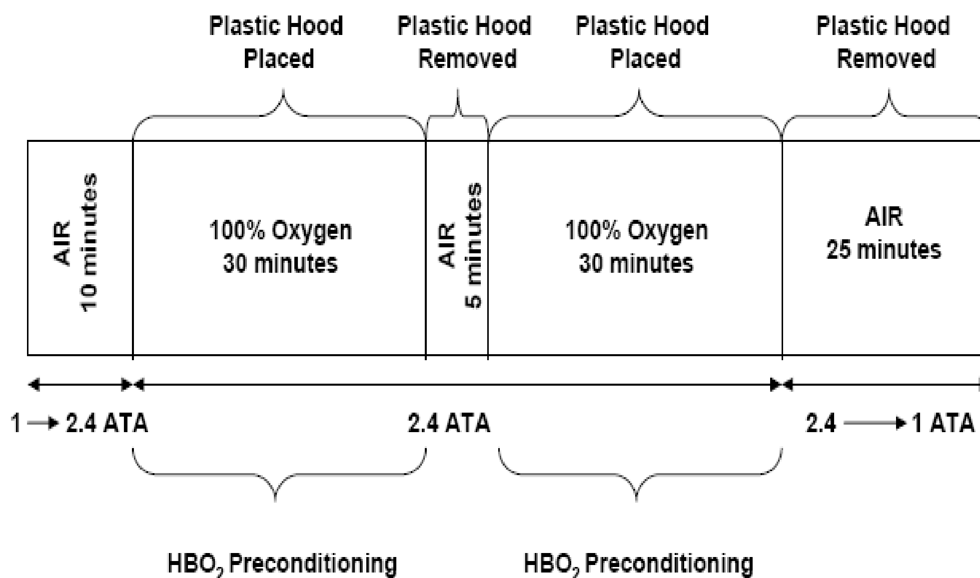
Where appropriate, the location of the data are displayed in tables, as ranges, medians, means and ln means. The p-values provided are for

the geometric means. The variability of the the means of the data are displayed in bar charts as the standard error of the mean.

2.4 Hyperbaric Oxygen Preconditioning Protocol

The pressure and duration for HBO_2 preconditioning was based on the optimum effect noted in a previous clinical study (Alex et al., 2005) involving HBO_2 and CABG. On the morning of surgery, patients randomised to the HBO_2 Group were transported to the Classic Hospital (Hull, UK) where they were preconditioned with HBO_2. The protocol for the HBO_2 treatment is depicted in *Figure 2.3*.

Figure 2. 3: HBO_2 Protocol



The HBO_2 preconditioning treatment consisted of pressurisations over 10 minutes from 1 ATA to 2.4 ATA. During this pressurisation period, the patients were breathing air. Once at 2.4 ATA, patients placed a clear plastic hood over their heads and 100% oxygen was supplied for 30 minutes. This period was followed by a 5 minute interval out of the hood at 2.4 ATA, when the patients were breathing only air. After this interval, the hood was replaced and the patients again breathed 100% oxygen at 2.4 ATA for a further 30 minutes. The two 30 minute periods of 100% oxygen at 2.4 ATA effectively constitutes the treatment of HBO_2 preconditioning. Finally, the hood was removed and the chamber was depressurised over 25 minutes back to 1 ATA. During this depressurisation period, patients were again only breathing air. Treatment with HBO_2 preconditioning was completed approximately 2 hours prior to CPB. This duration between the end of HBO_2 preconditioning and the start of CPB was the minimum duration that this research unit could achieve, as the chamber for the administration of HBO_2 was not at the site where patients were having their CABG surgery.

2.5 Anaesthetic & Surgical Procedure

There were 8 anaesthetists and 2 surgeons who were part of this randomised control study. All of them adhered to the same anaesthetic and surgical technique.

2.5.1 Anaesthetic Procedure

Approximately 1 hour prior to CABG, while the patients were on the ward, all the patients were given an anoxylitic, which consisted of either Temazepam or Lorazepam. In the Control Group this was given following baseline venous blood sampling. In the HBO_2 Group, this was given after the post HBO_2 venous blood sample was taken.

In the anaesthetic room, once all the routine monitoring devices were attached to the patient, anaesthesia was induced using Fentanyl and Propofol. Following anaesthetic induction, a pulmonary artery (PA) catheter (Edward Life Sciences, Germany) was inserted to enable peri-operative haemodynamic monitoring. During the intra-operative period, Isoflurane and Ramifentanyl were used to maintain anaesthesia.

2.5.2 Surgical Procedure

Non-pulsatile (continuous flow) CPB was achieved using a Stockert SIII roller pump (Stockert Instrumente GmbH, Germany) together with a hollow fibre membrane oxygenator, an integral hard shell venous cardiotomy reservoir (Avant Phisio/M-Dideco, Italy) and a 38µm arterial line filter (Affinity352 Medtronic Inc, USA). During CPB, systemic hypothermia of 32°C was maintained. Intermittent ischaemic fibrillatory arrest was used as the intra-operative method for myocardial protection. Re-warming commenced during the distal anastamosis of the final coronary artery bypass graft. In this study, none of the patients were given Trasylol (serine protease inhibitor) as an anti-thrombolytic prior to CABG.

2.6 Study Data Collection & Measurements

The data collected and the measurements made during this study consisted of the following:

a. pre-operative and intra-operative patient data.
b. post operative patient AEs.
c. measurements of peri-operative haemodynamic parameters.
d. measurements of peri-operative venous blood serum biomarker concentrations.
e. measurements of the amount of intra-operative myocardial biomarkers.

The details of the data collected and the measurements made, will be discussed in the following sections.

2.6.1 Pre-Operative Patient Data Collection

Following screening, to determine suitability for this study, and obtaining informed consent, pre-operative patient data were collected as described in *Table 2.1*. Apart from serum Troponin-T, all these data are routinely collected as part of pre-operative assessment.

Table 2. 1: Pre-Operative Patient Data Catergories

Pre-Operative Patient Data	Age
	Sex
	Body Mass Index (BMI)
	History of unstable angina
	History of previous MI
	Severity of CAD
	Left ventricular function
	History of hypertension
	History of diabetes mellitus
	History of peripheral vascular disease
	EUROSCORE (Simple Additive Score) (Roques et al., 1999)
	Pre-HBO_2 Serum Troponin-T *(all patients)*
	Post HBO_2 Serum Troponin-T *(only patients in HBO_2 Group)*

These data were collected as these factors may affect the intra and post operative clinical outcomes of patients in this study.

The pre- and post operative measurement of serum Troponin-T was not routinely performed in the Cardiothoracic Surgical unit at Castle Hill Hospital, Hull. The pre- and post HBO_2 serum Tropinin-T measurements were used as a surrogate biomarker which enabled further assessment of the myocardial safety, with respect to myocardial injury, of HBO_2 preconditioning.

Serum Troponin-T from all time points were analysed by the Department of Biochemistry at the Hull & East Yorkshire NHS Trust within 4 hours of collection. This analysis was done using an electrochemiluminescence immunoassay (ELICA) (Troponin T STAT, Roche Diagnostics Ltd, Burgess Hill, UK) performed on the Roche Elecsys 2010 analyser in accordance with the manufacturer's protocol. The measuring range of the kit is 0.01-25.00ng/ml. The analytical sensitivity (lower detection limit) and specificity of the kit is 0.01ng/ml and, between 0.001%-0.1%, respectively. According to the European Society of Cardiology (ESC) and the American College of Cardiology (ACC), MI is diagnosed when the levels of cardiac Troponin are above the $99^{th.}$ percentile of the reference limit in the healthy population (Alpert J. S., 2000). According to the manufacturor, the $99^{th.}$ percentile for Troponin-T in the healthy population, measured using the Troponin-T STAT kit, is <0.01ng/ml. Furthermore, it has also been recommended that the Troponin-T concentration that is used to diagnose an MI must take in to account an imprecision (coefficient of variation) at the 99th. percentile that is equal to or less than 10% (Alpert JS, 2000) and this coefficiant of variation must also be taken into account when determining the serum Troponin-T concentration that will be used as a medical diagnostic guide (Apple FS, 2001). According to the manufacturor of the Troponin-T STAT kit, this would make a serum Troponin-T concentration of 0.03ng/ml as the medical diagnostic guide for diagnosing an MI. It is the lowest limit of quantification that can be reproducibly measuresed with a coefficient of variation of 10%.

2.6.2 Intra-Operative Patient Data Collection

Table 2.2 shows the routine categories of patient data that were collected during surgery:

Table 2. 2: Intra Operative Patient Data Categories

Intra Operative Patient Data	Myocardial ischaemic time
	CPB time
	Intra-operative blood loss

The intra-operative myocardial ischaemic time was measured to determine the length of time, during CABG, when the myocardium was exposed to ischaemia and thus susceptible to myocardial injury. The time spent on CPB was measured to determine the length of time patients were exposed to the hypothermia of 32°C and the non-pulsatile flow CPB. Intra-operative blood loss was measured by determining the volume of blood collected by the Yanker Suckers and the cell savers, as a measure of the amount of intra-operative bleeding experienced by patients during this study.

2.6.3 Post-Operative Patient Adverse Event Data

Table 2.3 shows the patient AE data that were collected following the CABG surgery, during the in-hospital stay:

Table 2. 3: Post Operative Patient Adverse Event Data

Type of AE	Measurement
Cardiovascular	Troponin-T ≥ 0.03ng/ml
	Proportion with low cardiac output syndrome
	Proportion with atrial fibrillation
	Proportion with MI
	Proportion with inotrope use
	Proportion needing cardiovascular supportive therapy (IABP*, pacing)
	Volume of post operative blood loss
	Volume of post operative blood transfusion
Pulmonary	Duration mechanical ventilation
	Duration of endotracheal inbubation
	Proportion with pneumothorax
	Proportion with pleural effusion
	Proportion with chest infection
	Proportion needing pulmonary supportive therapy (BIPAP¥, CPAP§)
Renal	Proportion with serum creatinine > 200mmols/l
	Proportion with needing renal supportive therapy (CVVH†)
Neurological	Proportion with confusion
	Proportion with transient ischemic attack (TIA)
	Proportion with stroke
Gastrointestinal	Proportion with diarrhoea
	Proportion with ischaemic bowel
Microbiological Infections	Proportion with superficial sternal wound infections
	Proportion with deep sternal wound infections
	Proportion with leg wound infections
ICU Stay	Number of hours
Sternal Re-wiring	Proportion needing sternal re-wiring
Mortality	Proportion of dead patients

*IABP-Intra-Aortic Balloon Pump;
¥BIPAP- Biphasic Positive Airway Pressure;
§CPAP-Continuous Positive Airway Pressure;
†CVVH- Continuous Venovenous Haemofiltration

Apart from the measurement of serum Troponin-T ≥ 0.03ng/ml, the above AE data were routinely collected post operative data at the Cardiothoracic Surgical unit in Castle Hill Hospital, Hull. This AE data enabled descriptive estimates of the post operative safety profile of each group and, enabled simple statistical comparisons between the groups. Post operative serum creatinine was measured by the Department of Biochemistry at the Hull & East Yorkshire NHS Trust using Beckman Coulter (UK) Ltd (High Wycombe, UK) Unicel DxC800 (Synchron Clinical Systems) modular creatinint (Jaffe) method.

In the hospital where this study was conducted, and as such for this study, patients requiring the used of inotropes, intra or post operatively, were identified as experiencing low cardiac output syndrome. Atrial fibrillation (AF) in this study was defined as an electrocardiogram (ECG) tracing without ‘p’ waves at a rate of ≥ 120 per minute.

2.6.4 Peri-Operative Haemodynamic Measurements

During the peri-operative period, haemodynamic measurements were taken using the PA catheter which was inserted following anaesthetic induction. The haemodynamic parameters and the time points at which they were measured are displayed in *Table 2.4*.

Table 2. 4: Peri-Operative Haemodynamic Parameters

Time Points								
		Intra-operative		**Post-Operative**				
		Post Anaesthetic Induction	5 minutes post CPB	2 hours post CPB	4 hours post CPB	8 hours post CPB	12 hours post CPB	24 hours post CPB
Haemodynamic Parameters	Heart Rate (HR)	X	X	X	X	X	X	X
	Mean Arterial Pressure (MAP)	X	X	X	X	X	X	X
	Stroke Volume (SV)	X	X	X	X	X	X	X
	Cardiac Output (CO)	X	X	X	X	X	X	X
	Cardiac Index (CI)	X	X	X	X	X	X	X
	Mean Pulmonary Artery Pressure (MPAP)	X	X	X	X	X	X	X
	Pulmonary Capillary Wedge Pressure (PCWP)	X	X	X	X	X	X	X
	Pulmonary Vascular Resistance (PVR)	X	X	X	X	X	X	X
	Pulmonary Vascular Resistance Index (PVRI)	X	X	X	X	X	X	X
	Systemic Vascular Resistance (SVR)	X	X	X	X	X	X	X
	Systemic Vascular Resistance Index (SVRI)	X	X	X	X	X	X	X
	Left Ventricular Stroke Work (LVSW)	X	X	X	X	X	X	X
	Left Ventricular Stroke Work Index (LVSWI)	X	X	X	X	X	X	X
	Right Ventricular Stroke Work (RVSW)	X	X	X	X	X	X	X
	Right Ventricular Stroke Work Index (RVSWI)	X	X	X	X	X	X	X

Measurement of these haemodynamic parameters enabled the assessment of the effects of HBO_2 preconditioning on peri-operative myocardial function (HR, MAP, SV, CO, CI, MPAP, LVSW, LVSWI, RVSW and RVSWI), systemic vascular function (MAP, SVR and SVRI) and pulmonary vascular function (MPAP, PCWP, PVR and PVRI).

2.6.5 Peri-Operative Serum Biomarker Sampling

During the peri-operative period, two 5ml samples of venous blood was drawn from each patient at the time points shown in *Table 2.5* for serum biomarker analysis.

Table 2. 5: Peri-Operative Venous Blood Sampling

	Time Points					
	Pre-HBO_2	Post HBO_2	5 minutes on CPB	5 minutes post IRI	2 hours post CPB	24 hours post CPB
Control Group	X	†	X	X	X	X
HBO_2 Group	X	X	X	X	X	X

†*The Control Group did not have any blood taken at the post HBO_2 time point*

In this study, the baseline blood samples from each group were not taken at the same time. In the Control Group, a baseline (this will be classified as the 'pre-HBO_2' time point in the Control group) blood sample was taken approximately 1 hour prior to anaesthetic pre-medication. In the HBO_2 Group, a baseline 'pre-HBO_2' blood sample was taken approximately 1 hour prior to HBO_2 preconditioning. As the Control Group of patients did not have any intervention or HBO_2 preconditioning prior to anaesthesia and CABG, no

blood sample was taken at the 'post HBO_2' time point in this study. In the HBO_2 Group, the 'post HBO_2' blood sample was taken approximately 1 hour after HBO_2. Only the HBO_2 Group had blood taken pre and post HBO_2 preconditioning to allow for assessment of changes in serum biomarkers following HBO_2 preconditioning. The blood sample at the time point '5 minutes on CPB' was taken 5 minutes following the onset of CPB. This '5 minutes on CPB' blood sample allowed for the assessments of the early changes in serum biomarkers following the initiation CPB, prior to operative IRI, in both groups, when compared to the blood sample taken at the 'pre-HBO_2' time point. The blood sample taken at the time point '5 minutes post IRI' was taken 5 minutes following the final release of the aortic cross clamp from the aorta i.e. after the final anastamosis of the final bypass graft to the coronary artery (distal anastamosis) was complete. This '5 minutes post IRI' blood sample allowed for the assessment of changes in serum biomarkers as a result of the effects of operative IRI in both groups when compared to blood samples taken at earlier time points in this study. The blood samples taken at the time point '2 hours post CPB' and '24 hours post CPB', allowed for the assessment of the delayed changes in serum biomarkers following the termination CPB in both groups when compared with blood samples taken at earlier time points in this study.

Each of the two 5ml blood sample taken was placed in an amber top BD Vacutainer Tube (SST11 Advance). One tube was analysed for serum Troponin-T and the other was used for the analysis of serum soluble adhesion molecule biomarkers. The tube for serum soluble adhesion molecule analysis

was centrifuged, within 4 hours of collection, at 1200*g* for 15 minutes at 2ºC in a Rotanta 96R centrifuger (Hettich Zentrifugen, Tuttingen, Germany). The supernatant (i.e. the serum) was subsequently pipetted into labelled 1.5ml propylene tubes and stored at -80ºC for later serum biomarker analysis.

2.6.6 Peri-Operative Serum Biomarkers Assessment

The methods used for the assessment of the biomarkers investigated in this study are described below.

2.6.6.1 Peri-Operative Serum Troponin-T Assessment

The pre- and post operative measurement of serum Troponin-T was not routinely performed in the Cardiothoracic Surgical unit at Castle Hill Hospital, Hull. The pre- and post HBO_2 serum Tropinin-T measurements were used as a surrogate biomarker which enabled further assessment of the myocardial safety, with respect to myocardial injury, of HBO_2 preconditioning. Serum Troponin-T from all time points were analysed by the Department of Biochemistry at the Hull & East Yorkshire NHS Trust within 4 hours of collection. This analysis was done using an electrochemiluminescence immunoassay (ELICA) (Troponin T STAT, Roche Diagnostics Ltd, Burgess Hill, UK) performed on the Roche Elecsys 2010 analyser in accordance with the manufacturer's protocol. The measuring range of the kit is 0.01-25.00ng/ml. The analytical sensitivity (lower detection limit) and specificity of the kit is 0.01ng/ml and, between 0.001%-0.1%,

respectively. According to the European Society of Cardiology (ESC) and the American College of Cardiology (ACC), MI is diagnosed when the levels of cardiac Troponin are above the $99^{th.}$ percentile of the reference limit in the healthy population (Alpert J. S., 2000). According to the manufacturor, the $99^{th.}$ percentile for Troponin-T in the healthy population, measured using the Troponin-T STAT kit, is <0.01ng/ml. Furthermore, it has also been recommended that the Troponin-T concentration that is used to diagnose an MI must take in to account an imprecision (coefficient of variation) at the 99th. percentile that is equal to or less than 10% (Alpert JS, 2000) and this coefficiant of variation must also be taken into account when determining the serum Troponin-T concentration that will be used as a medical diagnostic guide (Apple FS, 2001). According to the manufacturor of the Troponin-T STAT kit, this would make a serum Troponin-T concentration of 0.03ng/ml as the medical diagnostic guide for diagnosing an MI. It is the lowest limit of quantification that can be reproducibly measuresed with a coefficient of variation of 10%.

2.6.6.2 **Peri-Operative Serum Soluble Adhesion Molecule Assessment**

In this study, the serum soluble adhesion molecule biomarkers that were assessed were soluble E-Selectin (sE-Selectin), soluble P-Selectin (sP-Selectin), soluble ICAM-1 (sICAM-1) and soluble PSGL-1 (sPSGL-1). These serum biomarkers were measured as they are adhesion molecules that are known to be shed (Gearing and Newman, 1993, Hayward et al., 1999, Hillis et al., 2002, Pigott et al., 1992, Ushiyama et al., 1993) from the surfaces of the vascular endothelium (sE-Selectin, sP-Selectin and sICAM-1) or cleaved off the surfaces of neutrophils (sPSGL-1) (Gardiner et al., 2001, Lefer et al., 1998) following a period of oxidative stress such as IRI. Following the discussion in *section 1.2.1*, as intact adhesion molecules on the surfaces of the vascular endothelium and neutrophils are required for endothelial-neutrophil adhesion, it is reasonable to suggest that the presence of the soluble form of these adhesion molecules, which are no longer attached to the endothelial or neutrophilic surfaces, provides an indirect measure of the reduced adhesiveness of those surfaces. Furthermore, following on from the discussion in *section 1.2.1.3*, it is reasonable to suggest that the more of these intact adhesion molecules that are shed or cleaved off the surfaces of the vascular endothelium and the neutrophils into the circulation, the less likely is it for neutrophils to be able to adhere to the vascular endothelium and vice-versa. This has the potential to limit neutrophil mediated vascular injury.

To measure these serum soluble adhesion molecules, the required serum samples were removed from the -80ºC freezer and thawed prior to use. Each sample was analysed for the four different serum soluble adhesion molecules in the sequence of sE-Selectin, sP-Selectin, sPSGL-1 and sICAM-1. As such, samples from each time point for sE-Selectin analysis experienced one freeze-thaw cycle, the samples for sP-Selectin analysis experienced two freeze-thaw cycles, the samples for sPSGL-1 analysis experienced three freeze-thaw cycles and the samples for sICAM-1 analysis experienced four freeze-thaw cycles.

All samples for serum soluble adhesion molecule measurement were analysed using a quantitative sandwich ELISA procedure. The ELISA kits for sE-selectin, sP-selectin and sICAM-1 were purchased from R&D Systems (R&D Systems Inc, Minneapolis, USA) while those for sPSGL-1 analysis were purchased from Bender MedSystems (Bender MedSystems, Vienna, Austria).

The ELISA assay procedure was performed according to the methods recommended by the manufacturer. This method was similar for all the four types of assays. As such, for the purpose of describing the ELISA method that was used, the general steps involved in the serum sE-Selectin ELISA assay procedure will be described in the following section. A summary of the differences in between sE-Selectin ELISA assay procedure and the ELISA assay procedure for sP-Selectin, sICAM-1 and sPSGL-1, will be described in the subsequent sections.

2.6.6.2.1 **ELISA Assay Procedure For sE-Selectin**

All the reagents used in this procedure were provided by the manufacturer (R&D Systems Inc, Minneapolis, USA) and were brought to room temperature prior to use.

Sample Preparation

All samples were diluted 20 fold by adding 15μl of sample to 285μl of Sample Diluent.

Reagent Preparation

The wash buffer was made by diluting 20ml of the wash buffer concentrate with 480ml distilled water. The sE-Selectin conjugate concentrate (sheep polyclonal antibody to recombinant human sE-Selectin conjugated to horseradish peroxidase in buffer with preservative) was then diluted by pipetting 250μl of the concentrate into a bottle containing 11ml of Conjugate Diluent (horseradish peroxidase conjugate concentrate). All the provided standards were reconstituted by pippeting 1ml of distilled water into each of the bottles containing the standards. The reconstituted standards were then allowed to sit at room temperature for 10 minutes before use. The concentration of each of the reconstituted standards was stated on each of the bottles containing the standards *(Table 2.6).*

Table 2. 6: Concentration of sE-Selectin Standards

Standards	sE-Selectin Concentration (ng/ml)
5	10.56
4	7.91
3	4.88
2	2.54
1	0.57
0	0

Assay Procedure

Using the 96 well microplate (12 strips, 8 wells per strip, coated with a mouse monoclonal antibody to human sE-Selectin), 100µl of sE-Selectin Standard 5 was pipetted in duplicate in wells A1 & B1. This was followed by 100µl sE-Selectin Standard 4 in wells A2 & B2, 100µl sE-Selectin Standard 3 in wells A3 & B3, 100µl sE-Selectin Standard 2 in wells A4 & B4, 100µl sE-Selectin Standard 1 in wells A5 & B5 and 100µl sE-Selectin Standard 0 in wells A6 & B6. Wells A7, A8, B7 and B8 were left blank *(Figure 2.4)*. In this study, all the blank wells were left empty and not used during the assay procedure.

100µl of a particular patient's diluted sample, from an appropriate study time point, was then added in duplicates to the respective wells in strips C to L of the ELISA microplate *(Figure 2.4)*. 100µl of the diluted sE-Selectin Conjugate was then added to all the wells in strips A to L (except for the blank wells). The microplate was then covered with a plate sealer and allowed to incubate for 1.5 hours at room temperature on a rotator set at 100 revolutions

per minute (rpm). The plate was then washed six times with 300µl of the previously made up wash buffer using a Thermolab Systems Wellwash 4MK2 autowasher. Once washed and ensuring that all the liquid in each well was removed, 100µl of the Substrate Solution (tetramethylbenzidine-TMB) was added to each well (except for the blank wells). The micoplate was then once again covered with a new plate sealer and allowed to incubate, in the dark, at room temperature for approximately 15 minutes on a rotator set at 100rpm. Once the most concentrated standard had reached a deep blue colour, 100µl of the provided Stop Solution (Sulfuric Acid) was added to each well (except for the blank wells). Within 30 minutes of adding the Stop Solution, the spectophotometric reading from each well was then determined using the Anthos 2010 microtitre plate reader (Anthos Labtec Instrument GmdH, Austria) together with the Stingray software (Stingray Software Inc, USA). A primary wavelength of 450nm was used during the spectophotometric analysis with a correction for optical imperfections in the plate set at 620nm.

According to the manufacturer (R&D System), the intra-assay coefficient of variation for sE-Selectin ELISA microplate was between 4.7% and 5% while the inter-assay coefficient of variation was between 5.7% and 8.8%. The minimum detectable dose of sE-Selectin was 0.1ng/ml.

Figure 2. 4: Schematic Representations of ELISA Microplate Wells Containing ELISA Standards & Samples At Each Time Point In Duplicates

	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**	**L**
1	Standard 5	Standard 5	Pre HBO_2	Pre HBO_2	5 minutes On CPB	5 minutes On CPB	2 hours Post CPB	2 hours Post CPB	Pre HBO_2	Pre HBO_2	5 minutes On CPB	5 minutes On CPB
2	Standard 4	Standard 4	Post HBO_2	Post HBO_2	5 minutes Post Ischaemic Reperfusion	5 minutes Post Ischaemic Reperfusion	24 hours Post CPB	24 hours Post CPB	Post HBO_2	Post HBO_2	5 minutes Post Ischaemic Reperfusion	5 minutes Post Ischaemic Reperfusion
3	Standard 3	Standard 3	5 minutes On CPB	5 minutes On CPB	2 hours Post CPB	2 hours Post CPB	Pre HBO_2	Pre HBO_2	5 minutes On CPB	5 minutes On CPB	2 hours Post CPB	2 hours Post CPB
4	Standard 2	Standard 2	5 minutes Post Ischaemic Reperfusion	5 minutes Post Ischaemic Reperfusion	24 hours Post CPB	24 hours Post CPB	Post HBO_2	Post HBO_2	5 minutes Post Ischaemic Reperfusion	5 minutes Post Ischaemic Reperfusion	24 hours Post CPB	24 hours Post CPB
5	Standard 1	Standard 1	2 hours Post CPB	2 hours Post CPB	Pre HBO_2	Pre HBO_2	5 minutes On CPB	5 minutes On CPB	2 hours Post CPB	2 hours Post CPB	Pre HBO_2	Pre HBO_2
6	Standard 0	Standard 0	24 hours Post CPB	24 hours Post CPB	Post HBO_2	Post HBO_2	5 minutes Post Ischaemic Reperfusion	5 minutes Post Ischaemic Reperfusion	24 hours Post CPB	24 hours Post CPB	Post HBO_2	Post HBO_2
7			Pre HBO_2	Pre HBO_2	5 minutes On CPB	5 minutes On CPB	2 hours Post CPB	2 hours Post CPB	Pre HBO_2	Pre HBO_2	5 minutes On CPB	5 minutes On CPB
8			Post HBO_2	Post HBO_2	5 minutes Post Ischaemic Reperfusion	5 minutes Post Ischaemic Reperfusion	24 hours Post CPB	24 hours Post CPB	Post HBO_2	Post HBO_2	5 minutes Post Ischaemic Reperfusion	5 minutes Post Ischaemic Reperfusion

Legend 2. 1: Legend Colour for *Figure 2.4*

Well containing sE-Standard	**Well containing sample from Patient No. 7**	**Well containing sample from Patient No. 21**	**Well containing sample from Patient No. 17**	**Well containing sample from Patient No. 16**	**Well containing sample from Patient No. 23**	**Well containing sample from Patient No. 53**	**Well containing sample from Patient No. 56**	**Blank Wells**

2.6.6.2.2 ELISA Assay Procedure For sP-Selectin

The reagents, methods used and the steps involved in the ELISA assay procedure for sP-Selectin was similar to that used for sE-Selectin apart from

a) the first incubation period was only 1 hour in duration.

b) the microplate was washed only 3 times on the autowasher.

The intra-assay coefficient of variation for sP-Selectin ELISA microplate was between 4.9% and 5.1% while the inter-assay coefficient of variation was between 7.9% and 9.9%. The minimum detectable dose of sP-Selectin was 0.5ng/ml.

2.6.6.2.3 ELISA Assay Procedure For sICAM-1

The reagents, methods used and the steps involved in the ELISA assay procedure for sICAM-1 was exactly the same as for sE-Selectin. The intra-assay coefficient of variation for sICAM-1 ELISA microplate was between 3.3% and 4.8% while the inter-assay coefficient of variation was 6% between 10.1%. The minimum detectable dose of sICAM-1 was 0.35ng/ml.

2.6.6.2.4 ELISA Assay Procedure For sPSGL-1

This assay procedure also involved a 20 fold dilution of the samples. While the general methods and steps for the ELISA assay procedure for sPSGL-1 was similar to that of sE-Selectin, there were some differences. These differences involved the following:

Reagent Preparation

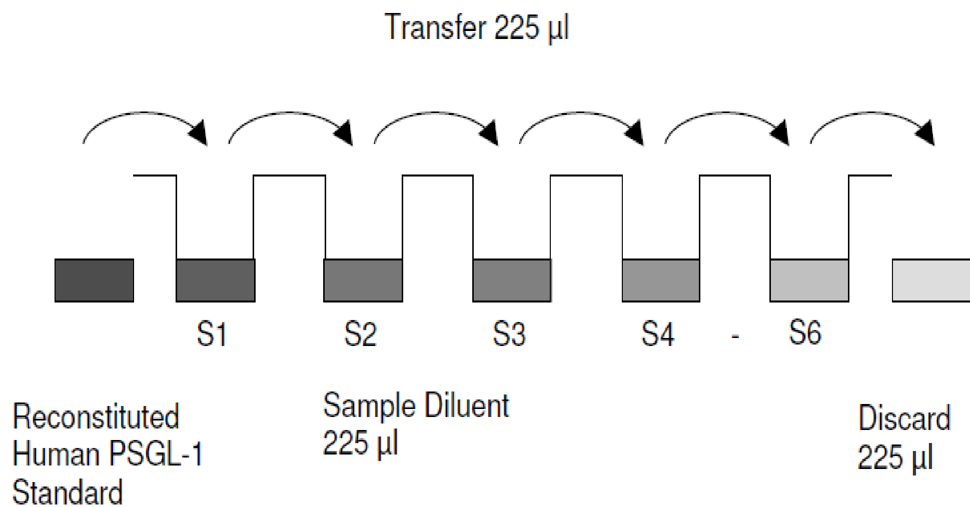
50ml of the manufacturer provided wash buffer concentrate was mixed with 950ml of distilled water to make up a 1liter of 20 fold dilution of the wash buffer. 5ml of the manufacturer provided Assay Buffer concentrate was then mixed with 95ml of distilled water to make up a 100ml 20 fold dilution of the Assay Buffer.

A 100 fold dilution of Biotin Conjugate solution was then made by mixing 0.06ml of Biotin Conjugate (anti-human sPSGL-1 monoclonal antibody) to 5.94ml of the prepared Assay Buffer. After this, a 200 fold dilution of the Streptavidin-Horseradish Peroxidase (HRP) concentrate was made by adding 60µl of Streptavidin-HRP concentrate to 12 ml of Assay Buffer. Then the sPSGL-1 standard was reconstituted by the addition of a volume of distilled water that was stated on the bottle of the sPSGL-1 standard. The concentration of the reconstituted standard was 100u/ml.

Once the standard was reconstituted, a series dilution of the standard was then made. This was done by firstly labelling 6 propylene tubes as S1, S2, S3, S4, S5, S6, respectively, one for each diluted standard. Then a 2 fold serial dilution of the provided sPSGL-1 standards was made by firstly pipetting 225µl of Sample Diluent into each tube. Then, 225µl of the

reconstituted standard was pipetted into the first tube, labelled S1. The contents of S1 was mixed (concentration of standard 1 = 50u/ml). Following this, 225µl of the diluted standard from S1 was pipette into the second tube, labelled S2. This was mix thoroughly before the next transfer *(Figure 2.5)*. This step was repeated 4 more times to create a standard dilution series with concentrations ranging from 50 to 1.6U/ml. The sample diluent serves as blank.

Figure 2. 5: Series Dilution of sPSGL-1 Standard



(Figure was adapated from Bender MedSystems PSGL-1 ELISA instruction book)

Assay Procedure

The prepared sPSGL-1 standards were pipetted in duplicates into columns A and B (rows 1 to 6), as in *Figure 2.4*, of the 96 well ELISA microplate (coated with monoclonal antibodies to human sPSGL-1). Wells A7 A8, B7 and B8 were left blank. 100µl of the diluted patient sample, from an appropriate time point, was then respectively added in duplicates to all wells, except the standard and blank wells. 50µl of the diluted Biotin-Conjugate was then added to all wells, except the blank wells. The microplate was then covered with a plate cover and left to incubate for 2 hours at room temperature on a rotator set at 100rpm.

After this incubation, the microplate was washed 3 times with 300µl of the manufacturer provided wash buffer. 100µl of the diluted Streptavidin-HRP was added to all wells (except for the blank wells). The microplate was covered with a plate cover and allowed to incubate at room temperature on a rotator set at 100rpm for a further 1 hour.

Then the plate was washed again as before. 100µl of the Substrate Solution (TMB) was then added to all wells (except for the blank wells). The micoplate was once again covered with a new plate sealer and allowed to incubate, in the dark, at room temperature for 10 minutes on a rotator set at 100rpm.

Once the most concentrated standard had reached a deep blue colour, 100µl of the manufacturer provided Stop Solution (Sulfuric Acid) was added to each well (except for the blank wells). The spectophotometric reading from each well was then determined using Anthos 2010 microtitre plate reader

(Anthos Labtec Instrument GmdH, Austria) together with the Stingray software (Stingray Software Inc, USA). A primary wavelength of 450nm was used during the spectophotometric analysis with a correction for optical imperfections in the plate set at 620nm. The intra-assay coefficient of variation for sPSGL-1 ELISA microplate was 3.2% while the inter-assay coefficient of variation was 6.6%. The limit of detection of this assay was 0.99u/ml.

2.6.6.3 Correction For Haemodilution Of Serum Biomarkers

Haemodilution is a standard practice during CPB (Liam et al., 1998). This is known to result in moderate to severe reduction of the serum soluble adhesion molecule (Williams et al., 1998) and Troponin-T (Licker et al., 2005) concentrations. As such, a correction for the effects of haemodilution on the measured serum biomarker concentration in the venous blood samples taken during the intra and post operative periods was required to determine their absolute concentration serum. Such a correction was also done in a similar previous study (Alex et al., 2005). In this study, this correction was done using a formula described by Taylor *et al* (Taylor et al., 1976). The haematocrit measurement in the venous blood samples taken during the intra and post operative time points, that was required for use in this formula, was measured using a GEM 3000 analyser (Instrumentation Laboratory).

Formula for correction of hemodilution:

Corrected Serum Soluble Adhesion Molecule/Troponin-T concentration (ng/ml)=
Adhesion Molecule Concentration at Sampling Time (ng/ml) x (Pre-Op Hct)/(Hct at time of sampling)

(Hct=Haematocrit)

2.6.7 Intra-Operative Myocardial Biopsy

In this study, a myocardial biopsy of the right atrium was taken from each patient, at the time points shown in *Table 2.7*.

Table 2. 7: Intra-Operative Time Points For Right Atrial Biopsy

	Time Points			
	Post Induction	5 minutes on CPB	5 minutes post IRI	5 minutes post CPB
Control Group	X	X	X	X
HBO_2 Group	X	X	X	X

Myocardial biopsies were taken by excising a piece of right atrium from the part of the atrium above the atrial purse string suture that was securing the atrial venous cannula in the right atrium and inferior vena cava. Each biopsy measured approximately 0.5cm x 0.5cm.

The myocardial biopsy taken at time point 'post induction' was taken after anaesthetic induction, median sternotomy and, cannulation of the aorta and the right atrium, prior to the onset of CPB. This biopsy provided a baseline for the assessment of myocardial biomarkers. The myocardial biopsy taken at time point '5 minutes on CPB' was taken 5 minutes following the

onset of CPB to enable the assessments of the early changes in the myocardial biomarkers as a result of CPB, prior to operative IRI, in both groups when compared to the biopsy taken at the time point 'post induction'. The myocardial biopsy taken at the time point '5 minutes post IRI' was taken 5 minutes following the final release of the aortic cross clamp from the aorta i.e. after the final anastamosis of the final bypass graft to the coronary artery (distal anastamosis) was complete. This biopsy allowed for the assessment of the changes in myocardial biomarkers as a result of the operative IRI in both groups when compared to biopsies taken earlier in the intra-operative period. The final myocardial biopsy taken at the time point '5 minutes post CPB' was taken 5 minutes following the termination of CPB to enable the assessment of the changes in myocardial biomarkers in comparison with the changes prior to the onset of CPB.

Each of the myocardial biopsy was placed in a 2ml cryo-vial (Nunc) containing RNA-Later (Qiagen) for immediate stabilisation of RNA and was processed according to manufacturer's recommendations for RNA stabilisation. The cryo-vials were then stored in a 4ºC fridge for 24 hours. After this period, in a sterile Class 2 Biological Safety Cabinet (Faster, SLS, Nottingham), the RNA-Later was removed from the cryo-vial and discarded. The biopsy was left in the cryo-vial and stored in a -80ºC for later myocardial biomarker analysis.

2.6.7.1 Intra-Operative Myocardial Biomarker Assessment

In this study, the myocardial biomarkers that were assessed were eNOS (Cabigas et al., 2006a) and Hsp72 (Hutter et al., 1996, Iwaki et al., 1993, Marber et al., 1995, Plumier et al., 1995) as they have been shown to be cardioprotective in models of IRI.

The required myocardial biopsies for eNOS or Hsp72 measurements, were removed from the -80ºC freezer. Before the samples could thaw, in a sterile Class 2 Biological Safety Cabinet (Faster, SLS, Nottingham), a small specimen from the biopsy was quickly excised. The remaining biopsy was returned to the -80ºC freezer. The myocardial specimen was allowed to completely thaw. Once thawed, the myocardial specimen was weighed and the weight was recorded. Each myocardial biopsy was analysed in the sequence of eNOS first then, followed by Hsp72. As such, the biopsies, from each time point, for myocardial eNOS analysis experienced one freeze-thaw cycle while those for myocardial Hsp72 analysis experienced two freeze-thaw cycles.

A quantitative Enzyme Linked ImmunoSorbent Assay (ELISA) test was used for measuring myocardial eNOS and myocardial Hsp72. The ELISA kits for the measurement of myocardial eNOS were purchased from R&D Systems (R&D Systems Inc, Minneapolis, USA) and those for myocardial Hsp72 ELISA were purchased from Stressgen Bioreagents (Victoria, BC, Canada).

2.6.7.2 Myocardial Specimen Lysis

The lysis of the myocardial specimens was done according to the recommendations of the manufacturer for the respective ELISA kits.

In a sterile Class 2 Biological Safety Cabinet (Faster, SLS, Nottingham), using separate petri dishes, each myocardial specimen was teased apart and disrupted in 1ml of the manufacturer provided lysis buffer using two size 11 blades. The specimens were then homogenised by passing the lysis buffer containing the disrupted specimen, 5 times through a 20G needle and syringe. The homogenate was then transferred into two separate 1.5ml propylene tube and centrifuged at 300*g* for 5 minutes. The supernatant (lysate) from the propylene tube was then removed and transferred into another labelled 1.5ml propylene tube. The lysates were then frozen at -80ºC for later analysis.

2.6.7.3 Myocardial Lysate eNOS ELISA Assay Procedure

All the reagents used in this procedure were provided by the manufacturer (R&D Systems Inc, Minneapolis, USA). The preparation of the reagents and the ELISA assay procedure were carried out according to the methods recommended by the manufacturer.

Sample Preparation

The required propylene tubes containing myocardial lysate samples for analysis, were removed from the -80ºC freezer and allowed to thaw. 100μl of the lysate from each propylene tube was then transferred to a fresh propylene tube for use in the myocardial eNOS ELISA procedure. The remaining myocardial lysate sample was immediately returned to the -80ºC freezer.

For this myocardial eNOS ELISA procedure, the sample lysate did not require dilution. The above procedure was repeated for all the sample lysates that were required for eNOS ELISA analysis.

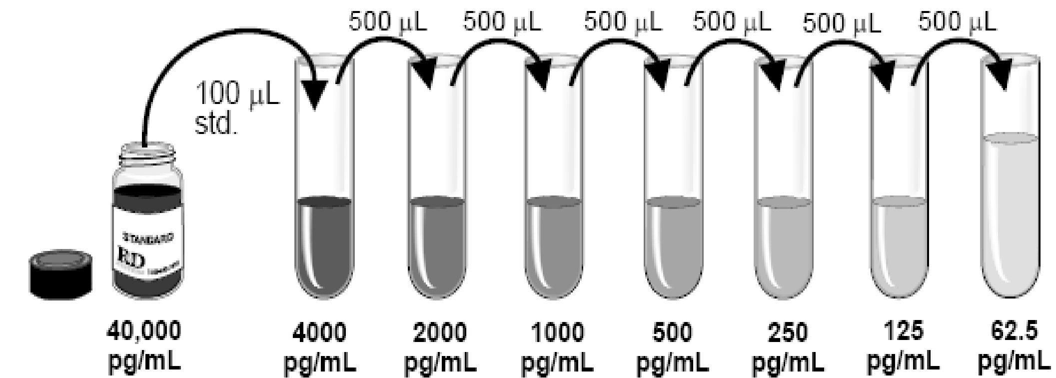
Reagent Preparation

All reagents were first brought to room temperature. 500ml wash buffer was made by diluting 20ml of the provided Wash Buffer Concentrate with 480ml of distilled water.

The substrate solution for this ELISA assay procedure was made by mixing equal volumes of the provided Colour Reagents A (stabilised hydrogen peroxide) & B (stabilised chromogen- tetramethylbenzidine). This was done 15 minutes prior to addition of the substrate solution to the wells. This substrate solution was light sensitive and needed to be covered from the light.

The eNOS standard (40ng of recombinant human eNOS) was then reconstituted with 1ml of distilled water. This reconstitution produced a stock solution of 40,000pg/ml. The standard was allowed to sit for 15 minutes before a series dilution was made. This was done by pipetting 900µl of Calibrator Diluent RD5K (a buffered protein base) into the first propylene tubes *(Figure 2.6)*. 500µl of Calibrator Diluent RD5K was then pipetted into propylene tube 2, 3, 4, 5, 6 and 7. Then, 100µl of stock was added to propylene tube 1. After mixing the 1000µl content of propylene tube 1, 500µl form propylene tube 1 was transferred to propylene tube 2 and mixed. Following this, 500µl of the content from propylene tube 2 was transferred to propylene tube 3. This procedure was continued until propylene tube 7 was reached. Propylene tube 7 ended up with a volume of 1000µl. The 4000pg/ml standard, in propylene tube 1, served as the high standard. Calibrator Diluent RD5K serves as the zero standard (0pg/ml).

Figure 2. 6: Series Dilution of eNOS Standard Stock



Propylene tube: 1 2 3 4 5 6 7

(Figure was adapated from R&D Systems eNOS ELISA instruction book)

<u>*Assay Procedure*</u>

In this myocardial eNOS ELISA assay procedure, the manufacturer provided 96 well microplate (12 strips, 8 wells per strip) was used. The wells of this plate were coated with a monoclonal antibody against eNOS. 100µl of Assay Diluent RD1W (a buffered protein base) was added to each well. 100µl of eNOS Standard from propylene tube 1 was pipetted, in duplicates, into wells A1 & B1. This was followed by pipetting 100µl eNOS Standard from propylene tube 2 into wells A2 & B2, 100µl eNOS Standard from propylene tube 3 into wells A3 & B3, 100µl eNOS Standard from propylene tube 4 into wells A4 & B4, 100µl eNOS Standard from propylene tube 5 into wells A5 & B5, 100µl eNOS Standard from propylene tube 6 into wells A6 & B6 and 100µl eNOS Standard from propylene tube 7 into wells A7 & B7. Wells A8 and B8 were left blank. 100µl of sample lysate was then added to all wells except the standard and blank wells *(Figure 2.4)*.

The microplate was covered with an adhesive strip and incubated for 2 hours at room temperature on a horizontal microplate shaker set at 500rpm. The plate was then washed 3 times with 400µl, of the previously made up, manufacturer wash buffer using a Thermolab Systems Wellwash 4MK2 autowasher. Once washed, and ensuring that all the liquid in each well was removed, 200µl of eNOS Conjugate (polyclonal antibody against eNOS conjugated to horseradish peroxidase) was added to each well. The microplate was then covered with a new adhesive seal prior to incubation for a further 2 hours at room temperature on a horizontal microplate shaker set at 500rpm. Then the plate was washed 3 times with 400µl of the wash buffer using a Thermolab Systems Wellwash 4MK2 autowasher. After this second wash step, 200µl of Substrate Solution was added to each well. The microplate was then incubated for 30 minutes on a bench top while protecting it from light. Once the most concentrated standard had reached a deep blue colour, 50µl of the manufacturer provided Stop Solution (Sulfuric Acid) was added to all the wells. Within 30 minutes of adding the Stop Solution, the spectophotometric reading from each well was then determined using Anthos 2010 microtitre plate reader (Anthos Labtec Instrument GmdH, Austria) together with the Stingray software (Stingray Software Inc, USA). A primary wavelength of 450nm was used during the spectophotometric analysis with a correction for optical imperfections in the plate set at 540nm.

The intra-assay coefficient of variation for the eNOS ELISA microplate was between 3.7% and 4.9% while the inter-assay coefficient of variation was between 3.6% and 7.4%. The maximum detectable concentration of eNOS is typically less than 25ρg/ml.

The quantity of eNOS in each milligram of the right atrial biopsy, at each time point, was then determined using the following formula:

Quantity of eNOS in Right Atrial Specimen (pg/mg) = [eNOS Concentration at time of sampling (ng/ml) X Lysate Buffer Volume(ml)] / Right Atrial Specimen Weight (mg)

2.6.7.4 Myocardial Lysate Inducible Hsp70 (Hsp72) ELISA Assay Procedure

All the reagents used in this procedure were provided by the manufacturer (Stressgen Bioreagents; Victoria, BC, Canada). The preparation of the reagents and the ELISA assay procedure were carried out according to the methods recommended by the manufacturer.

Sample Preparation

The required propylene tubes containing myocardial lysate samples for analysis, were removed from the -80ºC freezer and allowed to thaw. 15µl of the lysate from each propylene tube was then transferred to fresh propylene tube for use in the Hsp72 ELISA procedure. The remaining myocardial lysate sample was immediately returned the -80ºC freezer.

285µl of the provided Sample Diluent, was then added to the propylene tube containing the 15µl of lysate sample. This created a 20 fold dilution of the sample lysate that was to be used. The above procedure was repeated for all the sample lysates that were required Hsp72 ELISA analysis.

<u>*Reagent Preparation*</u>

All reagents were brought to room temperature.

a. *Series Dilution of Anti-Human Hsp70 Standard.*

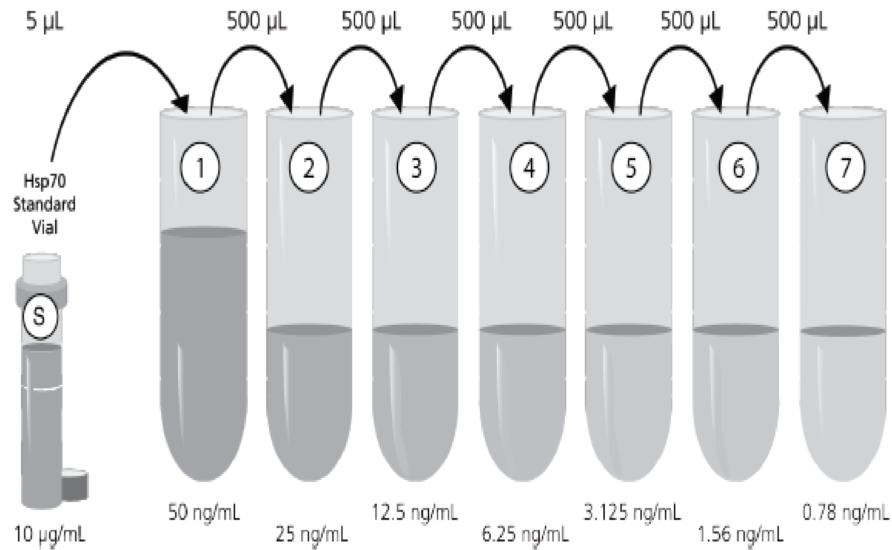
The provided Recombinant Hsp70 Standard (10µg/ml stock solution of inducible Hsp70 protein) was used to generate a series dilution of Hsp70 Standard concentration, ranging from 0.78 to 50ng/ml. This was done in the following manner. Seven 1.5ml propylene tubes were respectively labelled with the following standard values:

i. Propylene tube 1: 50ng/ml
ii. Propylene tube 2: 25ng/ml
iii. Propylene tube 3: 12.5ng/ml
iv. Propylene tube 4: 6.25ng/ml
v. Propylene tube 5: 3.125ng/ml
vi. Propylene tube 6: 1.56ng/ml
vii. Propylene tube 7: 0.78ng/ml

995µl of Sample Diluent was added to propylene tube 1. 500µl of Sample Diluent was added to propylene tube 2, 3, 4, 5, 6 and 7. 5µl of Hsp70 Standard stock solution was added to propylene tube 1 and was mixed thoroughly. 500µl of mixture from propylene tube 1 was then transferred to propylene tube 2 and mixed thoroughly. The dilution series was then completed by transferring 500µl from propylene tube 2 to propylene tube 3 and so forth until propylene tube 7 was reached to generate the standard dilutions *(Figure 2.7)*. Propylene tube 7 ended up with a volume of 1ml. Finally, 500µl

of Sample Diluent was added to another propylene tube (propylene tube 8). This served as the blank assay (0ng/ml).

Figure 2. 7: Series Dilution of Hsp70 Standard Stock



Propylene tube: 1 2 3 4 5 6 7

(Figure was adapated from Stressgen Hsp72 ELISA instruction book)

b. Preparation of Anti-Hsp70 Biotin Conjugate

22µl of the provided Anti-Hsp70 Biotin Conjugate (Rabbit polyclonal antibody specific for inducible Hsp70) was diluted in 11ml of Anti-Hsp70 Biotin Conjugate Diluent (a buffer) in a propylene tube and mixed.

c. Preparation of Avidin HRP Conjugate

22µl of Avidin-HRP Conjugate(Horseradish Peroxidise conjugated anti-rabbit IgG) was mixed with 11ml of Avidin-HRP Conjugate Diluent in a propylene tube.

d. Wash Buffer Preparation

100ml of the provided wash buffer concentrate was diluted with 900ml of distilled water to make a 10 fold dilution of the wash buffer.

Assay Procedure

The manufacturer provided 96 well microplate (12 strips, 8 wells per strip) was used. The wells were pre-coated with mouse monoclonal antibody specific for inducible Hsp70. 100µl of Hsp70 Standard from propylene tube 1 was placed in duplicate in wells A1 & B1. This was followed by 100µl Hsp70 Standard from propylene tube 2 into wells A2 & B2, 100µl Hsp70 Standard from propylene tube 3 into wells A3 & B3, 100µl Hsp70 Standard from propylene tube 4 into wells A4 & B4, 100µl Hsp70 Standard from propylene tube 5 into wells A5 & B5, 100µl Hsp70 Standard from propylene tube 6 into wells A6 & B6 and 100µl Hsp70 Standard from propylene tube 7 into wells A7 & B7. Wells A8 and B8 were left blank. 100µl of sample lysate was then added all well except the standard and blank wells *(Figure 2.4)*.

The microplate was covered with an adhesive seal and incubated for 2 hours at room temperature on a horizontal microplate shaker set at 500rpm. Then the plate was washed six times with 300µl of the previously made up manufacturer wash buffer using a Thermolab Systems Wellwash 4MK2 autowasher. Once washed and ensuring that all the liquid in each well was removed, 100µl of Anti-Hsp70 Biotin Conjugate was added to each well. The microplate was covered with an adhesive seal and incubated for 1 hour at room temperature on a horizontal microplate shaker set at 500rpm.

The plate was then washed six times with 300µl of the wash buffer using a Thermolab Systems Wellwash 4MK2 autowasher. Once washed and again ensuring that all the liquid in each well was removed, 100µl of Avidin-HRP Conjugate was added to each well. The microplate was covered with an adhesive seal and incubated for 1 hour at room temperature on a horizontal microplate shaker set at 500rpm. The plate was washed again six times with 300µl of the wash buffer using a Thermolab Systems Wellwash 4MK2 autowasher. Again after ensuring all the liquid in each well was removed, 100µl of the provided TMB Substrate Solution was added to each well and the plate was left to incubate at room temperature for 10 minutes.

Once the most concentrated standard had reached a deep blue colour, 50µl of Acid Stop solution (Sulfuric Acid) was added to all the wells in the same order the TMB Substrate was added. Within 30 minutes of adding the Stop Solution, the spectophotometric reading from each well was then determined using Anthos 2010 microtitre plate reader (Anthos Labtec Instrument GmdH, Austria) together with the Stingray software (Stingray Software Inc, USA). A primary wavelength of 450nm with a correction for optical imperfections in the plate set for 540nm, was used.

According to manufacturer (Stressgen Bioreagent), the intra-assay and the inter-assay coefficient of variation for the Hsp70 ELISA microplate was <10%, respectively.

The quantity of inducible Hsp70 (Hsp72) in each milligram of the right atrial tissue specimen at each time point was then determined using the following formula

Quantity of Hsp72 in Right Atrial Specimen (ng/mg) = [Hsp72 Concentration at time of sampling (ng/ml) X Lysate Buffer Volume (ml)] / Right Atrial Specimen Weight (mg)

3. The Effects Of HBO_2 Preconditioning On Peri-Operative Cardiovascular Efficacy & Clinical Safety

3.1 Introduction

In 1965, a case report was published describing a case of low cardiac output syndrome, following complex cardiac surgery (and therefore post IRI), which improved after treatment with HBO_2 (Yacoub and Zeitlin, 1965). This interesting case report was followed up in 1971, by the publication of a randomised control study involving HBO_2 and patients who had suffered an AMI (Thurston, 1971). In that study, in addition to the unit's protocol for treatment of AMI, 127 patients who had suffered an AMI were randomly allocated either to a group where patients were treated intermittently for the first 48 hours with HBO_2 (n=58) or to a group where patients were treated with oxygen via a face mask (n=69). It was observed that patients with a history of cardiovascular disease and patients who presented with cardiogenic shock or heart failure, performed clinically better following treatment with HBO_2. In particular, it was observed that some arrhythmias appeared to resolve during treatment with HBO_2, occasionally only to return in air at atmospheric pressure but, correctable again when the pressure was raised. There was also less in-

hospital mortality in the HBO_2 group compared to the control group (19% vs. 24.6%) despite the HBO_2 group having patients with slightly more severe MIs. In 1973, this same group published the results of an another similar, but larger, randomised control study (Thurston et al., 1973). The primary objective of that study was to determine the effects of HBO_2 on mortality following recent AMI. In that study, 103 and 105 patients were randomised to the HBO_2 and control groups, respectively. Both groups of patients were treated using the unit's protocol for AMI. However, the HBO_2 group of patients also received 100% oxygen at 2 ATA for 2 hours followed by air for 1 hour in a repeating cycle, day and night, for 48 hours. It was observed that in the HBO_2 group, compared to the control group, there was a reduction in mortality 3 weeks after an AMI and a reduction in significant dysrrhythmias (complete heart block, ventricular fibrillation and asystole). That study concluded that the reduction in major adverse coronary events after an AMI in the HBO_2 group justified the routine use of HBO_2 in selected cases of AMI. While the findings of that study indicated the favorable use of HBO_2 post AMI to reduce mortality, the results were however not statistically significant.

Since those randomised controlled studies of the early 1970s, there had been a paucity in the number of reported clinical studies investigating the effects of HBO_2 on myocardial ischaemia and reperfusion. This was probably driven by the lack of statistically significant clinical findings and a lack of understanding of the biochemical mechanisms of action of HBO_2 in models of ischaemia and reperfusion. However, from the 1980s

onwards, following a series of studies demonstrating the beneficial effects of HBO_2 in experimental models of IRI (Nylander et al., 1985, Sterling et al., 1993, Nylander et al., 1987, Yamada et al., 1995, Chen et al., 1998, Kim et al., 2001, Choi et al., 2006, Tjarnstrom et al., 2001), there appeared to be a steady resurgence in the interest of administering HBO_2 to limit ischaemia-reperfusion associated myocardial injury.

In the earliest clinical study investigating the effects of HBO_2 on myocardial function following IRI, a study was designed to determine the potential for HBO_2 to produce an improvement in myocardial function in the hibernating myocardium (Swift et al., 1992). That study found that, compared to those patients who were not treated with HBO_2 post AMI, patients who were also treated with HBO_2 consisting of 100% oxygen at 2.0 ATA for 30 minutes following an AMI, had improved myocardial contraction as observed by echocardiography and, demonstrated reversible ischaemia as determined by Single Photon Emission Computer Tomography (SPECT). The interesting findings from that study were later followed by the first study from 'HOT-MI' group (Shandling et al., 1997) which was conducted to assess the safety of treating patients, who recently suffered from an AMI, with HBO_2. In the that study by the 'HOT-MI' group, 66 patients with an AMI were randomised to treatment with HBO_2, which consisted of 100% oxygen at 2 ATA for 60 minutes, in addition to recombinant tissue plasminogen activator (rTPA) (n=32) or treatment with rTPA alone (n=34). The results showed that in patients who were also treated with HBO_2, there was a 35% reduction in mean

creatine phosphokinase (CK) ($p=0.03$) at 12 and 24 hours post MI and, a reduction in time to pain relief and ST segment resolution post MI. The ejection fraction (EF) on discharge of the HBO_2 group of patients was 52.4% while that of the control group's was 47.3%. The study concluded that adjunctive HBO_2 was a feasible and safe treatment for patients who had suffered from an AMI. This group later validated their findings by conducting a second similar, but larger randomised controlled study. In that later study (Stavitsky et al., 1998), 112 patients who were admitted with an AMI were randomised to treatment with either HBO_2 and either rTPA or streptokinase (STK) or, to a group that was treated with only rTPA or STK. In that second study by the 'HOT MI' group, it was observed that patients in the HBO_2 group had a CK that was 7.5% lower than the control group at 12 and 24 hours post AMI. Additionally, the HBO_2 group of patients also experienced a shorter time to pain relief. The left ventricular ejection fraction (LVEF) on discharge in the HBO_2 group was also better than the control group, 48.4% compared to 43.4%, respectively.

The 'HOT-PI' study (Sharifi et al., 2004) was conducted to assess whether using HBO_2 as an adjunct to percutaneous coronary intervention (PCI) in patients who presented with unstable angina or AMI, could reduce clinical re-stenosis. In that randomised control study, 33 and 36 patients were randomised to the HBO_2 and control group, respectively. All the patients presented with unstable angina or AMI and were treated according to the unit's protocol for AMI. The patients randomised to the HBO_2 group also

received HBO_2 treatment 2 hours before or immediately after PCI followed by another treatment less than 18 hours after the first HBO_2 treatment. The HBO_2 treatment consisted of 100% oxygen at 2 ATA for 90 minutes. In that study, 8 months post treatment for AMI, the HBO_2 group not only had a significant reduction of composite adverse cardiac events ($p=0.001$), which included mortality, MI, CABG or revascularization of target lesion, but there was also a significant reduction in both the revascularization of the previous target lesion ($p<0.003$) and the recurrence of angina ($p<0.003$). However, it was also found that while collectively there was a significant reduction in composite adverse cardiac event, the reduction in mortality rate in the HBO_2 group was not statistically significant. That finding was in keeping with findings from earlier clinical studies (Sharifi et al., 2002, Thurston, 1971, Thurston et al., 1973).

In 2005, the Cochrane Collaborative (Bennett et al., 2005) conducted a meta-analysis to review the effects of clinical treatment with HBO_2 following an acute coronary syndrome (ACS). The meta-analysis comprised of 6 randomised control trials (Sharifi et al., 2004, Dekleva et al., 2004, Stavitsky et al., 1998, Shandling et al., 1997, Swift et al., 1992, Thurston et al., 1973) consisting of a total of 536 patients (n=273 were treated with HBO_2, n=263 were not treated with HBO_2). This meta-analysis showed that while there was a trend towards significance, there was no statistically significant decrease in the risk of death in patients who were also treated with HBO_2 ($p=0.08$). There was however a significant reduction in the time to pain relief following the onset of angina ($p<0.0001$) in this group of patients. The

Cochrane Collaborative Group concluded that despite HBO_2 treatment following ACS, in individual trials, having the potential to significantly reduce the risk of major adverse coronary events ($p=0.03$) and some dysrrhythmias ($p=0.01$), particularly complete heart block ($p=0.02$), it did not demonstrate the potential to significantly reduce mortality following an ischaemic reperfusion event such as ACS. This they determined was due to study flaws in those trials such as modest patient numbers, methodological shortcomings and poor reporting. As such, until an appropriately powered study of high methodological rigor is conducted to identify those cardiac patients who can be expected to benefit from HBO_2 treatment, the routine application of HBO_2 treatment in clinical practices where IRI is a common occurrence, remains to be justified.

3.2 Objectives

The primary cardiovascular efficacy objective for this part of this clinical study was to determine if systemically treating CAD patients with HBO_2 preconditioning, involving two episodes of 30 minutes of 100% oxygen at 2.4 ATA separated 5 minutes apart, which was completed approximately 2 hours prior to on CPB CABG, is capable of remotely preconditioning the myocardium by leading to better post operative improvement in myocardial function, as measured by left ventricular stroke work (LVSW) at the time point 24 hours post CPB. Additionally, the secondary objectives for this part of the study was to estimate the effect of HBO_2 preconditioning on:

a. other peri-operative cardiovascular efficacy parameters, as measured by the haemodynamic parameters at the pre-specified time points listed in *Table 2.4*.

b. patient outcomes with respect to post operative safety, as measured by the AEs listed in *Table 2.3*.

A *post hoc* analysis of the cost effectiveness HBO_2 preconditioning was also conducted.

3.3 Methods

Peri-operative (pre, intra and post operative) patient data were collected as described in *section 2.6.1-2.6.3* and peri-operative haemodynamic data were collected using a PA catheters as described in *section 2.6.4*.

3.4 Results

Table 3.1 shows the baseline patient characteristics and *Table 3.2* shows the intra operative disposition of the patients recruited to this randomised controlled study. The patient ages and gender were similar between the groups. The Control Group had a higher mean pre-operative risk score with a Euroscore of 3.78 while the HBO_2 Group had a EUROSCORE of 2.83. The Control Group also had a slightly higher number of patients with unstable angina, previous MI, hypertension and diabetes compared to the HBO_2 Group. There were however slightly more patients with peripheral vascular disease in the HBO_2 Group compared to the Control Group. The majority of patients in the Control Group (n=20; 50%) and HBO_2 Group (n=22; 55%) had CABG x 3. More patients in the HBO_2 Group, compared to the Control Group, had CABG x 1 (n=2; 5% vs. n=1; 2.5%) and CABG x 3 (n=22; 55% vs. n=20; 50%). However, more patients in the Control Group (n=5; 12.5%), compared to the HBO_2 Group, had CABG x 4 (n=3; 7.5%). The Control Group, compared to the HBO_2 Group, had a slightly longer mean myocardial ischaemic time (29.2 minutes vs. 27.6 minutes) and a slightly longer mean CPB time (65.8 minutes vs. 62.5 minutes).

Table 3. 1: Baseline Patient Characteristics

Variable	Control Group (n=40)	HBO_2 Group (n=41)
Age (years; mean)	69	65
Men	29 (72.5%)	33 (80.5%)
BMI	28.8	28.2
Pre-op Euroscore (mean)	3.78	2.83
Unstable Angina	3 (7.5%)	1 (2.5%)
Previous MI	20 (51.3%)	16 (41.0%)
Left Main Stem Disease	13 (32.5%)	13 (31.7%)
1 Diseased Coronary Artery	0	1 (2.4%)
2 Diseased Coronary Arteries	7 (17.5%)	8(19.5%)
33 Diseased Coronary Arteries	33(82.5%)	32(78.0%)
Left Ventricular Function ≥ 50%	31 (79.5%)	33 (82.5%)
Left Ventricular Function 30% - 50%	7 (17.9%)	7 (17.5%)
Hypertension	30 (75.0%)	25 (62.5%)
Diabetes Mellitus	5 (12.5%)	3 (7.5%)
Peripheral Vascular Disease	1 (2.5%)	3 (7.5%)

Table 3. 2: Intra Operative Patient Disposition

Variable	Control Group (n=40)	HBO_2 Group (n=41)
CABG X 1	1 (2.5%)	2 (5.0%)
CABG X 2	13 (32.5%)	13 (32.5%)
CABG X 3	20 (50.0%)	22 (55.0%)
CABG X 4	5 (12.5%)	3 (7.5%)
CABG X 5	1 (2.5%)	1 (2.5%)
Myocardial Ischaemia Time (minutes; mean)	29.2	27.6
Cardiopulmonary Bypass Time (minutes; mean)	65.8	62.5

3.4.1 Effects Of HBO_2 Preconditioning On Peri-Operative Haemodynamic Parameters

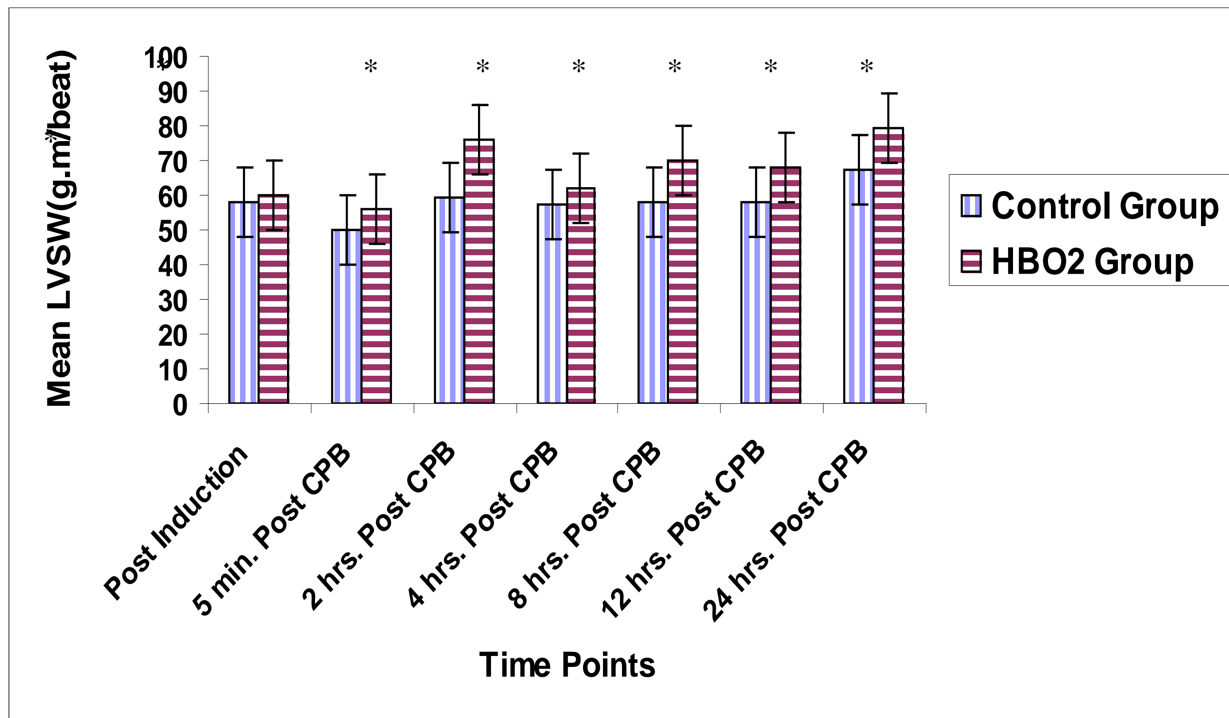
In this study, only 22 patients (54%) in the HBO_2 Group and 25 patients in the Control Group (63%) had their haemodynamic parameters measured. This was due to an insufficient number of monitoring equipment required for measuring haemodynamic parameters. This insufficiency occurred because the haemodynamic monitoring equipment was required for the management of other more critical ICU patients. As 46% (n=19) patients in the HBO_2 Group and 37% (n=15) patients in the Control Group did not have any peri-operative haemodynamic measurements, and no imputation of data were carried out in this study, only an on-treatment analysis of the haemodynamic measurement was done. This was because, due to the large numbers of patients without peri-operative hemdynamic measurements, an intention to treat analysis would reveal results that were not clinically meaningful.

Due to the large number of peri-operative haemodynamic measurements that were recorded during this study, only the results of the primary endpoint (LVSW) and, results which showed statistical significance, are reported. In this study, only LVSW, LVSWI, SV, PVR and PVRI show statistically significant results.

a. Peri-Operative Mean LVSW & LVSWI

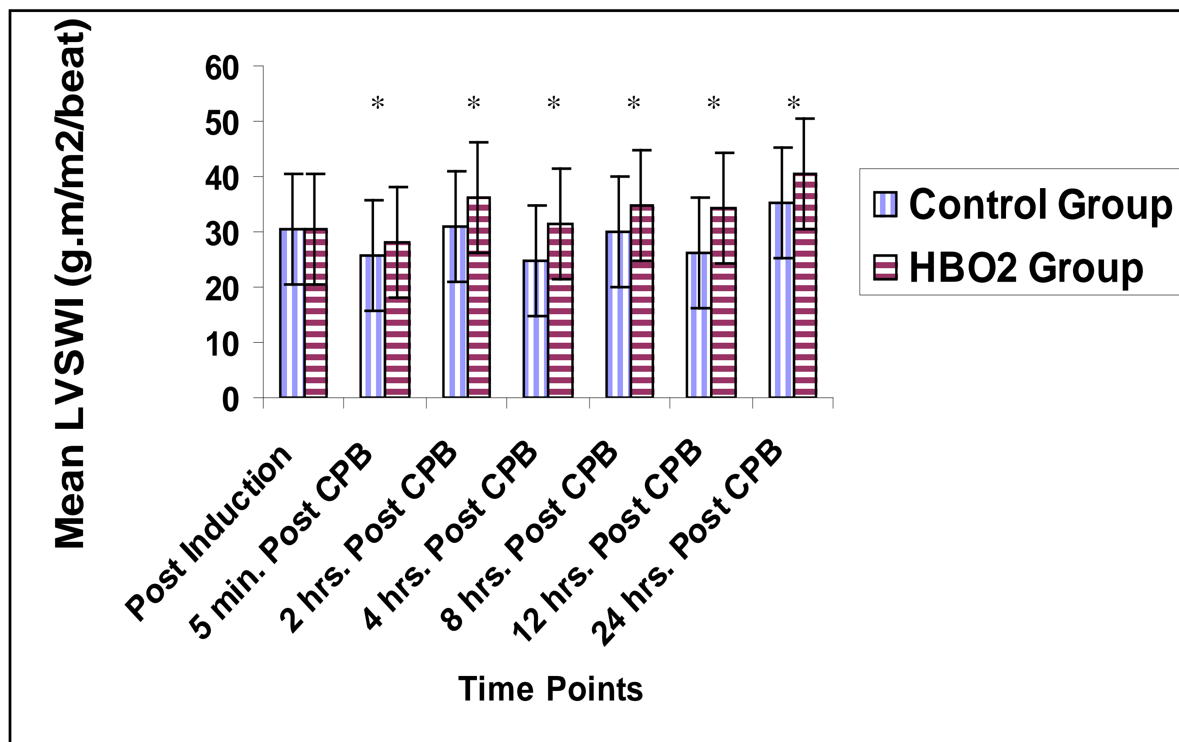
In this study, in both groups, between the baseline ‘post induction’ time point and the time point ‘24 hours post CPB’, there were general increases in the mean LVSW and mean LVSWI in both the groups. At all time points following the baseline ‘post induction’ time point, the mean LVSW and mean LVSWI were higher in the HBO_2 Group. In this study, it was determined that at all the time points following the ‘post induction’ time point, there were statistically significant improvements in both the mean LVSW (p=0.005; Geometric Mean Estimate & 95% Confidence Interval: 1.28 & 1.05, 1.31) *(Figure 3.1)* and the mean LVSWI (p=0.02; Geometric Mean Estimate & 95% Confidence Interval: 1.12 & 1.02, 1.24) *(Figure 3.2)* in the HBO_2 Group, compared to the Control Group. A summary of the results are provided in *Table 3.3*. In this table, the data are displayed as both mean and median values. As the means and medians are not the same at each time point, this indicates that the data were skewed. To normalise the data and enable parametric statistical analysis, the mean values were transformed into natural logarithmic (ln) values.

Figure 3. 1: Bar Chart of Peri-Operative Mean LVSW



HBO_2 Group, n=22; Control Group, n=25
Bars chart showing mean values and error bars for the standard error of the mean;
***** *$p<0.05$ following repeated measures of ANOVA analysis*

Figure 3. 2: Bar Chart of Peri-Operative Mean LVSWI



HBO_2 Group, n=22; Control Group, n=25
Bars chart showing mean values and error bars for the standard error of the mean;
***** *$p<0.05$ following repeated measures of ANOVA analysis*

Table 3. 3: Peri-Operative LVSW & LVSWI

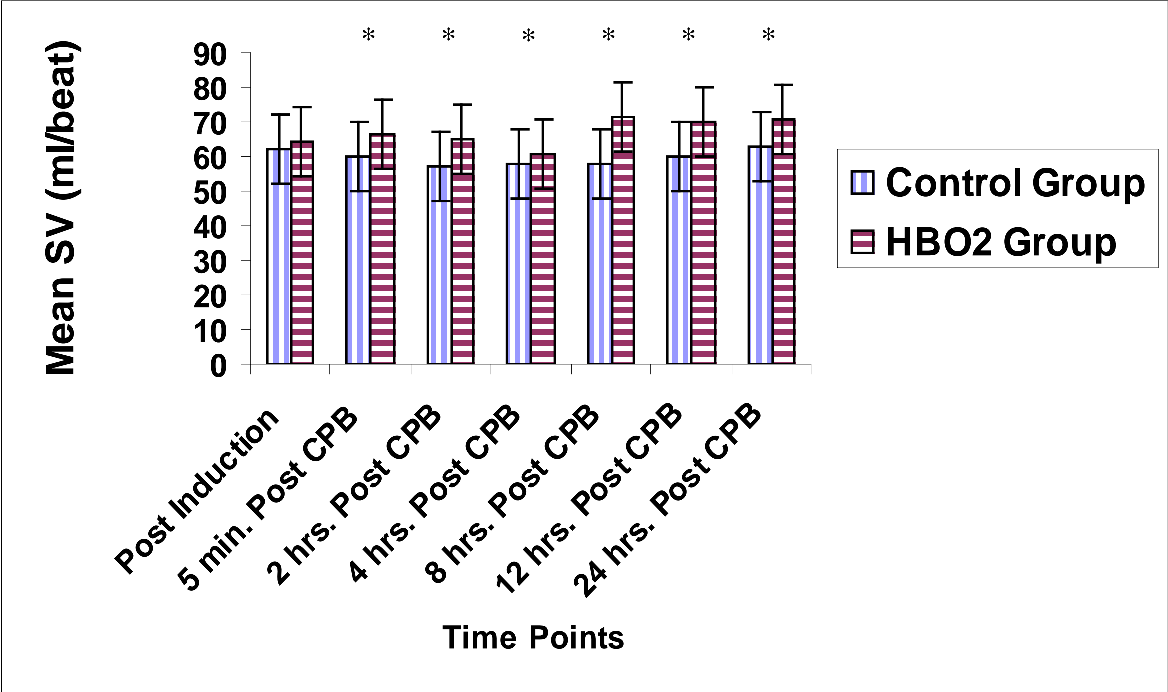
		LVSW (g.m/beat)		**LVSWI** ($g.m/m^2/beat$)	
		Control Group [n=25][†]	HBO_2 Group [n=22][†]	Control Group [n=25][†]	HBO_2 Group [n=22][†]
Post Induction	Range	32-111	29-89	18-55	15-43
	Median	59	62	30	32
	Mean	58	60	31	30
	Mean Ln	4.1	4.1	3.4	3.4
	Geometric Mean Estimate & 95% Confidence Interval[¥] (p-value[*])	p=0.08		p=0.08	
5 minutes post CPB	Range	33-73	35-82	18-37	20-42
	Median	50	55	24	29
	Mean	50	60	26	30
	Mean Ln	3.9	4.1	3.3	3.4
	Geometric Mean Estimate & 95% Confidence Interval[¥] (p-value[*])	1.28 & 1.05-1.31 (p=0.005)		1.12 & 1.02- 1.24 (p=0.02)	
2 hours post CPB	Range	30-115	51-129	16-54	26-76
	Median	61	72	30	36
	Mean	59	76	31	36
	Mean Ln	4.1	4.3	3.4	3.6
	Geometric Mean Estimate & 95% Confidence Interval[¥] (p-value[*])	1.28 & 1.05-1.31 (p=0.005)		1.12 & 1.02- 1.24 (p=0.02)	
4 hours post CPB	Range	35-87	33-111	19-45	18-56
	Median	54	59	39	30
	Mean	54	62	25	31
	Mean Ln	4.0	4.1	3.2	3.4
	Geometric Mean Estimate & 95% Confidence Interval[¥] (p-value[*])	1.28 & 1.05-1.31 (p=0.005)		1.12 & 1.02- 1.24 (p=0.02)	
8 hours post CPB	Range	29-90	34-129	16-40	18-65
	Median	55	65	30	33
	Mean	58	70	30	35
	Mean Ln	4.1	4.2	3.4	3.6
	Geometric Mean Estimate & 95% Confidence Interval[¥] (p-value[*])	1.28 & 1.05-1.31 (p=0.005)		1.12 & 1.02- 1.24 (p=0.02)	
12 hours post CPB	Range	35-93	35-98	19-49	19-51
	Median	63	65	32	35
	Mean	58	68	26	34
	Mean Ln	4.1	4.2	3.2	3.5
	Geometric Mean Estimate & 95% Confidence Interval[¥] (p-value[*])	1.28 & 1.05-1.31 (p=0.005)		1.122 & 1.02- 1.24 (p=0.02)	
24 hours post CPB	Range	35-91	48-144	19-48	21-79
	Median	63	73	33	39
	Mean	67	80	35	48
	Mean Ln	4.2	4.4	3.6	3.9
	Geometric Mean Estimate & 95% Confidence Interval[¥] (p-value[*])	1.28 & 1.05-1.31 (p=0.005)		1.122 & 1.02- 1.24 (p=0.02)	

[†] *This is an on treatment analysis;*
Statistical analysis using repeated measures of ANOVA;
[¥]*95% Confidence Intervals and p values are only given where the result is statistically significant;*
**p values are for geometric means*

b. Peri-Operative Mean SV

In this study, in both groups, between the baseline 'post induction' time point and the time point '24 hours post CPB', there was a general increase in mean SV in both groups. At all time points following the baseline 'post induction' time point, the mean stroke volume (SV) was higher in the HBO_2 Group. At all the time points following the 'post induction' time point, there was a statistically significant improvement in SV ($p=0.01$; Geometric Mean Estimate & 95% Confidence Interval: 1.13 & 1.03, 1.25) *(Figure 3.3)* in the HBO_2 Group compared to the Control Group. A summary of the results are provided in *Table 3.4*. In this table, the data are displayed as both mean and median values. As the means and medians are not the same at each time point, this indicates that the data were skewed. To normalise the data and enable parametric statistical analysis, the mean values were transformed into natural logarithmic (ln) values.

Figure 3. 3: Bar Chart of Peri-Operative Mean SV



HBO_2 Group, n=22; Control Group, n=25
Bars chart showing mean values and error bars for the standard error of the mean;
***** *$p<0.05$ following repeated measures of ANOVA analysis*

Table 3. 4: Peri-Operative SV

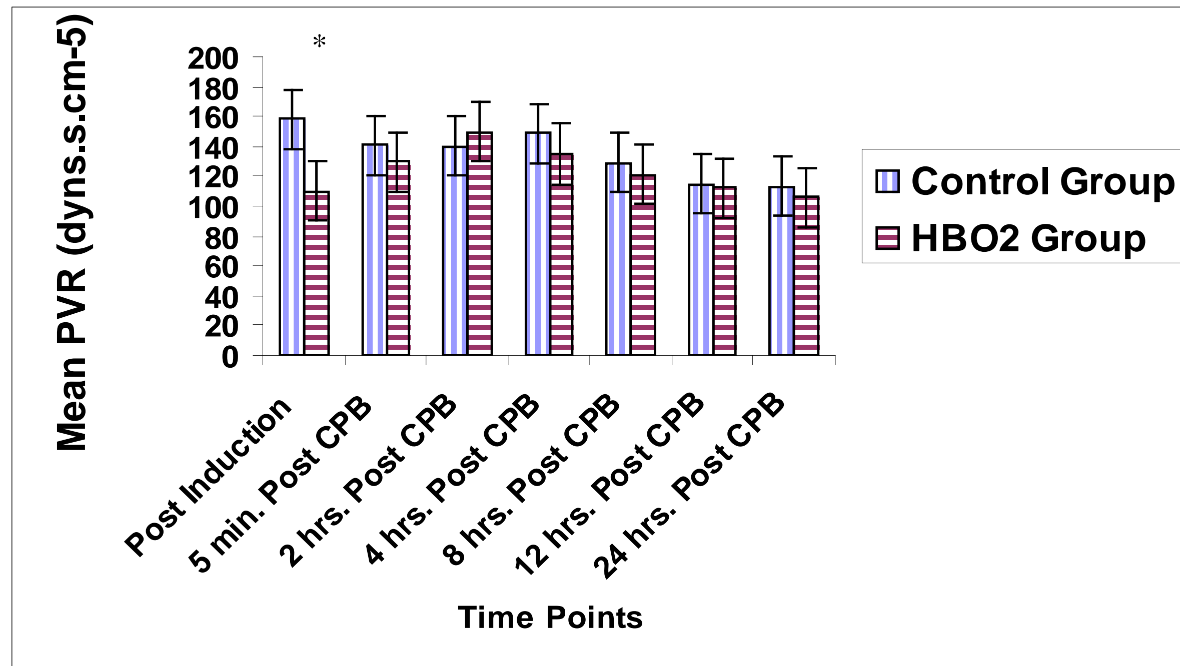
		SV (ml/beat)	
		Control Group [n=25][†]	HBO_2 Group [n=22][†]
Post Induction	Range	32-91	37-94
	Median	59	62
	Mean	62	65
	Mean Ln	4.1	4.2
	Geometric Mean Estimate & 95% Confidence Interval[¥] (p-value[*])	p=0.07	
5 minutes post CPB	Range	39-86	43-94
	Median	60	63
	Mean	60	66
	Mean Ln	4.1	4.2
	Geometric Mean Estimate & 95% Confidence Interval[¥] (p-value[*])	1.13 & 1.03-1.25 (p=0.01)	
2 hours post CPB	Range	26-99	47-81
	Median	57	63
	Mean	57	65
	Mean Ln	4.0	4.2
	Geometric Mean Estimate & 95% Confidence Interval[¥] (p-value[*])	1.13 & 1.03-1.25 (p=0.01)	
4 hours post CPB	Range	33-92	35-94
	Median	53	60
	Mean	58	60
	Mean Ln	4.1	4.1
	Geometric Mean Estimate & 95% Confidence Interval[¥] (p-value[*])	1.13 & 1.03-1.25 (p=0.01)	
8 hours post CPB	Range	38-97	52-137
	Median	58	64
	Mean	58	72
	Mean Ln	4.1	4.3
	Geometric Mean Estimate & 95% Confidence Interval[¥] (p-value[*])	1.13 & 1.03-1.25 (p=0.01)	
12 hours post CPB	Range	48-110	41-92
	Median	64	68
	Mean	68	68
	Mean Ln	4.2	4.2
	Geometric Mean Estimate & 95% Confidence Interval[¥] (p-value[*])	1.13 & 1.03-1.25 (p=0.01)	
24 hours post CPB	Range	43-99	44-115
	Median	63	76
	Mean	63	75
	Mean Ln	4.1	4.3
	Geometric Mean Estimate & 95% Confidence Interval[¥] (p-value[*])	1.13 &1.03-1.25 (p=0.01)	

[†] *This is an on treatment analysis;*
Statistical analysis using repeated measures of ANOVA;
[¥]*95% Confidence Intervals and p values are only given where the result is statistically significant;*
**p values are for geometric means*

c. Peri-Operative Mean PVR & PVRI

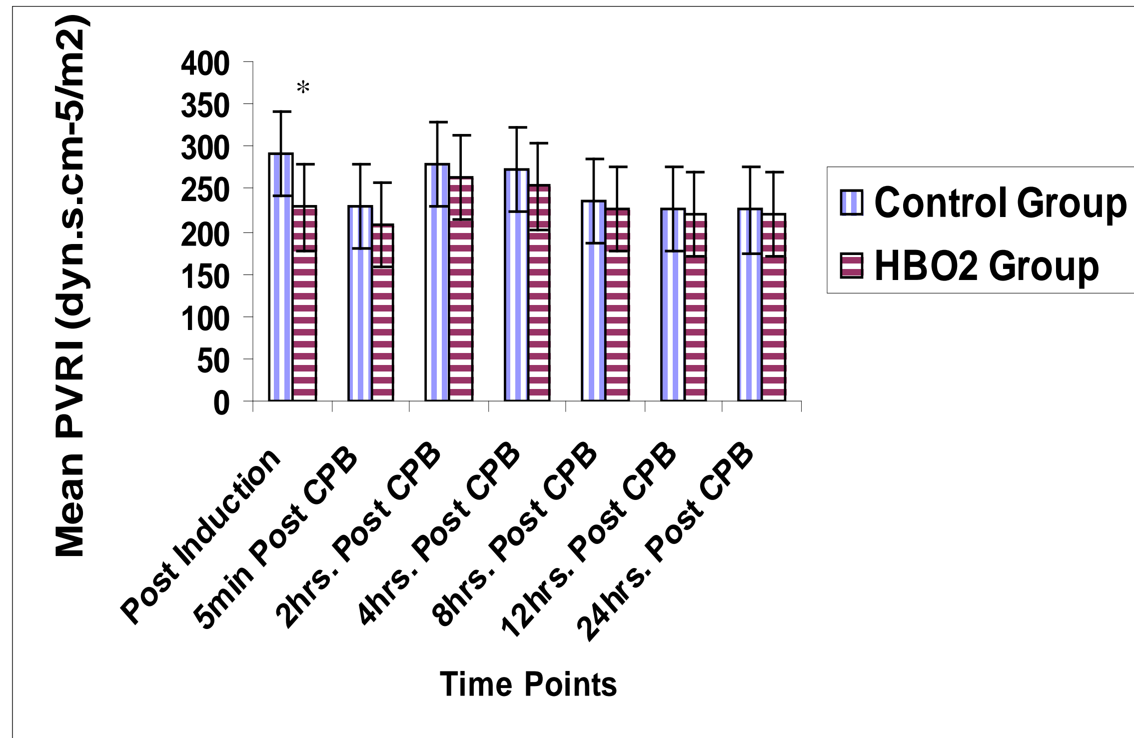
In this study, in both groups, between the baseline 'post induction' time point and the time point '24 hours post CPB', there was a general decrease in the mean PVR and PVRI in both groups. At all time points following the baseline 'post induction' time point, both the mean PVR and PVRI were lower in the HBO_2 Group. In this study it was determined that at the baseline 'post induction' time point, there was a statistically significant decrease in PVR (p=0.03; Geometric Mean Estimate & 95% Confidence Interval: 1.55 & 1.05, 2.29) *(Figure 3.4)* and the PVRI (p=0.05; Geometric Mean Estimate & 95% Confidence Interval: 1.49 & 1.02, 2.12) *(Figure 3.5)* in the HBO_2 Group compared to the Control Group. A summary of the results are provided in *Table 3.5*. In this table, the data are displayed as both mean and median values. As the means and medians are not the same at each time point, this indicates that the data were skewed. To normalise the data and enable parametric statistical analysis, the mean values were transformed into natural logarithmic (ln) values.

Figure 3. 4: Bar Chart of Peri-Operative Mean PVR



HBO_2 Group, n=22; Control Group, n=25
Bars chart showing mean values and error bars for the standard error of the mean;
** p<0.05 following repeated measures of ANOVA analysis*

Figure 3. 5: Bar Chart of Peri-Operative Mean PVRI



HBO_2 Group, n=22; Control Group, n=25
Bars chart showing mean values and error bars for the standard error of the mean;
***** *$p<0.05$ following repeated measures of ANOVA analysis*

Table 3. 5: Peri-Operative PVR & PVRI

		PVR (dyns.cm[5])		PVRI (dyns.cm[5]/m^{2})	
		Control Group [n=25][†]	HBO_2 Group [n=22][†]	Control Group [n=25][†]	HBO_2 Group [n=22][†]
Post Induction	Range	72-303	21-309	68-530	42-571
	Median	150	100	281	208
	Mean	158	110	292	228
	Mean Ln	5.1	4.7	5.7	5.4
	Geometric Mean Estimate & 95% Confidence Interval[¥] (p-value[*])	1.55 & 1.05-2.29 (p=0.03)		1.49 & 1.01, 2.19 (p=0.05)	
5 minutes post CPB	Range	46-855	12-218	45-349	65-432
	Median	125	101	212	129
	Mean	141	130	230	219
	Mean Ln	4.9	4.9	5.4	5.4
	Geometric Mean Estimate & 95% Confidence Interval[¥] (p-value[*])	0.08		0.08	
2 hours post CPB	Range	51-220	11-341	70-623	22-681
	Median	133	121	248	237
	Mean	140	150	280	263
	Mean Ln	4.9	5.0	5.6	5.6
	Geometric Mean Estimate & 95% Confidence Interval[¥] (p-value[*])	0.08		0.08	
4 hours post CPB	Range	24-1246	53-661	24-1246	53-661
	Median	166	168	227	258
	Mean	149	135	273	253
	Mean Ln	5.0	4.9	5.6	5.5
	Geometric Mean Estimate & 95% Confidence Interval[¥] (p-value[*])	0.08		0.08	
8 hours post CPB	Range	23-589	32-432	23-589	32-432
	Median	141	130	245	229
	Mean	129	121	236	226
	Mean Ln	4.9	4.8	5.5	5.1
	Geometric Mean Estimate & 95% Confidence Interval[¥] (p-value[*])	0.08		0.08	
12 hours post CPB	Range	80-408	27-543	80-408	27-543
	Median	121	123	239	229
	Mean	115	112	236	220
	Mean Ln	4.7	4.7	5.4	5.4
	Geometric Mean Estimate & 95% Confidence Interval[¥] (p-value[*])	0.08		0.08	
24 hours post CPB	Range	28-538	29-406	28-538	29-406
	Median	129	119	243	231
	Mean	113	106	225	219
	Mean Ln	4.7	4.6	5.4	5.4
	Geometric Mean Estimate & 95% Confidence Interval[¥] (p-value[*])	0.08		0.08	

[†] This is an on treatment analysis;
Statistical analysis using repeated measures of ANOVA;
[¥]95% Confidence Intervals and p values are only given where the result is statistically significant;
**p values are for geometric means*

3.4.2 Effects Of HBO_2 Preconditioning On Post Operative Clinical Safety

3.4.2.1 Cardiovascular Adverse Events

In this study, compared to the Control Group, in the HBO_2 Group, there was a 10.4% reduction in the proportion of patients with low cardiac output syndrome (p=0.4), an 8% reduction in the proportion of patients requiring the use of inotropes (p=0.1) and an 11% reduction in the proportion of patients with AF (p=0.6) *(Table 3.6)*. There were no incidences of the use of cardiovascular supportive therapy (IABP or pacing), MI or mortality in either group.

Table 3. 6: Post Operative Cardiovascular Adverse Events

Cardiovascular Adverse Event	**Control Group** (n=40)[†]	**HBO_2 Group** (n=41)[†]	**p- value**
Low cardiac output	10 (25%)	6 (14.6%)	0.4
Inotrope Usage	10 (25%)	7 (17%)	0.4
1. Adrenaline 2. Dopamine 3. Noradrenaline 4.Adrenaline + Dopamine 5. Adrenaline + Noradrenaline 6. Adrenaline + Milrinone 7.Adrenaline + Noradrenaline + Milrinone	2 3 0 1 2 1 1	1 3 2 0 1 0 0	
Atrial Fibrillation	10 (25%)	6 (14%)	0.4
Cardiovascular Supportive Therapy (IABP, pacing)	0	0	1.0
Myocardial Infarction	0	0	1.0
Mortality	0	0	1.0

Values are patient numbers with percentages in brackets;
[†]This is an intention to treat analysis;
Statistical analysis using Chi-Squared Test

Patients in the HBO_2 Group had a 57% reduction in intra-operative blood loss compared to patients in the Control Group (p=0.02). There were no significant difference between the groups with respect to post operative blood loss and blood transfusion. However, in the HBO_2 Group, compared to the Control Group, there was an 11.6% reduction in post operative (p=0.1) blood loss and a 34% reduction in post operative blood transfusion (p=0.4) *(Table 3.7)*.

Table 3. 7: Intra-Operative & Post Operative Blood Loss & Blood Transfusion

		Control Group (n=40)†	**HBO_2 Group** (n=41) †	**% Mean Reduction in HBO_2 Group**	**p-value**	**95% Confidence Intervals** *(for % change)*
Intra Operative Blood Loss (ml)	**Range**	0-1528	0-961	[(309-133)/ 309] X 100 = 57	0.02	-318, -32
	Mean	309	133			
Post Operative Blood Loss (ml)	**Range**	255-1295	325-1330	[(727-643)/ 727] X 100 = 11.6	0.1	-31, 200
	Mean	727	643			
Total blood transfusion (ml)	**Range**	0-612	0-610	[(138-91)/ 138] X 100 =34	0.4	-63, 159
	Mean	138	91			

†*This is an intention to treat analysis*
Statistical analysis using Independent Sample t-test

3.4.2.2 Pulmonary Adverse Events

Patients in the HBO_2 Group had a mechanical ventilation time and an endotracheal intubation time that was 30 minutes longer compared to patients in the Control Group. Neither of these results were significant *(Table 3.8).*

Table 3. 8: Post Operative Duration of Ventilation & Intubation

	Control Group (n=40)[†]		**HBO_2 Group** (n=41)[†]		**p-value**
	Range	**Mean**	**Range**	**Mean**	
Mechanical Ventilation Time (hours)	1-13.5	2.5	0-18	3.0	0.2
Duration of Intubation (hours)	1.5-14	3.0	0.5-18.5	3.5	0.2

[†]*This is an intention to treat analysis;*
Statistical analysis using Chi-Squared Test

In this study, compared to the Control Group, in the HBO_2 Group, there was a 12.7% reduction in the proportion of patients with pulmonary AEs (p=0.3) *(Table 3.9)*. There were no incidences of pneumothoraces, pleural effusions or the use of pulmonary supportive therapy in the HBO_2 Group, all of which were increased in the Control Group. While there was a 5.1% reduction in chest infections in the HBO_2 Group (p=0.9), there was one patient (2.4%) in this group with a chest infection associated with pleural effusion.

Table 3. 9: Post Pulmonary Adverse Events

	Control Group (n=40)[†]	**HBO_2 Group** (n=41)[†]	**p-value**
Pulmonary Adverse Events	9 (22.5%)	4 (9.8%)	0.3
1. Pneumothorax	1 (2.5%)	0	1
2. Pleural Effusion	2 (5%)	0	0.9
3. Chest Infection	4 (10%)	2 (4.9%)	0.9
4. Pulmonary atelectasis requiring pulmonary supportive therapy (BiPAP/CPAP)	1 (2.5%)	0	1
5. Chest infection + Pleural Effusion	0	1 (2.4%)	1
6. Other	1 (2.5%)	1 (2.4%)	1

Values are patient numbers with percentages in brackets;
[†]*This is an intention to treat analysis;*
Statistical analysis using Chi-Squared Test;
BiPAP=Biphasic Positive Airway Pressure; CPAP=Continous Positive Airway Pressure

3.4.2.3 Renal Adverse Events

In this study, there were no incidences of serum creatinine > 200mmol/l in the HBO_2 while in the Control Group, there were two patients (5%) *(Table 3.10).* There were no incidences requiring the use of renal supportive therapy in either group.

Table 3.10: Post Operative Renal Adverse Events

Renal Adverse Event	**Control Group** (n=40)[†]	**HBO_2 Group** (n=41)[†]	**p-value**
Creatine>200mmols/l	2 (5%)	0	0.9
Renal Supportive Therapy (CVVH)	0	0	1.0

Values are patient numbers with percentages in brackets;
[†]This is an intention to treat analysis;
Statistical analysis using Chi-Squared Test

3.4.2.4 Neurological Adverse Events

In this study, there was one patient (2.5%) who developed transient confusion associated with blurred vision in the Control Group. There were no incidences of TIAs or strokes in both groups *(Table 3.11)*.

Table 3.11: Post Operative Neurological Adverse Events

Neurological Adverse Event	**Control Group** (n=40)†	**HBO_2 Group** (n=41)†	**p-value**
Confusion + blurred vision	1 (2.5%)	0	1.0
TIA	0	0	1.0
Stroke	0	0	1.0

Values are patient numbers with percentages in brackets;
†This is an intention to treat analysis;
Statistical analysis using Chi-Squared Test

3.4.2.5 Gastrointestinal Adverse Events

In this study, both the HBO_2 and the Control Group had similar proportions of patients who experienced gastrointestinal AEs *(Table 3.12)*. There was one patient (2.5%) in the Control Group who developed *Clostridia Difficiele* diarrhoea while there were none in the HBO_2. There were no incidences of ischaemic bowel in either group.

Table 3.12: Post Operative Gastrointestinal Adverse Events

Variable	**Control Group** (n=40)†	**HBO_2 Group** (n=41)†	**p-value**
Gastrointestinal Adverse Event	1 (2.5%)	1 (2.4%)	1.0
1. *Clostridia Difficiele* Diarrhoea	1	0	1.0
2. Ischaemic Bowel	0	0	1.0
3. Other	0	1	1.0

Values are patient numbers with percentages in brackets;
†This is an intention to treat analysis;
Statistical analysis using Chi-Squared Test

3.4.2.6 Microbiological Adverse Events

Compared to the Control Group, in the HBO_2 Group there was a 7.6% reduction in the proportion of patients who experienced microbiological AEs (p=0.8) *(Table 3.13).* There were no incidences of superficial sternal or deep sternal wound infections in the HBO_2 Group, of which there were three patients (7.3%) and one patient (2.5%), respectively in the Control Group. There was however, one patient (2.4%) who developed leg wound infection in the HBO_2 Group while there were none in the Control Group. There were no incidences of patients requiring sternal wound re-wiring secondary to deep sternal wound infection in the HBO_2 while there was one (2.5%) patient in the Control Group.

Table 3.13: Post Operative Microbiological Adverse Events

	Control Group (n=40)†	**HBO_2 Group** (n=41)†	**p-value**
Microbiological Adverse Event *(based on bacterial culture results)*	4 (10%)	1 (2.4%)	0.8
1. Superficial sternal wound infections	3 (7.3%)	0	0.8
2. Deep sternal wound infections	1 (2.5%)	0	1.0
3. Leg wound infections	0	1 (2.4%)	1.0
Sternal infection requiring re-wiring	1 (2.5%)	0	1.0

Values are patient numbers with percentages in brackets;
†This is an intention to treat analysis;
Statistical analysis using Chi-Squared Test

3.4.2.7 Post Operative ICU Length of Stay

In this study, after adjusting for longest aortic cross clamp and CPB time, there was a statistically significant reduction in the post operative ICU length of stay of patients in the HBO_2 Group compared to those in the Control Group (p=0.05; Geometric Mean Estimate & 95% Confidence Interval for the difference: 1.18 and 0.02, 7.96). On the average, patients in the HBO_2 Group spent 4 hours less in ICU. A summary of the results are provided in *Table 3.14*. In this table, the data are displayed as both mean and median values. As the means and medians are not the same at each time point, this indicates that the data were skewed. To normalise the data and enable parametric statistical analysis, the mean values were transformed into natural logarithmic (ln) values.

Table 3.14: Post Operative ICU Length of Stay

		Control Group (n=40)[†]	**HBO_2 Group** (n=41)[†]	**Geometric Mean Estimate & 95% Confidence Interval (p-value [*])**
Length of ICU Stay (hours)	**Total**	1051	850	1.18 & 0.99, 1.39 (p=0.05)
	Range	21-76	6-28	
	Median	24	18	
	Mean	18	14	
	Mean Ln	2.89	2.64	

**p values are for geometric means;*
[†]This is an intention to treat analysis;
Statistical analysis using Independent Sample t-test

3.4.3 Cost Effectiveness of HBO_2 Preconditioning

The *post hoc* cost effectiveness analysis of HBO_2 preconditioning is shown in *Table 3.15*. For this study, the HBO_2 treatment as a whole was estimated to be approximately £200 per patient. The cost for the use of the ICU bed per day at Castle Hill Hospital (Hull & East Yorkshire NHS Trust) was estimated to be approximately £2,000. Using these cost estimates, it was calculated that patients treated with HBO_2 prior to CABG saved approximately £292.63 per patient, in terms of ICU bed cost. This equated to an estimated savings of approximately £73.16 per saved ICU hour.

Table 3.15: Summary of Cost

Item	Cost (£)
HBO_2 Treatment	200
HBO_2 treatment for 34 patients	6,800
ICU bed per day *(24 hours)*	2000
ICU bed per hour	2000/24 = 83.33
ICU stay for 35 Control patients (A) *(see Table 3.14)*	83.33 x 1051 = 87,579.83
ICU stay for 34 HBO_2 patients *(see Table 3.14)*	83.33 x 850 = 70,830.50
HBO_2 treatment + ICU stay for 34 patients (B)	6,800 + 70,830.50 = 77,630. 50
Total Savings for HBO_2 patients (A-B=C) *(savings made by 34 HBO_2 patients who spent a total of 1051-850=201 hours less in ICU; see Table 3.14)*	87,579.83 - 77,630. 50 = 9,949.33
Savings per HBO_2 patient in ICU (C/34=D)	9,949.33/34 = 292.63
Savings per mean ICU hour saved (*see Table 3.14)* **in each HBO_2 patient (D/4)**	292.63/4 = 73.16

*This is an on treatment analysis

3.5 Discussion

3.5.1 Peri-Operative Cardiovascular Efficacy of HBO_2 Preconditioning

In this study it was observed that following the termination of CPB, in the HBO_2 Group, there were statistically significant increases in the mean LVSW *(Figure 3.1)*, LVSWI *(Figure 3.2)* and SV *(Figure 3.3)*. At present, there are no other published clinical studies that have determined the effects of HBO_2 preconditioning on myocardial function following IRI. These findings, however, do not corroborate with the findings of an experimental study of HBO_2 preconditioning prior to IRI (Radice et al., 1997). In that study, it was found that preconditioning rats with 100% O_2 at 2.5 ATA for 1 hour, 3 hours or 6 hours, prior to ischaemia of 40 minutes and reperfusion of 20 minutes, resulted in a time proportional increase in the heart resting tension (left ventricular end-diastolic pressure). This reflects an increased degree of ventricular stiffness and depression of myocardial mechanics. In that study, it was suggested that the findings were in keeping with the observation by others that high oxygen tension (100%) increased isometric systolic tension and alters coronary flow as a result of oxygen toxicity (Daniell and Bagwell, 1968). This may well be the explanation for the findings in that experimental study but is one that cannot be used in the current HBO_2 clinical study which demonstrated haemodynamic benefits from HBO_2 preconditioning. In this clinical HBO_2 preconditioning protocol, the possibility of oxygen toxicity was mitigated by having a 5 minute air break between the two 30

minute periods of 100% O_2 at 2.4 ATA. This HBO_2 preconditioning protocol has also been safely used before (Alex et al., 2005) with no report of any cardiopulmonary decompensation or oxygen toxicity. By reducing the duration of exposure to HBO_2 that is intermittent in nature and limiting the compression pressure to a level that is known to be clinically safe, the results from this clinical study indicate that preconditioning CAD patients with HBO_2 prior to CABG may have the potential to enhance myocardial mechanical function post CABG and IRI.

An indirect measure of support for the haemodynamic findings of the current clinical study comes from clinical studies where HBO_2 was administered following the ischaemic-reperfusion event. Dekleva *et.al* conducted a randomised control study to assess the benefits of HBO_2, after thrombolysis, on the ventricular function and remodelling in patients who had suffered from an AMI (Dekleva et al., 2004). 74 patients with first time AMI were randomly assigned to HBO_2 treatment consisting of 100% oxygen at 2 ATA for 60 minutes in addition to STK or treatment with just STK alone. In that study, it was observed that there was a significant decrease in end-systolic volume index from the first day to the third week after HBO_2 treatment, in HBO_2 group of patients compared with control group of patients. This was accompanied by a lack of changes in end diastolic volume index in the HBO_2 group compared to increased values in the control group. The EF was also significantly improved in the HBO_2 group while it decreased in the control group of patients 3 weeks after AMI. That study concluded that adjunctive HBO_2 after thrombolysis in patients with an AMI, had a favourable effect on left ventricular function and the remodelling process. Further support comes from the 'HOT MI' study group (Shandling et al., 1997, Stavitsky et al.,

1998). In the studies conducted by that group, it was observed that in the patients who were also treated with HBO_2 following an AMI, there was an improvement in post MI EF. The 'HOT-MI' clinical studies demonstrated that HBO_2, as a drug to adjunct thrombolysis, was capable improving systolic function while at the same time arresting any further deterioration in diastolic function following AMI. Furthermore, it showed that in the absence of HBO_2 to adjunct thrombolysis, the control group went on to develop systolic and diastolic dysfunction post AMI. More recently, it was also observed that in diabetic patients who were being treated for non-healing lower extremity ulcers, after 10 HBO_2 treatment sessions, these patients had an improved diastolic function (Aparci et al., 2008). Those clinical studies, together with the results from this current study, further support the premise that HBO_2 treatment, pre and post myocardial ischaemia and reperfusion may be capable of improving myocardial function.

In this study, it was also observed that prior to the onset of CPB (i.e. at the time point 'post induction'), the PVR *(Figure 3.4)* and PVRI *(Figure 3.5)* were significantly lower in the HBO_2 Group compared to in the Control Group. This reduction in PVR and PVRI suggest that preconditioning CAD patients with HBO_2 prior to CABG and IRI has the potential to reduce pulmonary vascular resistance and as such, improve pulmonary vascular blood flow. Physiologically, this finding is in keeping with what is known about pulmonary vascular reactivity following exposure to hyperoxia i.e. in response to hyperoxia, the pulmonary vessels dilate thus allowing improved blood flow. Furthermore, it also possible that these observations may be related to changes in the kinetics of sPSGL-1 and, this shall be discussed later in *section 5*.

3.5.2 Safety of HBO_2 Preconditioning

3.5.2.1 Cardiovascular Safety

While there were no statistically significant differences between the groups with respect to cardiovascular AEs, the proportion of patients with low cardiac output syndrome, AF or requiring inotrope use was lower in the HBO_2 *(Table 3. 6)*. This suggests that preconditioning CAD patients with HBO_2 prior to on CPB CABG is a relatively safe procedure that does not lead significant post operative cardiovascular AEs.

3.5.2.2 Pulmonary Safety

In this study, it was observed that patients in the HBO_2 Group had a post operative duration of mechanical ventilation and endotracheal intubation that was 30 minutes longer compared to patients in the Control Group *(Table 3.8)*. These findings were not statistically significant and indicates that preconditioning CAD patients with HBO_2 prior to on CPB CABG does not lead to prolonged post operative pulmonary support. This, in addition to the observation that this group of patients had a lower PVR and PVRI *(Table 3.4)* prior to CPB and, post operatively there was a lower proportion of patients who experienced combined pulmonary AEs *(Table 3.9)*, suggests that this modality of treatment is a relatively safe procedure that does not lead significant post operative pulmonary AEs.

3.5.2.3 Renal, Neurological & Gastrointestinal Safety

In this study, the proportion of patients with renal and neurological AEs were lower in the HBO_2 Group while the proportion of patients with gastrointestinal AEs were similar between the groups *(Tables 3.10-3.12)*. While none of these findings were statistically significant, it does suggest that preconditioning CAD patients with HBO_2 prior to on CPB CABG is a relatively safe procedure that does not lead significant post operative renal, neurological and GI AEs.

3.5.2.4 Microbiological Safety

In this study, the proportions of patients with wound infections was lower in the HBO_2 Group. In particular, while the Control Group had 4 cases of sternal wound infection, HBO_2 Group had none. While there are a number of studies (Barili et al., 2007, De Feo et al., 2001, Lappa et al., 2003, Petzold et al., 1999) that have shown that treatment with HBO_2 was a useful adjunct to promote sternal wound healing following post operative sternal wound infection, the results from this study suggests that HBO_2 treatment prior to median sternotomy may also be capable of limiting post operative sternal wound infections *(Table 3.13)*. Perhaps this may have been due to the induction of prophylactic anti-microbial effects of treatment with HBO_2. This is plausible because oxygen and HBO_2 have been described to have anti-microbial properties (Park et al., 1992).

3.5.2.5 Post Operative Length of ICU Stay

In this study it was observed that patients who were preconditioned with HBO_2 prior to CABG and IRI had a significantly shorter post operative length of stay in ICU compared to patients in the Control Group. Age, CPB time, pre-CABG chronic COPD and pre-CABG pulmonary function are known to affect ICU length of stay (Nakasuji et al., 2005, Rosenfeld et al., 2006). In this study, the ages of patients in both groups were similar *(Table 3.1)*. While the Control Group had slightly longer mean ischaemic and CPB times *(Table 3.2)*, the potential confounding effect of these were mitigated by performing a multivariable linear regression analysis which adjusted for longest cross aortic clamp and CPB times. Patients with COPD were excluded from this study as they are more susceptible to pneumothoraces and this risk is increased in a HBO_2 chamber where there are changes in pressure during the compression and decompression stages of the HBO_2 treatment. As patients in both groups were of similar ages and appropriate statistical analysis had been performed to adjust for the potential confounding effect of time on CPB, it would be reasonable to suggest that HBO_2 preconditioning was, in part, the cause for the shorter ICU stay that was observed in the HBO_2 Group. This may have been the result of the HBO_2 Group of patients having lower incidences of post operative AEs.

3.5.3 Cost Effectiveness

In this clinical study, the *post hoc* cost effectiveness analysis revealed that preconditioning CAD patients with HBO_2 prior to CABG and IRI was a cost effective means of reducing post operative length of stay in ICU. This was found to be the case despite the slightly longer mechanical ventilation and intubation time in this group of patients. The saving made by the HBO_2 Group of patients were based on the cost per hour for the use of an ICU bed. This analysis did not take into account the added cost of any post operative AEs and the treatments required to resolve these AEs or, the staff and infra-structure cost. As the proportion of patients with post operative AEs was lower in the HBO_2 Group than in the Control Group, it is quite likely that the overall savings made by the HBO_2 Group, in addition to the savings made from the shorter post operative ICU length of stay, may have been greater. The health technology assessment involving the cost effectiveness of HBO_2 as a modality of treatment, has been reviewed by others (Guo et al., 2003b), particularly in the area of wound management (Chuck et al., 2008, Guo et al., 2003a, McEwen and Smith, 1997, Treweek and James, 2006). It is clear from these assessments that HBO_2 is a cost effective treatment. This clinical study is the first to attempt to assess the cost effectiveness of HBO_2 in a clinical model of CABG and IRI. While it was shown to be cost effective, no assessment was made of the possible 'Quality Added Life Years' (QUALY) that may have been made as a result of HBO_2 preconditioning. However, based on the clinical study by Alex *et.al*, involving HBO_2 preconditioning and CABG patients,

which found that patients who were preconditioned with HBO_2 prior to CABG had significantly less post operative neurocognitive dysfunction (Alex et al., 2005), it is quite possible that this modality of treatment may have the capacity to increase QUALY in patients following CABG. To accurately determine the cost effectiveness and QALY associated with the use of HBO_2 in this group of patients, health technology assessment models will need to be developed in collaboration with health economists.

3.6 Conclusion

This study met its primary cardiovascular efficacy endpoint and demonstrated that preconditioning CAD patients with HBO_2 prior to CABG led to a significant improvement in LVSW 24 hours post CPB. As such, it can be concluded that HBO_2 preconditioning in this group of patients leads to an improvement in myocardial function following CABG.

As this study was not designed to determine the effects of HBO_2 preconditioning with respect to all the other clinical endpoints, which were all secondary endpoints, despite some statistically significant results, no definitive conclusions can be made with regards to the effects of HBO_2 preconditioning on these endpoints. Where there were findings of statistical significance, this only provided a sensitivity analysis to support the estimates for any numerical differences between the groups. However, based on some of the estimates of the differences between the groups with respect to the clinical secondary endpoints, it is possible to make a few comments with regards to these differences.

In addition to the observed improvement in LVSW 24 hours post CPB, in this study it was also observed that treating CAD patients with HBO_2 prior to CABG, also leads to significant improvements in the LVSWI and SV 24 hours post CPB. These data supports the hypothesis that this modality of pre-treatment is capable of improving myocardial function in this group of patients. Furthermore, HBO_2 preconditioning also led to significant reductions in PVR and PVRI prior to CPB. This indicates that preconditioning

CAD patients with HBO_2 prior to CABG has the potential to improve pulmonary vascular blood flow prior to surgery.

This study also demonstrated that despite the longer post operative mechanical ventilation and intubation times, the patients who were preconditioned with HBO_2 had a significantly shorter post-operative length of stay in ICU. As such, it is reasonable to suggest that HBO_2 preconditioning was in part, the cause for the shorter ICU stay. It was also observed that HBO_2 preconditioning, in this group of patients, was a safe modality of treatment as it was associated with a smaller proportion of patients experiencing post operative cardiovascular, pulmonary, renal, neurological and microbiological AEs. Where cost effectiveness was concerned, it was determined that the shorter length of post operative ICU stay amongst patients preconditioned with HBO_2, was also associated with savings in hospital ICU bed cost.

The main limitation of this study, that may have an impact on the observed clinical outcomes, is that patients in both groups had variable durations of intra-operative ischaemia, reperfusion and CPB and, a variety of ischaemic-reperfusion cycles as patients had a variable number of coronary artery bypasses *(Table 3.1)*. While it would have been difficult to standardise the duration of ischaemia, reperfusion and CPB in a clinical study, perhaps one way to limit the variability in these durations was to limit the study population to patient who were only going to have a particular number of coronary artery bypass grafts.

4. Effects of HBO_2 Preconditioning on A Surrogate Biomarker of Myocardial Injury: Serum Troponin-T

4.1 Introduction

To date, there are no other experimental or clinical studies where the effects of HBO_2 on serum Troponin-T had been investigated. However, the 'HOT-MI' study group (Shandling et al., 1997) found that in AMI patients who were treated with HBO_2 in addition to routine therapy, there was a 35% reduction in mean CK at 12 and 24 hours post MI. In a later similar study by the 'HOT-MI' group of investigators (Stavitsky et al., 1998), it was again observed that, compared to a control group, in AMI patients who were also treated with HBO_2 in addition to routine therapy, the mean CK was 7.5% lower at 12 and 24 hours post AMI.

4.2 Objective

The objective for this part of this clinical study was to assess the myocardial safety of systemically preconditioning CAD patients with one session of HBO_2 preconditioning, involving two episodes of 30 minutes of 100% oxygen at 2.4 ATA. Treatment with HBO_2 preconditioning was completed approximately 2 hours prior to on CPB CABG. The assessment of the myocardial safety of this modality of treatment was done by determining the degree of myocardial injury as measured by the concentration of the surrogate biomarker of myocardial injury, serum Troponin-T at the pre-specified time points *(Table 2.5)*.

4.3 Methods

The time points for the collection of venous blood samples in this study and the method used for the measurement of serum Troponin-T have already been described in *section 2.6.5*. The measurement for serum Troponin-T was done by the Department of Biochemistry at the Hull & East Yorkshire NHS Trust within 4 hours of collection using an ELICA method as described in *section 2.6.6.1*. Correction for the effects of hemodilution of serum Troponin-T during CPB, was done as described in *section 2.6.6.3*.

4.4 Results

a. Pre- & Post HBO_2 Mean Concentration of Serum Troponin-T in the HBO_2 Group

In this study, any value for Troponin-T that was between 0 and 0.03ng/ml was reported as <0.03ng/ml by the Department of Biochemistry at the Hull & East Yorkshire NHS Trust. This is because, as described in *section 2.6.1*, the medical diagnostic guide for diagnosing an MI using the Troponin-T STAT kit is a Troponin-T concentration of 0.03ng/ml and greater. Furthermore, for clinical purposes, 0.03ng/ml is the lowest limit of quantification that can be reproducibly measuresed using this kit. In this study, in the HBO_2 Group of patients, the pre- and post HBO_2 serum Troponin-T concentrations, in all patients, were reported as <0.03ng/ml. As in this study there were no plans for data imputation, it was not possible to determine the range, median, mean, ln mean or statistical significance of serum Troponin-T measurements from both these time points in this group of patients *(Table 4.1)*.

Table 4. 1: Pre & Post HBO_2 Mean Concentration of Serum Troponin-T in the HBO_2 Group

HBO_2 Group (n=41) †					
Concentration of Serum Serum Troponin-T(ng/ml)					
	Range	**Median**	**Mean**	**Ln Mean**	**p-value**
Pre-HBO_2	*Unable to determine*	*Unable to determine*	*Unable to determine*	*Unable to determine*	-
1 hour post HBO_2	*Unable to determine*	*Unable to determine*	*Unable to determine*	*Unable to determine*	

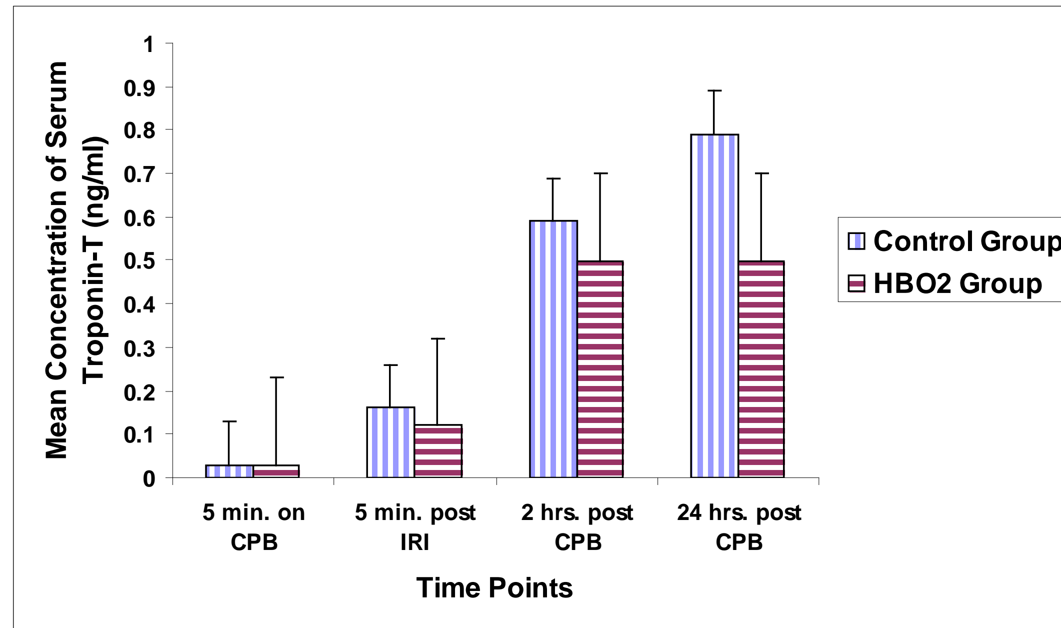
†*This is an intention to treat analysis*

b. Peri Operative Mean Concentration of Serum Troponin-T Between The Groups

Just as in the HBO_2 Group of this study, in the Control Group of patients, the serum Tropinin-T taken at the time point labelled 'pre-HBO_2', were also reported as <0.03ng/ml. Again, as no imputation of data were done during this study, serum Troponin-T values that were reported as <0.03ng/ml were not used for statistical analysis. As such, for the purposes of this part of this study, when comparing serum Troponin-T between the groups, the time point which was taken as the baseline for serum Troponin-T analysis was the time point '5 minutes on CPB'.

In this study, after adjusting for longest aortic cross-clamp time and CPB time, no statistically significant changes in serum Troponin-T were found between the groups at any of the time points. It was observed, however, that at all time points, apart from at the time point '5 minutes on CPB', the mean concentration of serum Troponin-T was lower in the HBO_2 Group *(Figure 4.1)*. In both groups, the mean concentration of serum Troponin-T appeared to rise from the time point '5 minutes on CPB' up to the time point '2 hours post CPB'. After this time point, the mean concentration of serum Troponin-T continued to rise in the Control Group but remained unchanged in the HBO_2 Group. A summary of the results are provided in *Table 4.2*. In this table, the data are displayed as both mean and median values. As the means and medians are not the same at each time point, this indicates that the data were skewed. To normalise the data and enable parametric statistical analysis, the mean values were transformed into natural logarithmic (ln) values.

Figure 4. 1: Bar Chart of Peri-Operative Mean Concentration of Serum Troponin-T



HBO_2 Group, n=41; Control Group, n=44;
Bars chart showing mean values and error bars for the standard error of the mean

Table 4. 2: Peri-Operative Concentration of Serum Troponin-T

Serum Troponin-T (ng/ml)									
	Control Group (n=40)[†]				**HBO_2 Group** (n=41)[†]				
	Range	**Median**	**Mean**	**Ln Mean**	**Range**	**Median**	**Mean**	**Ln Mean**	**p-value***
Pre-HBO_2	*Unable to determine*	*Unable to determine*	*Unable to determine*	-	*Unable to determine*	*Unable to determine*	*Unable to determine*	-	-
5 minutes on CPB	0.03-0.11	0.03	0.03	-3.4	0.02-0.15	0.03	0.03	-3.4	0.90
5 minutes post IRI	0.03-0.59	0.10	0.16	-1.9	0.03-0.34	0.1	0.12	-2.1	0.61
2 hours post CPB	0.22-1.50	0.22	0.59	-0.5	0.17-1.43	0.5	0.50	-0.7	0.99
24 hours post CPB	0.14-7.62	0.14	0.79	-0.2	0.10-2.90	0.49	0.50	-0.7	0.99

[†]*This is an Intention to treat analysis;*
Statistical analysis using repeated measures of ANOVA;
**p-values are for geometric means;*

4.5 Discussion

In this study, it was observed that 1 hour following HBO_2 treatment, serum from patients in HBO_2 Group did not show any clinically meaningful change in serum Troponin-T. This suggests that 1 hour following HBO_2 preconditioning, the oxidative stress of this modality of treatment did not cause any clinically significant myocardial injury. Furthermore, during the peri-operative period, it appeared that HBO_2 preconditioning induced a degree of cardioprotection by limiting the amount of myocardial injury and hence the release of Troponin-T into the circulation. In fact following the time point '2 hours post CPB', the serum concentration of Troponin-T in the HBO_2 Group appeared to plateau off while in the Control Group it continued to rise suggesting that there was no progressive myocardial injury in the HBO_2 Group after that time point but a continued to escalation of injury in the Control Group. As such, these observations provide some further support that in a group of patients with ischaemic heart disease, HBO_2 preconditioning is a safe modality of treatment. This in keeping with the findings of other randomised controlled studies (Shandling et al., 1997, Stavitsky et al., 1998).

As a modality of treatment that induces an oxidative stress, it is still not entirely clear how HBO_2 preconditioning and its generated ROS, paradoxically, limits myocardial injury and reduces the release of biomarkers of myocardial injury such as CK and Troponin-T during ischaemia and reperfusion. Clinical studies also have yet to find any correlation between ROS generation and, myocardial injury and Troponin-T release (Berg et al., 2006,

Berg et al., 2005, Berg et al., 2004). In a recent study, it was demonstrated that while oxidative stress does occur following IRI and CABG, this does not necessarily equate to cellular injury and clinical deterioration (Milei et al., 2007). While high doses (100-200μM) of ROS (such as H_2O_2) have been shown to have deleterious effects on the structure and function of the myocardium (Evans et al., 1995, Janero et al., 1991, Miki et al., 1988, Onodera et al., 1992), it has also been demonstrated in an experimental model, that low doses of H_2O_2 (between 1μM and 2 μM), added during reperfusion, can reduce myocardial infarct size (Ytrehus et al., 1995). In another experimental study, it was demonstrated 25μM of H_2O_2 administered during reperfusion, resulted in a beneficial effect on functional and metabolic recovery in isolated rat hearts (Hegstad et al., 1997). Coronary flow and, left ventricular developed pressure and relaxation, were improved in the group that was exposed to 25μM of H_2O_2 compared to the untreated control group. Furthermore, the addition of 25μM of H_2O_2 increased the levels of high energy phosphates (ATP) at the end of the reperfusion period compared to the control group. Additionally, it has also been demonstrated that low concentrations of H_2O_2 causes vasodilatation (Burke and Wolin, 1987). These beneficial ROS induced effects may account for the reduction in myocardial injury observed in the HBO_2 Group of this study, who were indirectly exposed to ROS during treatment with HBO_2 preconditioning prior to CABG and IRI. As myocardial protection via ROS preconditioning, has been successfully demonstrated by others (Das et al., 1999a), it is possible that the HBO_2 induced ROS preconditioned the myocardium to better tolerate the subsequent stress of IRI induced ROS.

4.6 Conclusion

In this clinical study, no statistically or clinically significant results were observed when determining the effects of HBO_2 preconditioning on serum Troponin-T. As such, and as per the statistical plan for analysing the secondary endpoints of this study, no definitive conclusions can be made with respect to the changes that were observed. However, based on the observed mean differences between the groups with respect to this secondary endpoint, it would not be unreasonable to further reinforce the suggestion that preconditioning CAD patients with HBO_2 prior to CABG is a safe as it did not exacebate the peri-operative myocardial injury and may have even contributed to limiting this injury post operatively.

The main limitations to this part of the study was the inability to accurately quantify the concentrations of serum Troponin-T for values <0.03ng/ml. As no imputation of data was done for this study, there were no available serum Troponin-T measurements at the time points 'pre-HBO_2' (both groups) and 'post HBO_2' (HBO_2 Group only), that could be used in the statistical analysis. As a result of this, the all the serum Troponin-T values at the time point '5 minutes on CPB' had to be used as the baseline measurement. This meant that there was no assessment of changes in serum Troponin-T between the pre-HBO_2 period and the post HBO_2 period or between the pre-CPB period and on-CPB period. In this study, there was also no assessment of ROS generation or, for the presence of its metabolites, as a result of HBO_2, ischaemia and reperfusion. As such, the correlation between ROS

generation and serum Troponin-T could not be determined. Furthermore, the duration of ischaemia, reperfusion and CPB and, the number of ischaemic-reperfusion cycles in both groups were not standardised in this clinical study and this may limit the value of the results from this study.

5. Effects of HBO_2 Preconditioning on Surrogate Biomarkers of Neutrophilic & Endothelial Adhesiveness: Serum Soluble Adhesion Molecules

5.1 Introduction

HBO_2 prior to the insult of IRI (HBO_2 preconditioning), has been shown to limit myocardial infarct size (Kim et al., 2001) and improve myocardial function (Dekleva et al., 2004). It appears that part of the mechanism by which HBO_2 limits IRI is by decreasing neutrophil sequestration (Tjarnstrom et al., 1999), decreasing the polarization expression of the neutrophil adhesion molecule, CD18 (Khiabani et al., 2008), and decreasing the expression of the endothelial cell adhesion molecule, ICAM-1 (Buras et al., 2000) which is a ligand for CD18. A clinical study involving two episodes of 30 minutes of 100% oxygen at 2.4 ATA at 24, 12, and 4 hours prior to on CPB CABG, showed that preconditioning with three sessions of HBO_2 prior to the insult of IRI during on CPB CABG, was capable of significantly attenuating the rise in plasma CD18 and sE-Selectin (Alex et al.,

2005). However, this study also found that HBO_2 preconditioning increased the expression of sICAM-1 and sP-selectin.

5.2 Objective

The objective of this part of this clinical study was to evaluate the effects of systemically preconditioning CAD patients with one session of HBO_2 preconditioning, involving two episodes of 30 minutes of 100% oxygen at 2.4 ATA, which was completed approximately 2 hours prior to on CPB CABG, on the on the expression of surrogate biomarkers (soluble adhesion molecules) of endothelial (sP-Selectin, sE-Selectin and sICAM-1), platelet (sP-Selectin) and neutrophilic (sPSGL-1) adhesiveness as measured by the concentration of these biomarkers at the pre-specified time points *(Table 2.5)*.

5.3 Methods

The time points for the collection of venous blood samples in this study and the method used for the measurement of serum soluble adhesion molecules have been described in *sections 2.6.5* and *2.6.6.2*, respectively. Correction for the effects of hemodilution on the concentration of serum soluble adhesion molecules during CPB, was done as described in *section 2.6.6.3*.

5.4 Results

From the 81 patients recruited to this study who had venous blood samples taken at 6 different time points for ELISA serum soluble adhesion molecule analysis, a total of 486 samples were analysed. Each sample was analysed in duplicates in a 96 well ELISA microplate. In this microplate, 80 wells were used to analyse a total of 40 samples. The 12 remaining wells were used to analyse, in duplicates, the standards for each serum soluble adhesion molecule. The remaining 4 wells on each plate were left as blanks.

As each ELISA microplate was only used to analyse 40 of the total 486 samples, 13 ELISA microplates were used for the analysis of each type of serum soluble adhesion molecules. As there were 4 different types of serum soluble adhesion molecules that were measured, a total of 52 ELISA microplates were used in total.

5.4.1 Serum Adhesion Molecule Results

Figure 5.1 shows an example of a set of spectrophotometric results that were obtained from an ELISA microplate following an ELISA assay procedure for a particular batch of samples for sE-Selectin analysis. As each sample analysed during the sE-Selectin ELISA assay procedure was analysed in duplicates on the ELISA microplate, the spectrophotometric results are also in duplicates. As part of this study, it had been pre-defined that if either of the spectrophotometric duplicates were more or less than 50% of the other's value, that pair of reading would be labelled as unreliable and omitted from the analysis. From *Figure 5.1* for example, the readings in E6 is greater than 50% of F6 and F6 is less than 50% of E6. As such, these readings were labelled as unreliable and were omitted from the analysis. This error may have occurred due to an operator error in pipetting. From all the 52 plates analysed, there were only 10 pairs of results that were unreliable and omitted from subsequent analysis.

The duplicates from each pair of spectrophotometric results were then averaged to determine the average spectrophotometric result for each sample that was analysed in a particular ELISA microplate. *Figure 5.2* shows the averaged spectrophotometric results for the sE-Selectin spectrophotometric results from *Figure 5.1*.

Using the manufacturer provided concentrations for each of the serum soluble adhesion molecule standards and by using the average of the duplicates of these standard's spectrophotometric results, a standard curve and

its equation was determined using Microsoft Excel 2003. *Figure 5.3* shows the standard curve obtained from the sE-Selectin ELISA microplate example that was used earlier to determine the spectrophotometric results.

Figure 5. 1: Spectrophotometric Readings From Serum sE-Selectin ELISA Plate

	A	B	C	D	E	F	G	H	I	J	K	L
1	2.57	2.22	0.56	0.42	0.39	0.35	0.2	0.2	0.45	0.48	0.27	0.26
2	1.77	1.82	0.49	0.43	0.37	0.35	0.54	0.56	0.44	0.42	0.28	0.25
3	1.28	1.19	0.39	0.32	0.44	0.38	0.40	0.42	0.39	0.39	0.30	0.24
4	0.60	0.64	0.33	0.32	0.30	0.28	0.36	0.37	0.36	0.42	0.29	0.28
5	0.18	0.18	0.40	0.46	0.22	0.15	0.29	0.30	0.4	0.43	0.41	0.24
6	0.06	0.06	0.29	0.29	0.65	0.15	0.28	0.27	0.42	0.45	0.31	0.26
7			0.46	0.34	0.29	0.16	0.83	0.45	0.31	0.32	0.25	0.23
8			0.53	0.43	0.27	0.38	0.45	0.49	0.31	0.33	0.24	0.24

Legend 5. 1: Legend Colour for *Figure 5.1*

Spectrophotometric reading for sE-Selectin Standard	**Spectrophotometric reading for sE-Selectin for Patient No. 7**	**Spectrophotometric reading for sE-Selectin for Patient No. 21**	**Spectrophotometric reading for sE-Selectin for Patient No.17**	**Spectrophotometric reading for sE-Selectin for Patient No.16**	**Spectrophotometric reading for sE-Selectin for Patient No. 23**	**Spectrophotometric reading for sE-Selectin for Patient No. 53**	**Spectrophotometric reading for sE-Selectin for Patient No. 56**	**Blank Wells**

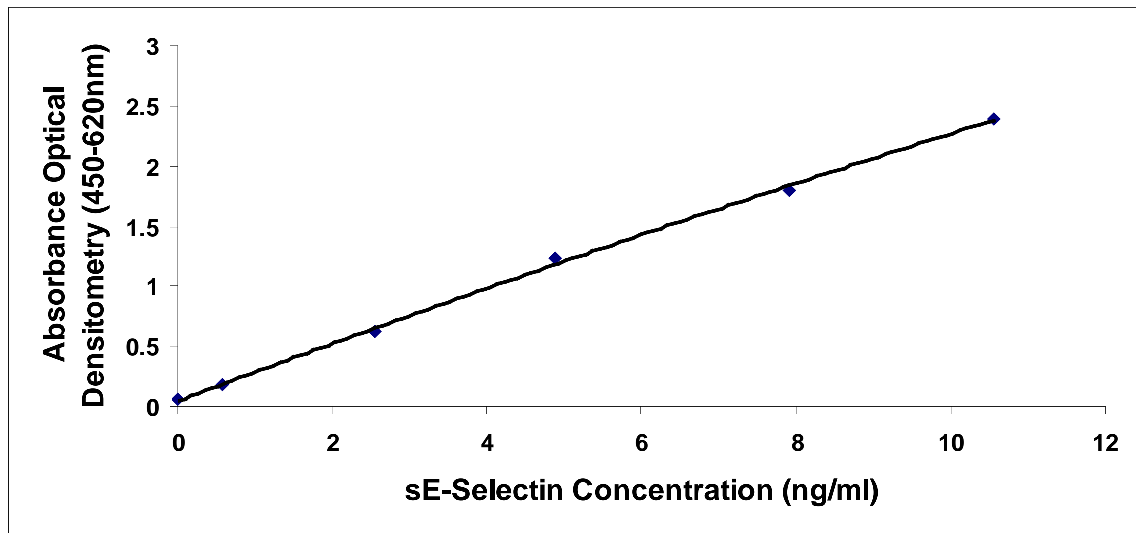
Figure 5. 2: Averages Of Spectrophotometric Reading For Each Patient From Each Time Point

2.40	0.49	0.37	0.2	0.46	0.27
1.80	0.46	0.36	0.55	0.43	0.26
1.23	0.35	0.41	0.41	0.39	0.27
0.62	0.32	0.29	0.37	0.39	0.29
0.18	0.43	0.19	0.29	0.41	0.32
0.06	0.29	Omitted	0.27	0.43	0.28
	0.40	0.22	0.64	0.31	0.24
	0.48	0.32	0.47	0.32	0.24

Legend 5. 2: Legend Colour for *Figure 5.2*

Spectrophoto-metric reading for sE-Selectin Standard	**Spectrophotometric reading for sE-Selectin for Patient No. 7**	**Spectrophotometric reading for sE-Selectin for Patient No. 21**	**Spectrophotometric reading for sE-Selectin for Patient No.17**	**Spectrophotometric reading for sE-Selectin for Patient No.16**	**Spectrophotometric reading for sE-Selectin for Patient No. 23**	**Spectrophotometric reading for sE-Selectin for Patient No. 53**	**Spectrophotometric reading for sE-Selectin for Patient No. 56**	**Blank Wells**

Figure 5. 3: Example of a Standard Curve For Serum sE-Selectin



The equation obtained for this standard curve was:

$$y = -0.002x^2 + 0.242x + 0.0467.$$

The r^2 value for this standard curve was 0.99. Using this standard curve, the concentration of sE-Selectin at each time point was determined using Microsoft Excel 2003.

This method of determining the serum soluble adhesion molecule concentration was also used during the analysis of all the other ELISA microplates in this study. The r^2 values for all the standard curves obtained ranged from 1.0 to 0.97.

In this study, following the ELISA assay procedure and spectrophotometry analysis, where necessary, a correction for sample dilution during the assay procedure was calculated. In addition to this, to correct for the effect of hemodilution during CPB, a further correction for the concentration of serum soluble adhesion molecule during the intra-operative period was calculated as described in *section 2.6.6.3.*

The results will be discussed in the sequence of sPSGL-1, sP-Selectin, sE-Selectin and sICAM-1, as this sequence of serum soluble adhesion molecule discussion enables a more logical discussion of the findings.

5.4.2 Serum sPSGL-1 Results

a. Pre- & Post HBO_2 Mean Concentration of Serum sPSGL-1 in the HBO_2 Group

In this study, approximately 1 hour following HBO_2 preconditioning, there was a statistically significant increase in the mean concentration of serum sPSGL-1 (p=0.03) *(Table 5.1)*.

Table 5. 1: Pre & Post HBO_2 Mean Concentration of Serum sPSGL-1 in the HBO_2 Group

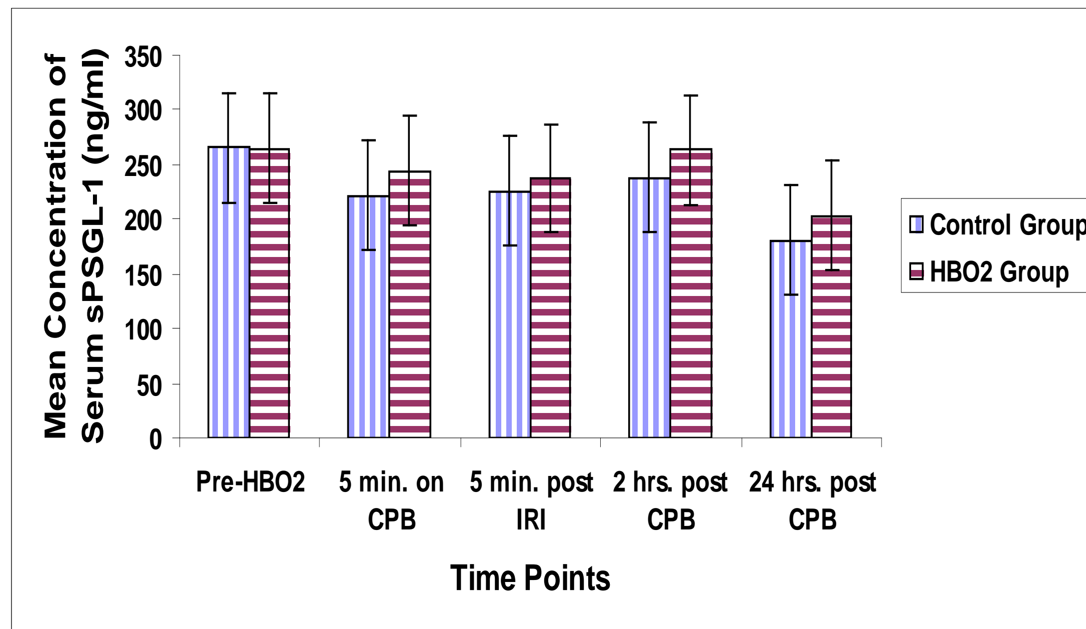
HBO_2 Group (n=41)[†]			
Concentration of Serum sPSGL-1 (ng/ml)			
	Range	**Mean**	**p-value**
Pre-HBO_2	12-1187	265	0.03
1 hour post HBO_2	149-875	278	

[†]This is an intention to treat analysis
Statistical results obtained using Wilcoxon Signed Rank Test

b. Peri Operative Mean Concentration of Serum sPSGL-1 Between The Groups

In this study, after adjusting for longest aortic cross-clamp time and CPB time, none of the changes at any of the time points between the groups were statistically significant. In both groups *(Figure 5.4)*, there was a decrease in the mean concentration of serum sPSGL-1 from the time point 'pre-HBO_2' to the time point '5 minutes on CPB'. At the time point '5 minutes on CPB', the concentration of of sPSGL-1 was higher in the HBO_2 Group. During the period of IRI, (i.e. from the time point '5 minutes on CPB' to the time point '5 minutes post IRI'), there was a small decrease in the concentration of sPSGL-1 in the HBO_2 Group while in the Control Group there was a small increase. Despite this decrease, at the time point '5 minutes post IRI', the concentration of serum sPSGL-1 was higher in the HBO_2 Group than in the Control Group. From the time point '5 minutes post IRI' to the time point '2 hours post CPB', the concentration of sPSGL-1 increased in both groups. At the time point '2 hours post CPB', the concentration of serum sPSGL-1 continued to remain higher in the HBO_2 Group. From the time point '2 hours post CPB' up to the time point '24 hours post CPB', in both groups, the concentration of sPSGL-1 decreased but remained higher in the HBO_2 Group. A summary of the results are provided in *Table 5.2*. In this table, the data are displayed as both mean and median values. As the means and medians are not the same at each time point, this indicates that the data were skewed. To normalise the data and enable parametric statistical analysis, the mean values were transformed into natural logarithmic (ln) values.

Figure 5. 4: Bar Chart of Peri-Operative Mean Concentration of Serum sPSGL-1



HBO_2 Group, n=41; Control Group, n=40
Bars chart showing mean values and error bars for the standard error of the mean

Table 5. 2: Peri-Operative Concentration of Serum sPSGL-1

Peri-Operative Concentration of Serum sPSGL-1 (ng/ml)									
	Control Group (n=40)[†]				**HBO_2 Group** (n=41)[†]				
	Range	**Median**	**Mean**	**Ln Mean**	**Range**	**Median**	**Mean**	**Ln Mean**	**p-value***
Pre-HBO_2 *(baseline)*	24-611	211	265	5.6	12-1187	245	265	5.8	-
5 minutes on CPB	62-930	199	221	5.4	93-703	225	244	5.5	0.09
5 minutes post IRI	70-847	220	226	5.4	108-804	229	237	5.5	0.16
2 hours post CPB	78-485	214	238	5.5	94-1212	242	263	5.6	0.13
24 hours post CPB	78-485	180	180	5.2	80-605	190	204	5.3	0.13

[†]*This is an intention to treat analysis;*
Statistical analysis using repeated measures of ANOVA;
**p-values are for Geometric Means*

5.4.3 Serum sP-Selectin Results

a. Pre- & Post HBO_2 Mean Concentration of Serum sP-Selectin in the HBO_2 Group

In this study, approximately 1 hour following HBO_2 preconditioning, there was a very small, statistically non-significant, increase in the mean concentration of serum sP-Selectin *(Table 5.3)*.

Table 5. 3: Pre & Post HBO_2 Mean Concentration of Serum sP-Selectin in the HBO_2 Group

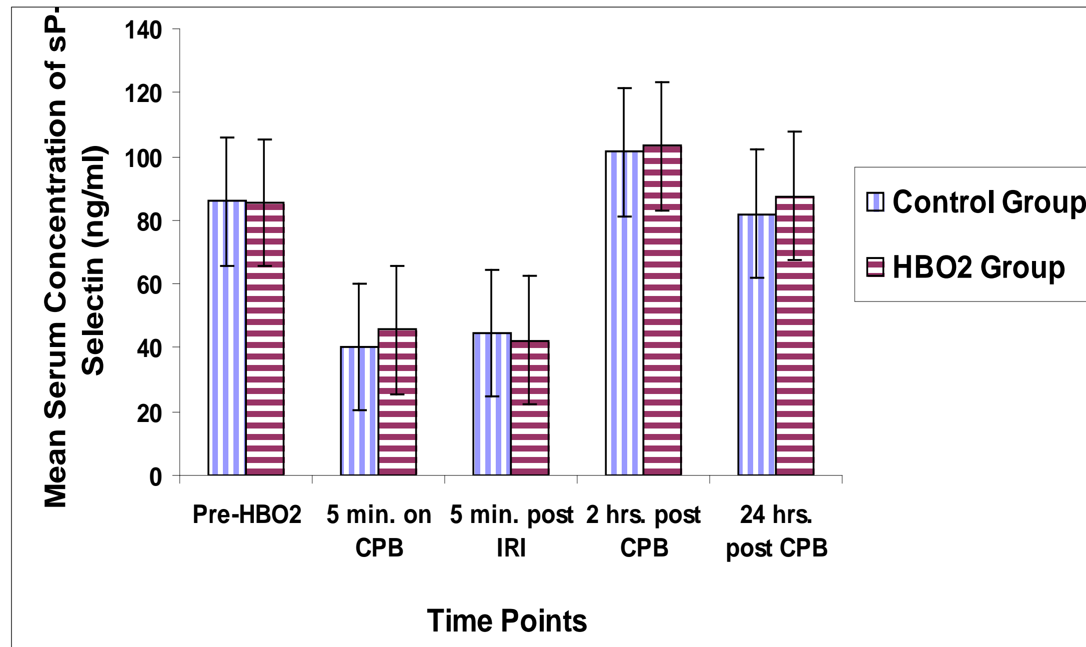
HBO_2 Group (n=41) †			
Concentration of Serum sP-Selectin (ng/ml)			
	Range	**Mean**	**p-value**
Pre-HBO_2	30-238	86	
1 hour post HBO_2	33-233	90	0.9

†This is an intention to treat analysis;
Statistical analysis using Wilcoxon Signed Rank Test

b. Peri Operative Mean Concentration of Serum sP-Selectin Between The Groups

After adjusting for longest aortic cross-clamp time and CPB time, none of the changes at any of the time points between the groups were statistically significant. In both groups *(Figure 5.5)*, between the time point 'pre-HBO2' and '5 minutes on CPB' the mean concentration of serum sP-Selectin decreased. At the time point '5 minutes on CPB', the mean concentration of serum sP-Selectin was higher in the HBO_2 Group. During the period of IRI (between time points '5 minutes on CPB' and '5 minutes post IRI'), there was a further small decrease in the mean concentration of serum sP-Selectin in the HBO_2 Group while in the Control Group, there was an increase. At the time point '5 minutes post IRI', the mean concentration of serum sP-Selectin was lower in the HBO_2 Group. In both groups, from the time point '5 minutes post IRI' to the time point '2 hours post CPB', the mean concentration of serum sP-Selectin increased. At time point '2 hours post CPB' the mean concentration of sP-Selectin was slightly higher in the HBO_2 Group. Following this, from the time point '2 hours post CPB' to '24 hours post CPB' the mean concentration of serum sP-Selectin decreased in both groups but remained higher in the HBO_2 Group. A summary of the results are provided in *Table 5.4.* In this table, the data are displayed as both mean and median values. As the means and medians are not the same at each time point, this indicates that the data were skewed. To normalise the data and enable parametric statistical analysis, the mean values were transformed into natural logarithmic (ln) values.

Figure 5. 5: Bar Chart of Peri-Operative Mean Concentration of Serum sP-Selectin



HBO_2 Group, n=41; Control Group, n=40
Bars chart showing mean values and error bars for the standard error of the mean

Table 5. 4: Peri-Operative Concentration of Serum sP-Selectin

Peri-Operative Concentration of Serum sP-Selectin (μg/ml)									
	Control Group (n=40)[†]				**HBO_2 Group** (n=41)[†]				
	Range	**Median**	**Mean**	**Ln Mean**	**Range**	**Median**	**Mean**	**Ln Mean**	**p-value***
Pre-HBO_2 *(baseline)*	30-238	91	86	4.0	50-232	89	86	4.4	-
5 minutes on CPB	1-114	39	40	3.5	11-178	39	46	3.8	0.36
5 minutes post IRI	7-139	43	45	3.6	14-175	38	42	3.7	0.61
2 hours post CPB	39-258	94	101	4.8	19-352	102	103	4.6	0.22
24 hours post CPB	22-296	72	82	4.1	52-502	74	88	4.5	0.82

[†]*This is an intention to treat analysis;*
Statistical analysis using repeated measures of ANOVA;
**p-values are for Geometric Means*

5.4.4 Serum sE-Selectin Results

a. Pre- & Post HBO_2 Mean Concentration of Serum sE-Selectin in the HBO_2 Group

In this study, approximately 1 hour following HBO_2 preconditioning, there was a no change in the mean concentration of serum sE-Selectin *(Table 5.5)*.

Table 5. 5: Pre & Post HBO_2 Mean Concentration of Serum sE-Selectin in the HBO_2 Group

HBO_2 Group (n=41)[†]			
Concentration of Serum sE-Selectin (ng/ml)			
	Range	**Mean**	**p-value**
Pre-HBO_2	3-54	16	0.3
1 hour post HBO_2	3-53	16	

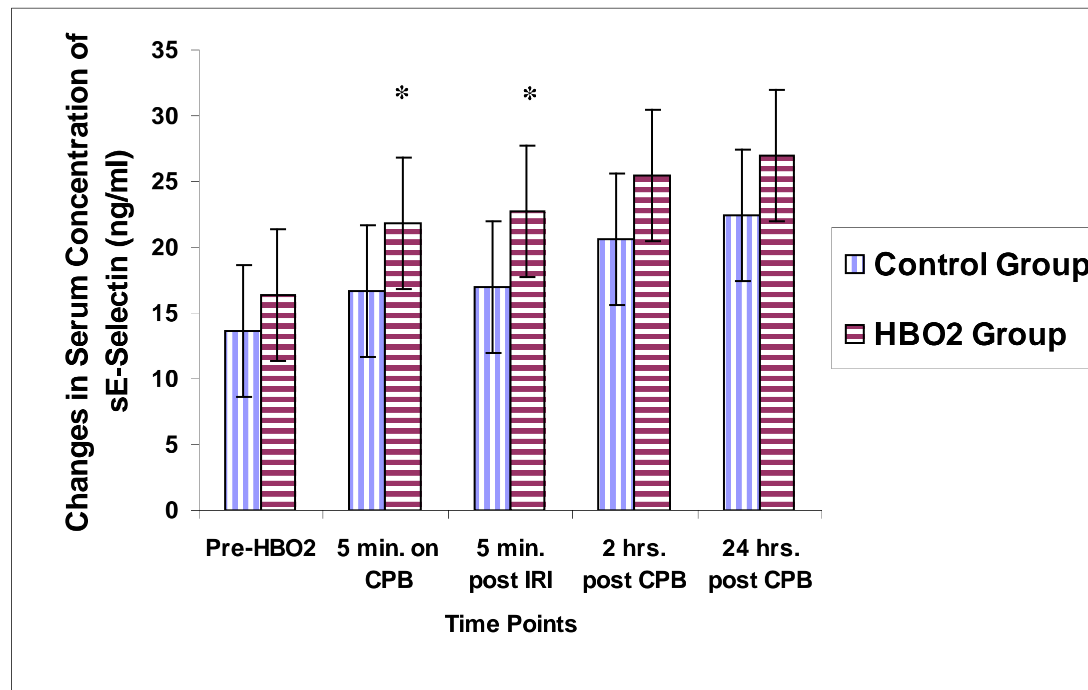
[†]*This is an intention to treat analysis;*
Statistical analysis using Wilcoxon Signed Rank Test

b. Peri Operative Mean Concentration of Serum sE-Selectin Between The Groups

At all time points, *Figure 5.6*, the mean concentration of serum sE-Selectin was higher in the HBO_2 Group compared to in the Control Group. From the time points 'pre-HBO_2' to '5 minutes on CPB', the mean concentration of serum sE-Selectin increased in both groups. At the time point '5 minutes on CPB', the mean concentration of serum sE-Selectin was higher in the HBO_2 Group. After adjusting for longest aortic cross-clamp time and CPB time, the mean concentration of serum sE-Selectin at the time point '5 minutes on CPB' was found to be significantly higher in the HBO_2 Group than in the Control Group (Geometric Mean Estimate & 95% Confidence Interval; 1.21 & 1.03, 1.42; p=0.02). During the period of IRI (between the time points '5 minutes on CPB' and '5 minutes post IRI'), there was a very small increase in the mean concentration of serum sE-Selectin in HBO_2 Group and no change in the Control Group. At the time point '5 minutes post IRI', the mean concentration of serum sE-Selectin was higher in the HBO_2 Group. After adjusting for longest aortic cross-clamp time and CPB time, the mean concentration of serum sE-Selectin at the time point '5 minutes post IRI' was found to be significantly higher in the HBO_2 Group than in the Control Group (Geometric Mean Estimate & 95% Confidence Interval; 1.26 & 1.04, 1.52; p=0.02). Between the time points '5 minutes post IRI' and '24 hours post CPB', the mean concentration of serum sE-Selectin increased in both groups and continued to remain higher in the HBO_2 Group. None of the changes between the the time points '5 minutes post IRI' and '24 hours post CPB' were

statistically significant. A summary of the results are provided in *Table 5.6*. In this table, the data are displayed as both mean and median values. As the means and medians are not the same at each time point, this indicates that the data were skewed. To normalise the data and enable parametric statistical analysis, the mean values were transformed into natural logarithmic (ln) values.

Figure 5. 6: Bar Chart of Peri-Operative Mean Concentration of Serum sE-Selectin



HBO_2 Group, n=41; Control Group, n=40;
Bars chart showing mean values and error bar for the standard error of the mean;
* *$p<0.05$ following repeated measures of ANOVA analysis*

Table 5. 6: Peri-Operative Concentration of Serum sE-Selectin

Peri-Operative Concentration of Serum sE-Selectin (ng/ml)									
	Control Group (n=40)[†]				**HBO_2 Group** (n=41)[†]				
	Range	**Median**	**Mean**	**Ln Mean**	**Range**	**Median**	**Mean**	**Ln Mean**	**p-value* (Geometric Mean Estimate & 95% Confidence Interval)**
Pre-HBO_2 *(baseline)*	3-39	11	14	2.6	4-53	13	16	2.8	-
5 minutes on CPB	4-48	13	17	2.8	5-66	19	22	3.1	0.02 1.21 & 1.03, 1.42
5 minutes post IRI	2-46	14	17	2.8	4-66	22	23	3.1	0.02 (1.26 & 1.04, 1.52)
2 hours post CPB	0.5-54	18	21	3.0	5-79	20	25	3.2	0.42
24 hours post CPB	6-75	19	23	3.1	4-138	22	27	3.3	0.42

[†]*This is an intention to treat analysis*
Statistical analysis using repeated measures of ANOVA
**p-values are for the Geometric Means*

5.4.5 Serum sICAM-1 Results

a. Pre- & Post HBO_2 Mean Concentration of Serum sICAM-1 in the HBO_2 Group

In this study, approximately 1 hour following HBO_2 preconditioning, there was a small, statistically non-significant increase in the mean concentration of serum sICAM-1 concentration *(Table 5.7)*.

Table 5. 7: Pre & Post HBO_2 Mean Serum sICAM-1 Concentrations in the HBO_2 Group

HBO_2 Group (n=41)[†]			
Concentration of Serum sICAM-1 (ng/ml)			
	Range	**Mean**	**p-value**
Pre-HBO_2	33-451	258	0.3
1 hour post HBO_2	35-464	261	

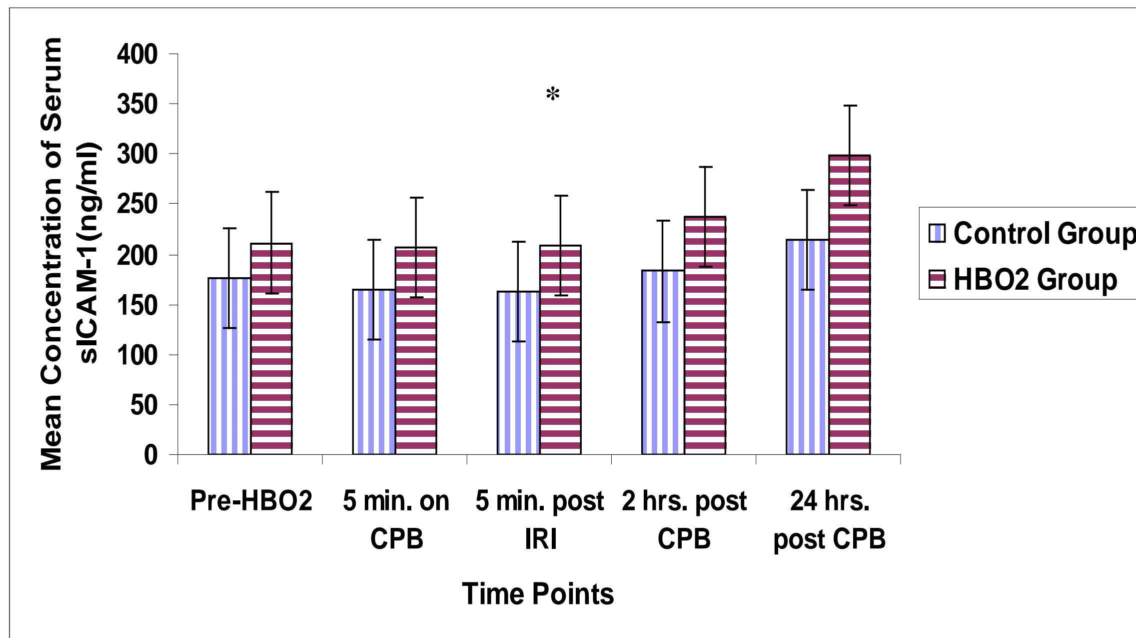
[†]*This is an intention to treat analysis*
Statistical analysis using Wilcoxon Signed Rank Test

b. Peri Operative Mean Concentration of Serum sICAM-1 Between The Groups

In this study, at all time points, the mean concentration of serum sICAM-1 was higher in the HBO_2 Group than in the Control Group *(Figure 5.7)*. In both groups, between the time points 'pre-HBO_2' and '5 minutes on CPB', there was a small decrease in the mean concentration of serum of sICAM-1. At the time point '5 minutes on CPB', the mean concentration of serum sICAM-1 was higher in the HBO_2 Group but this was not a statistically significant result. During the period of IRI, (i.e. from the time points '5 minutes on CPB' to 5 minutes post IRI), in the HBO_2 Group there was a small increase in the mean concentration of serum sICAM-1 while in the Control Group there was a small decrease. At the time point '5 minutes post IRI', the mean concentration of serum sICAM-1 was higher in the HBO_2 Group. At the time point '5 minutes post IRI', after correction for longest aortic cross-clamp time and CPB time, the HBO_2 Group had a significantly higher mean concentration of serum sICAM-1 compared to the Control Group (Geometric Mean Estimate & 95% Confidence Interval; 1.17 & 1.02, 1.34; p=0.03). From the time point '5 minutes post IRI' up to the time point '24 hours post CPB', the mean concentration of serum of sICAM-1 increased in both groups and remained higher in the HBO_2 Group. None of the changes between the time point '5 minutes post IRI' and the time point '24 hours post CPB' were statistically significant. A summary of the results are provided in *Table 5.8*. In this table, the data are displayed as both mean and median values. As the means and medians are not the same at each time point, this indicates that the

data were skewed. To normalise the data and enable parametric statistical analysis, the mean values were transformed into natural logarithmic (ln) values.

Figure 5. 7: Bar Chart of Peri-Operative Mean Concentration of Serum sICAM-1



HBO_2 Group, n=41; Control Group, n=40
Bars chart showing mean values and error bars for the standard error of the mean;
** p<0.05 following repeated measures of ANOVA analysis*

Table 5. 8: Peri-Operative Concentration of Serum sICAM-1

Peri-Operative Concentration of Serum sICAM-1 (ng/ml)									
	Control Group (n=40)†				**HBO2 Group** (n=41)†				
	Range	**Median**	**Mean**	**Ln Mean**	**Range**	**Median**	**Mean**	**Ln Mean**	**p-value* (Geometric Mean Estimate & 95% Confidence Interval)**
Pre-HBO_2 *(baseline)*	33-326	212	175	5.2	34-451	258	211	5.6	-
5 minutes on CPB	39-316	196	165	5.1	105-509	223	207	5.5	0.07
5 minutes post IRI	37-321	191	162	5.1	135-478	254	209	5.5	0.03 (1.17 & 1.02, 1.34)
2 hours post CPB	28-399	221	183	5.2	95-727	281	237	5.7	0.06
24 hours post CPB	40-442	246	214	5.3	151-1798	308	299	5.9	0.06

†This is an intention to treat analysis
Statistical analysis using repeated measures of ANOVA
**p-values are for Geometric Means*

5.5 Discussion

5.5.1 sPSGL-1

In the HBO_2 Group, 1 hour following HBO_2 preconditioning *(Table 5.1)*, there was a statistically significant increase in the mean concentration of serum of sPSGL-1 (p=0.03). A possible explanation for this may have been the oxidative stress that is known to be generated by treatment with HBO_2 (Benedetti et al., 2004, Conconi et al., 2003, Gregorevic et al., 2001, Thom, 2009). At present there are no other clinical studies involving HBO_2, CABG and the oxidative stress of ischaemia and reperfusion, that have investigated the kinetics of sPSGL-1 in this group of patients. However, in *in-vivo* (Dulkanchainun et al., 1998, Hayward et al., 1999) and *ex-vivo* (Dulkanchainun et al., 1998) models of IRI, the presence of sPSGL-1 appears to be associated with reduced myocardial (Hayward et al., 1999) and liver (Dulkanchainun et al., 1998) reperfusion injury and, the preservation of endothelial function (Hayward et al., 1999). These cellular protective effects were largely the result of reduced neutrophil infiltration (Dulkanchainun et al., 1998) and reduced neutrophil-endothelial cell interaction (Hayward et al., 1999). The latter appears to be the result of the ability for sPSGL-1 to inhibit the adhesion between neutrophil bound PSGL-1 and its endothelial bound ligand, P-Selectin by binding to either of these intact adhesion molecules (Hayward et al., 1999). This suggests that in this study, preconditioning CAD patients with HBO_2 prior to CABG and IRI, may have

had the potential to reduce neutrophil mediated ischaemia reperfusion endothelial injury as the circulation of this group of patients had an increased load of sPSGL-1 that maybe capable of inhibiting endothelial P-Selectin adhesion to neutrophil PSGL-1.

5.5.2 sE-Selectin

During the period of anaesthetic pre-medication, induction and, maintenance, prior to the onset of CPB (i.e. between the time point 'pre-HBO_2' and '5 minutes on CPB') *(Figure 5.6)*, there was an increase in the mean concentration of serum sE-Selectin in both groups with the concentration being higher in the HBO_2 Group at the time point '5 minutes on CPB'. At this time point '5 minutes on CPB', the concentration in the HBO_2 Groups was significantly greater than in the Control Group ($p=0.02$). A reason for this may be that by the time point '5 minutes on CPB', approximately 4 hours would have passed since the end of the HBO_2 treatment. This may have provided sufficient time for the oxidative stress of HBO_2 to not only stimulate the transcription of E-Selectin but also for its eventual expression on the vascular endothelium. Increased levels of ROS, as generated during HBO_2, have not only been shown to stimulate E-Selectin expression (Rupin et al., 2004, Russell et al., 2000) but also possibly cleave them off the surfaces of endothelial cells to produce sE-Selectin (Eguchi et al., 2005).

During the period of ischaemia and reperfusion (i.e. between the time point '5 minutes on CPB' and '5 minutes post IRI') *(Figure 5.6)*, in the HBO_2 Group there was a small increase in the serum concentration of sE-

Selectin while in the Control Group, there was no change. At the time point '5 minutes post IRI', the serum concentration was higher in the HBO_2 Group. At this time point, the HBO_2 Group had a significantly higher mean serum concentration of sE-Selectin (p=0.02). As ischaemia and reperfusion are generally known to cause an increase in serum sE-Selectin (Kalawski et al., 1998, Matata and Galinanes, 2000), one possible reason for only a small increase in the circulating load of sE-Selectin during the period of IRI in the HBO_2 Group could perhaps be because prior to the period of IRI, there was also a high circulating load of sPSGL-1. It has been reported (Takada et al., 1997) that sPSGL-1 is capable of limiting the transcription of E-Selectin by limiting cytokines, such as TNF-α, that are necessary for the induction of E-Selectin expression on the vascular endothelium. This may limit the amount of available endothelial bound E-Selectin that maybe shed into the circulation as a result of ischaemia and reperfusion. While it has been demonstrated that sPSGL-1 is capable of inhibiting the adhesion between neutrophil bound PSGL-1 and its endothelial bound ligand, P-Selectin (Hayward et al., 1999), it has also been suggested that the interaction between PSGL-1 and P-Selectin is required for sE-Selectin expression (Bodary et al., 2007). As in this study, in the HBO_2 Group of patients, there was a high circulating sPSGL-1 prior to IRI, it would be reasonable to suggest that this too may have indirectly led to an attenuation of sE-Selectin expression in this group of patients during this period.

5.5.3 sICAM-1

In this study, during the period of ischaemia and reperfusion (i.e. between the time point '5 minutes on CPB' and '5 minutes post IRI'), the mean concentration of serum sICAM-1 increased a little in the HBO_2 Group and decreased a little in the Control Group. At the time point '5 minutes post IRI', the concentration was significantly higher in the HBO_2 Group ($p=0.03$) compared to in the Control Group *(Figure 5.7)*. It is possible that the adjunctive oxidative effects of ischaemia and reperfusion in combination with the earlier oxidative stress of HBO_2, was the cause for this small increase in sICAM-1 in the HBO_2 Group. In an experimental model, where whole blood was exposed to the oxidative effects of lipopolysaccharise (LPS) followed by the oxidative stress of HBO_2 i.e. 2 consecutive adjunctive oxidative events, a significant increase in sICAM-1 was also observed (Fildissis et al., 2004). The decrease in sICAM-1 in the Control Group is in keeping with the observations of another clinical study (Hambsch et al., 2002).

5.6 Conclusion

In this clinical study, estimating the effects of HBO_2 preconditioning on the serum biomarkers were only the secondary endpoints of the study. As such, despite a mix of statistically significant and non-significant findings, and as pre the statistical plan for the analysis of the secondary endpoint of this study, no definitive conclusions can be made with respect to the changes that were observed for these endpoints. However, based on the findings of this study, it is possible to suggest that systemically preconditioning CAD patients with HBO_2 prior to CABG, has the potential to lead to an overall increase in the serum concentration of all the serum soluble adhesion molecules. This may be of potential benefit as it indirectly suggest vascular, platelet and neutrophilic adhesion molecules are being cleaved off their respective surfaces and bering shed into the circulation, thus limiting the degree of neutrophil and endothelial interaction and, hence the ensuing ischaemia reperfusion mediated injury.

The main limitations to this part of the study, was that there was no assessment of the generation of ROS or its metabolites as a result of HBO_2, ischaemia and reperfusion and, as such an assessment of the correlation with soluble adhesion molecule expression was not possible. Additionally, this study did not assess the expression of adhesion molecules on the surfaces of the endothelium and neutrophils. As such, it was again not possible to assess the correlation between the ROS generation as a result of HBO_2, ischaemia and reperfusion with the expression of endothelial and neutrophilic adhesion

molecules. Furthermore, the actual correlation between endothelial and neutrophilc adhesion molecule expression and the expression of the soluble form of these adhesion molecules in the serum was not possible. Finally, as the durations of ischaemia, reperfusion and CPB and, the number of ischaemic reperfusion cycles in this study were not standardised, there is always the possibility that a different set of results may have been obtained if it was possible to standardise these intervals in a clinical study.

6. Effects of HBO_2 Preconditioning on Biomarkers of Cardioprotection: Myocardial eNOS & Hsp72

6.1 Introduction

6.1.1 HBO_2 & eNOS

HBO_2 has been shown to increase the expression of eNOS (Atochin et al., 2003, Buras et al., 2000, Cabigas et al., 2006b, Xu et al., 2009) and NO (Boykin and Baylis, 2007, Demchenko et al., 2000, Gurdol et al., 2009, Thom et al., 2006, Thom et al., 2003, Wang et al., 2009a). Studies have also shown that inhibition of eNOS promotes the adherence of neutrophils to the microvasculature while increased concentrations of eNOS have an anti-adherent effect (Kubes et al., 1991, Lefer and Lefer, 1996). In an experimental model of endothelial ischaemia and reperfusion, the downregulation of ICAM-1, as a result of treatment with HBO_2, correlated with an increase in eNOS concentration (Buras et al., 2000). As this downregulation of ICAM-1 prevents neutrophils from adhering to endothelial cells, this suggests that the mechanism for HBO_2 mediated downregulation of ICAM-1, and the attenuation of IRI, may be modulated by eNOS and NO.

Recently, in a rat heart model of IRI, it was observed that preconditioning with HBO_2 not only resulted in a greater decrease in myocardial infarct size and increased the recovery of left ventricular diastolic pressure but also led to an increase in myocardial eNOS and nitrite plus nitrate content (Cabigas et al., 2006b). This study suggested that preconditioning with HBO_2 prior to ischaemia and reperfusion induced endogenous myocardial protective effects which involved the increased expression of eNOS and NO.

6.1.2 HBO_2 & Hsp72

Only a limited amount of knowledge is known about the effects of HBO_2 on Hsp72 and, even less is known about its effects on myocardial Hsp72. There are however, studies examining the effects of HBO_2 in non-cardiac models.

In an experimental model of ischaemic tolerance, HBO_2 consisting of 60 minutes of 100% oxygen at 2.0 ATA, was administered to gerbils either for a single session or every other day for 5 sessions (Wada et al., 1996). 2 days after HBO_2 pre-treatment, the gerbils were subjected to 5 minutes of forebrain ischaemia by occlusion of both common carotid arteries under anesthesia. Immunohistochemical staining for Hsp72 in the gerbil hippocampus, showed that 5 sessions of HBO_2 prior to ischaemia and reperfusion increased the amount of Hsp72 compared to that in the ischaemic control group and in the single HBO_2 pre-treatment group. They concluded that tolerance against ischaemic neuronal damaged can be induced by repeated

treatment with HBO_2 prior to ischaemia and this tolerance occurrs through the induction of Hsp72 synthesis. Another group later determined, in a rat model of cerebral ischaemia, that when HBO_2 treatment was administered after ischaemia, in comparison to the control group, there was a weaker induction of Hsp72 (Konda et al., 1996). These observations suggest that HBO_2 treatment prior to an ischaemic event induces better cellular protection compared to treatment after an ischaemic event. However, the importance of this observation is a little difficult to confirm as while pre-treatment with HBO_2 before cerebral ischaemia has been shown to increase Hsp72 (Wada et al., 1996), this finding was not observed in a rat liver model of IRI that was pre-treated with HBO_2 (Yu et al., 2005). In fact the experimental findings by Yu *et.al* were similar to the findings of a recent clinical study involving HBO_2 pre-treatment and patients undergoing CABG (Alex et al., 2005). In that clinical study, in CAD patients who were treated with 3 sessions of HBO_2, consisting of 100% oxygen at 2.4 ATA for 90 minutes, prior to on CPB CABG, there were lower levels of inducible Hsp70 (Hsp72) compared to the control group.

6.2 Objective

The objective of this part of this clinical study was to evaluate the effects of systemically preconditioning CAD patients with one session of HBO_2 preconditioning, involving two episodes of 30 minutes of 100% oxygen at 2.4 ATA, which was completed approximately 2 hours prior to on CPB CABG, on the expression of two myocardial biomarkers of cardioprotection, eNOS and Hsp72, as measured by the quantity of these biomarkers in specimens from right atrial biopsies taken at the pre-specified time points *(Table 2.7).*

6.3 Methods

The intra-operative time points at which right atrial biopsies were taken in this study and the methods used for the measurement of myocardial eNOS and Hsp72 have been described in *sections 2.6.7*.

6.4 Results

From the 81 patients randomised to this study who had intra-operative right atrial biopsies taken at 4 different time points for myocardial ELISA eNOS and Hsp72 analysis, there were a total of 324 right atrial tissue specimens that were analysed. As the myocardial lysate samples from each specimen was analysed in duplicates during the ELISA analysis, in each 96 well ELISA microplate, 80 wells were used to analyse the lysate from 40 different right atrial specimens. 14 wells from each microplate were used to

analyse the standards for either eNOS or Hsp72 in duplicates. The remaining 2 wells on each plate were left as blanks.

As each ELISA microplate was only used to analyse the lysate from 40 of the total of 324 right atrial specimens, 8 ELISA microplates each were used for the analysis of myocardial eNOS and Hsp72, respectively. As such, a total of 16 ELISA microplates were used in the ELISA analysis of all the myocardial biomarkers.

6.4.1 Myocardial Biomarker ELISA Results

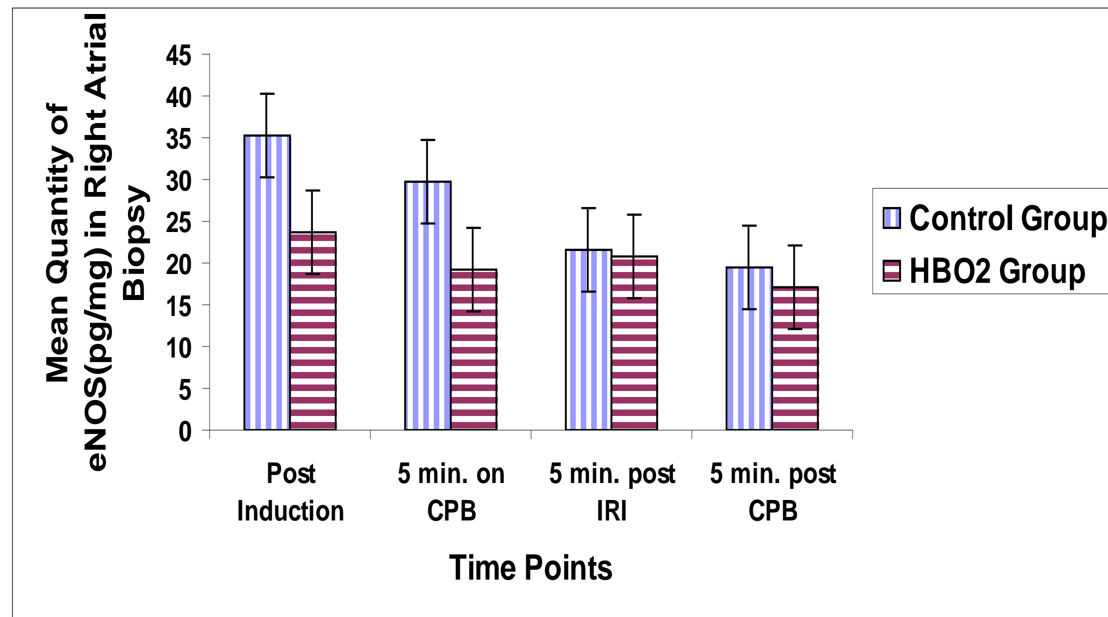
Spectrophotometric analysis for each of the myocardial biomarkers was done in a similar way as described for sE-Selectin in *section 5.4.1*. From the total of 16 ELISA microplates used for myocardial biomarker analysis, there were 3 pairs of unreliable spectrophotometric readings that were omitted from subsequent analysis.

Using the manufacturer provided concentrations for each of the myocardial biomarker standards and by using the average of the duplicates of these standard's spectrophotometric results, a standard curve and its equation was determined using Microsoft Excel 2003. R^2 values for all the standard curves for the myocardial biomarkers ranged from 1 to 0.98. Using the standard curve equation that was obtained, the concentration of the myocardial biomarker at each time point was determined. The quantity of each biomarker, in each milligram of the right atrial specimen that was analysed, from each time point, was then determined using the formula described in *sections 2.6.7.3 and 2.6.7.4*, respectively.

6.4.2 Myocardial eNOS ELISA Results

In this study, there were no statistically significant changes in the mean quantity of myocardial eNOS at any of the time points between the groups. At all time points, the mean quantity of myocardial eNOS was higher in the Control Group *(Figure 6.1)*. Between the time points ‘post induction’ and ‘5 minutes on CPB’, the mean quantity of myocardial of eNOS decreased in both groups. Between the time points ‘5 minutes on CPB’ and ‘5 minutes post IRI’, the mean quantity of myocardial eNOS increased slightly in the HBO_2 Group but decreased in the Control Group. Finally, between the time points ‘5 minutes post IRI’ and ‘5 minutes post CPB’, the mean quantity of myocardial eNOS decreased in both groups and remained lower in the HBO_2 Group. A summary of the results are provided in *Table 6.1.* In this table, the data are displayed as both mean and median values. As the means and medians are not the same at each time point, this indicates that the data were skewed. To normalise the data and enable parametric statistical analysis, the mean values were transformed into natural logarithmic (ln) values.

Figure 6. 1: Bar Chart of Intra-Operative Mean Quantity of Myocardial eNOS



HBO_2 Group, n=41; Control Group, n=40
Bars chart showing mean values and error bars for the standard error of the mean

Table 6. 1: Intra-Operative Quantity of Myocardial eNOS Atrial Biopsy

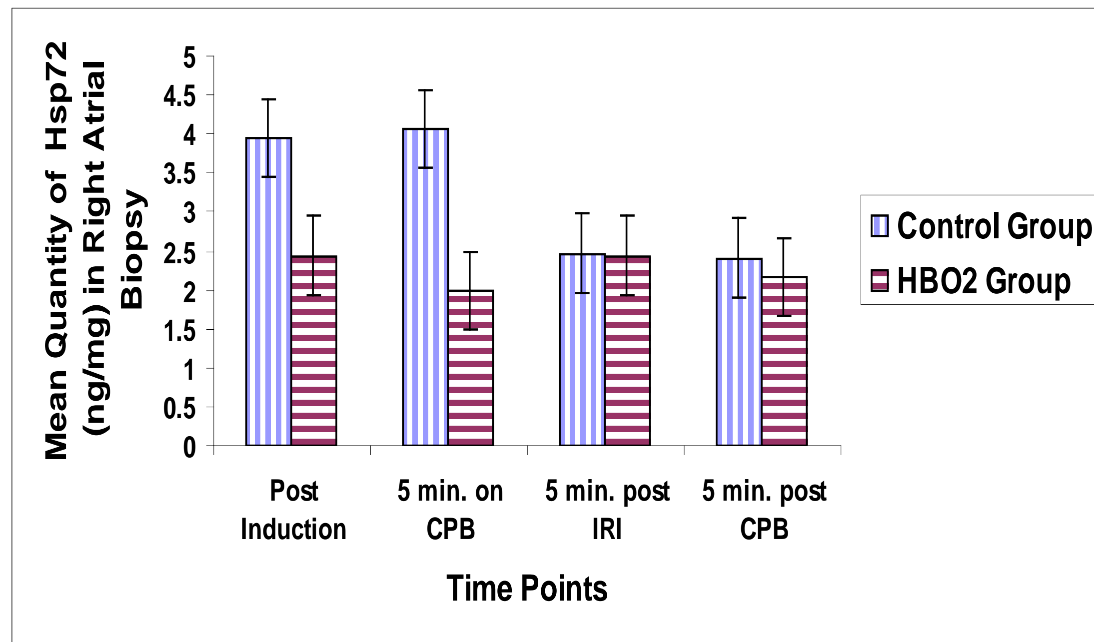
	Quantity of eNOS in the Right Atrial Biopsy (pg/mg)								
	Control Group (n=40)[†]				**HBO_2 Group** (n=41)[†]				
	Range	**Median**	**Mean**	**Mean ln**	**Range**	**Median**	**Mean**	**Mean ln**	**p-value***
Post induction *(baseline)*	5-416	17.2	35.3	3.7	3-146	18.9	23.7	3.4	0.9
5 minutes on CPB	4-257	15.6	29.7	3.5	5-89	12.6	19.3	3.1	0.8
5 minutes post IRI	4-272	14.5	21.6	3.2	2-73	15.7	20.7	3.2	1.0
5 minutes post CPB	1-255	11.8	19.4	3.1	3-93	13.6	17.1	3.0	1.0

[†]*This is an intention to treat analysis;*
Statistical analysis using repeated measures of ANOVA;
**p-values are for Geometric Means*

6.4.3 Myocardial Hsp72 ELISA Results

In this study, there were no statistically significant changes in the mean quantity of myocardial Hsp72 at any of the time points between the groups. At all time points, the mean quantity of myocardial Hsp72 was lower in the HBO_2 Group *(Figure 6.2)*. Between the time points 'post induction' and '5 minutes on CPB', there was a small decrease in the mean quantity of myocardial Hsp72 in the HBO_2 Group while in the Control Group there was a very small increase. Between the time points '5 minutes on CPB' and '5 minutes post IRI', there was a small increase in the mean quantity of myocardial Hsp72 in the HBO_2 Group while in the Control Group there was a decrease. Finally, between the time points '5 minutes post IRI' and '5 minutes post CPB', the mean quantity of myocardial Hsp72 decreased in both groups and remained lower in the HBO_2 Group. A summary of the results are provided in *Table 6.2*. In this table, the data are displayed as both mean and median values. As the means and medians are not the same at each time point, this indicates that the data were skewed. To normalise the data and enable parametric statistical analysis, the mean values were transformed into natural logarithmic (ln) values.

Figure 6. 2: Bar Chart of Intra-Operative Mean Quantity of Myocardial Hsp72



HBO_2 Group, n=41; Control Group, n=40
Bars chart showing mean values and error bars for the standard error of the mean

Table 6. 2: Intra-Operative Quantity of Myocardial Hsp72

	Quantity of Hsp72 in the Right Atrial Biopsy (ng/mg)								
	Control Group (n=40)[†]				**HBO_2 Group** (n=41)[†]				
	Range	**Median**	**Mean**	**Mean ln**	**Range**	**Median**	**Mean**	**Mean ln**	**p-value***
Post induction *(baseline)*	0.2-67.0	1.4	4.0	1.4	0.6-16.5	1.8	2.4	0.9	0.09
5 minutes on CPB	0.5-58.8	1.9	4.1	1.4	0.6-8.2	1.5	2.0	0.7	0.09
5 minutes post IRI	0.4-33.4	1.4	2.5	0.9	0.8-18.6	1.8	2.4	0.9	1.0
5 minutes post CPB	0.7-30.1	1.7	2.4	0.9	0.5-9.8	1.8	2.2	0.8	1.0

[†]*This is an intention to treat analysis;*
Stattistical analysis using repeated measures of ANOVA;
**p-values are for Geometric Means*

6.5 Discussion

6.5.1 Myocardial eNOS

In this study it was observed that following HBO_2 preconditioning, in the baseline right atrial biopsy taken at the time point 'post induction', the myocardium of patients in the HBO_2 Group had a lower mean quantity of myocardial eNOS compared to the Control Group *(Figure 6.1)*. While this was not a statistically significant finding, this finding suggests that preconditioning CAD patients with HBO_2 prior to CABG leads to a reduction in myocardial eNOS. This is the first time such a result has been observed in a clinical study. While there are no clinical studies to compare with, the findings of this study are contradictory to those of an experimental model of HBO_2 (Liu et al., 2008). In that mouse brain model, it was observed that exposure to HBO_2, consisting of twice daily 100% oxygen for 60 minutes at 2.4 ATA for 3 consecutive days, led to an increase in brain eNOS. However, in this experimental model, as the tissue being examined and, the dose and intervals for treatment with HBO_2 were different, this may provide some explanation for the differences seen between that experimental study and this clinical study. A reasonable possibility for the generally lower eNOS expression in the HBO_2 Group at the 'post induction' time point may be the negative feedback mechanism involving NO and eNOS (Abu-Soud et al., 2000, Assreuy et al., 1993, Grumbach et al., 2005, Santolini et al., 2001). During this feedback inhibition, a portion of the enzyme is trapped in a NO-bound form, thus

decreasing the portion of the enzyme that is able to participate in NO synthesis (Santolini et al., 2001). As HBO_2 in other studies has also been demonstrated to increase NO (Cabigas et al., 2006b) and NOS (Cabigas et al., 2006a, Buras et al., 2000), it is highly plausible that the negative feedback inhibition caused by an increased amount of NO as a result of HBO_2 in the HBO_2 Group of this study, may have led to partial inhibition eNOS thus reducing the presence of fully active, free, myocardial eNOS.

In the period between the 'post induction' time point and the time point '5 minutes on CPB', there was a decrease in the mean quantity of myocardial eNOS in both groups *(Figure 6.1)*. This decrease was greater in the HBO_2 Group resulting in a lower quantity of myocardial eNOS at the time point '5 minutes on CPB'. One possible explanation for this decrease in myocardial eNOS expression in both groups may lie in use of non-pulsatile flow (continuous flow) of blood during CPB in this study. The decrease in eNOS expression following the onset of CPB has also been described by others (Zhou et al., 2000). As it has been demonstrated that pulsatile flow (Li et al., 2005) and shear stress (Cheng et al., 2005, Nishida et al., 1992, Tao et al., 2006, Xiao et al., 1997) leads to an increase myocardial eNOS expression, it is quite possible that the loss of pulsatile flow, and as such a reduction in shear stress, following the onset of continuous flow during CPB in this study, may have led to the decrease in eNOS expression. It may also account for the general decreasing trend in myocardial eNOS expression in both groups of this study. Another possibility for the general decrease in eNOS expression may again be, as previously described, the negative feedback mechanism involving

NO and eNOS (Abu-Soud et al., 2000, Assreuy et al., 1993, Grumbach et al., 2005, Santolini et al., 2001).

During the period of ischaemia and reperfusion (i.e. between the time points ‘5 minutes post CPB’ and ‘5 minutes post IRI’) *(Figure 6.1)*, in the HBO_2 Group there was a small increase in myocardial eNOS quantity while in the Control Group there was a decrease. The increase in myocardial eNOS as a result of ischaemia and reperfusion following HBO_2 preconditioning has also been demonstrated by Cabigas *et al* (Cabigas et al., 2006b) in a rat model of IRI. It has been shown that eNOS expression may also be regulated by protein-protein interaction (Venema et al., 1996) and it has been suggested that this protein may be Hsp90 (Alderton et al., 2001, Garcia-Cardena et al., 1998). Cabigas *et al* (Cabigas et al., 2006b), in their experimental HBO_2 model of IRI, showed that following IRI, rats pre-treated with HBO_2 had an increase in the association of Hsp90 with eNOS and this occurred without any further increase in total Hsp90. This suggests that one possibility for the increase in myocardial eNOS expression during the period of ischaemia and reperfusion in the HBO_2 Group of this study, may be due to the protein-protein interaction between the HBO_2 induced myocardial eNOS and existing myocardial Hsp90, leading to a burst of further eNOS expression during this period in the HBO_2 Group. This observation, during the period of ischaemia and reperfusion, in the HBO_2 Group indicates that preconditioning CAD patients with HBO_2 prior to CABG may be capable of enhancing endogenous myocardial protection during ischaemia and reperfusion by increasing myocardial eNOS expression.

Following the period of ischaemia and reperfusion, between the time points ‘5 minutes post IRI’ and ‘5 minutes post CPB’ *(Figure 6.1)*, both groups again, showed a decrease in the mean quantity of myocardial eNOS and the final quantity of myocardial eNOS in the HBO_2 Group at the time point ‘5 minutes post CPB’ was lower than in the Control Group. It is possible that the decrease seen in the HBO_2 Group may be again due to the negative feedback effect of high levels of NO on myocardial eNOS following the period of ischaemia and reperfusion.

6.5.2 Myocardial Hsp72

In this study it was observed that following HBO_2 preconditioning, in the baseline right atrial biopsy taken at the time point 'post induction', the myocardium of patients in the HBO_2 Group had a lower mean quantity of myocardial Hsp72 compared to the Control Group *(Figure 6.2)*. Although this was not a statistically significant result, it does suggest that preconditioning CAD patients with HBO_2 prior to CABG leads to a reduction in myocardial Hsp72 expression. To date, this is the first and only clinical study that has demonstrated such as result. There are also no available experimental studies that have examined the tissue changes of Hsp72 before and after HBO_2 treatment, prior to IRI. As such, at present it is difficult to offer a reasonable explanation for this observation other than to suggest that perhaps, the initial oxidative stress of HBO_2 may have led to a degree of myocardial injury which required the chaperoning effect of Hsp72. This may have led to the reduced detection of myocardial Hsp72 in the HBO_2 Group of patients 1 hour following HBO_2 preconditioning. This explanation can only be confirmed if the actual concentration of serum Tropoin-T at the time points 'pre-HBO_2' and 'post HBO_2' are known. However, as the concentration of serum Tropoin-T at these time points were reported as <0.03ng/ml and, the absolute concentrations were not reported by the laboratory, it is not possible to determine if there were minor degrees (i.e. not clinically relevant) of the myocardial injury, that may have resulted from HBO_2 trearment, that could be correlated to changes in levels of myocardial Hsp72.

In the period between the time point ‘post induction’ and the time point ‘5 minutes on CPB’, there was a decrease in the mean quantity of myocardial Hsp72 in the HBO_2 Group while in the Control Group there was a very small increase *(Figure 6.1)*. A possible explanation for the decrease in Hsp72 in the HBO_2 Group at the time point ‘5 minutes on CPB’ may be the following. It has been documented that CPB causes myocardial damage (Cosgrave et al., 2006, Dahlin et al., 2003, Lehrke et al., 2004). As such, it could be possible that at this time point in the HBO_2 Group, the available myocardial Hsp72 may be chaperoning myocardial proteins that have unfolded as a result of 5 minutes of CPB. It has been suggested that any increase in the presence of unfolded proteins, shifts the equilibrium between Hsp and its transcription factor, Heat Shock Factor (HSF), to binding with the unfolded proteins (Abravaya et al., 1992, Morimoto, 1993). This may account for the reduced quantity of myocardial Hsp72 in the HBO_2 Group. Cellular ATP depletion has also been shown to induce Hsp72 expression in the recovering cells (Vogt et al., 2007). HBO_2 has been shown to preserve ATP levels (Chen et al., 1998, Nylander et al., 1987, Yamada et al., 1995) while CPB causes ATP depletion (Khuri et al., 1993). As such, it is possible to further speculate that the Hsp72 chaperoning of unfolding proteins during the initiation of CPB and, the loss of the ATP mediated stimulus for the induction of Hsp72 expression as a result of HBO_2 induced preservation of ATP, may have possibly led to the observed reduction in myocardial Hsp72 at the time point ‘5 minutes on CPB’

During the period of ischaemia and reperfusion (i.e. between the time points '5 minutes on CPB' and '5 minutes post IRI'), in the HBO_2 Group there was a small increase in the mean quantity of myocardial Hsp72 while in the Control Group there was a decrease. The finding in the HBO_2 Group of this study is similar to the previously described experimental findings of a HBO_2 IRI model (Wada et al., 1996) where the effects of repeated HBO_2 exposure on ischaemic tolerance in gerbil hippocampus was investigated. The findings from that experimental study and the of this clinical study, further suggest that HBO_2 preconditioning prior to ischaemia and reperfusion may be capable of increasing Hsp72 expression.

Following the period of ischaemia and reperfusion, between the time points '5 minutes post IRI' and '5 minutes post CPB' *(Figure 6.2)*, in both groups there was a decrease in the mean quantity of myocardial Hsp72. It could be possible that the decrease in myocardial Hsp72 in both the groups during this period may have been due to either, the further chaperoning of unfolded proteins as a result of IRI or post chaperoning structural changes to free Hsp72 (Kiang and Tsokos, 1998, Snoeckx et al., 2001) resulting in a reduction of free myocardial Hsp72.

6.6 Conclusion

In this clinical study, no statistically or clinically significant results were found when estimating the effects of HBO_2 preconditioning on the myocardial biomarkers of cardioprotection. As such, no definitive conclusions can be made with respect to the changes that were observed for these endpoints. Furthermore, as these were secondary endpoints, it was not part of the statistical plan of this study to make firm conclusions based on secondary endpoints. In keeping with this statistical plan, only an explanation for the results of the descriptive statistical were provided.

In this study, it was observed that following HBO_2 preconditioning, this group of patients, compared to the Control Group of patients, had a lower amount of both myocardial eNOS and Hsp72 during the peri-operative period. However, it was also observed that during the period of ischaemia and reperfusion, both these biomarkers increased in the HBO_2 Group but decreased in the Control Group. Despite these changes during ischaemia and reperfusion, the amount of both biomarkers remained lower in the HBO_2 Group when compared to in the Control Group. These results indirectly suggests *(see section 6.5)* that NO, from the catalytic action of eNOS, and Hsp72 may be functioning to protect the myocardium via their individual mechanisms of action, hence accounting for the limited pre-CPB detection of free eNOS and Hsp72 in the myocardium. Furthermore, as it was also observed that patients who were preconditioned with HBO_2 experienced an increase in eNOS and Hsp72 following ischaemia and reperfusion, this

possibly also suggest that HBO_2 preconditioning of this group of patients may be able to further induce the endogenous expression of these biomarkers to provide additional protection to the myocardium during the period of ischaemia and reperfusion.

The main limitation to this part of the study was that there was no assessment of myocardial NO production. As a result of this, the correlation between eNOS activity and NO production could not be determined. Additionally, as eNOS catalyses the production of NO, which in turn leads to a variety of haemodynamic changes, the correlation between NO production and the haemodynamic changes observed in this study also could not be determined. Finally, as was the case with the serum soluble adhesion molecules, as the durations of ischaemia, reperfusion, CPB and, the number of ischaemic reperfusion cycles in both groups of this study were not standardised, there is always the possibility that a different set of results for both eNOS and Hsp72 expression may have been obtained if it was possible to standardise these intervals in a clinical study.

7. Effects of HBO_2 Preconditioning on the Myocardial Molecular Expression of NOS & Hsp72

7.1 Introduction

CABG is associated with periods of ischaemia and reperfusion that leads to IRI (Venugopal et al., 2009). The induction however, of endogenous myocardial eNOS (Elrod et al., 2008, Han et al., 2008b, Iwase et al., 2007, Kim et al., 2007) and Hsp72 (Radford et al., 1996, Vahlhaus et al., 2005) have been shown to protect the myocardium against IRI.

Experimental models of IRI have demonstrated that treatment with HBO_2 prior to (Cabigas et al., 2006b, Kim et al., 2001) and, during ischaemia and reperfusion (Sterling et al., 1993) is capable of limiting myocardial infarct size as a result of IRI. It has also been demonstrated that HBO_2 preconditioning prior to ischaemia and reperfusion is also capable of increasing myocardial eNOS expression (Cabigas et al., 2006a). While currently no work has been published on the effects of HBO_2 and, ischaemia and reperfusion on myocardial Hsp72 expression, experimental work in gerbil brain tissue (Wada et al., 1996) has demonstrated that HBO_2 preconditioning prior to ischaemia and reperfusion is capable of increasing cerebral Hsp72.

7.2 Objective

The objective of this part of the study was to determine a method for the quantification of myocardial eNOS, iNOS and Hsp72 mRNA in myocardial tissue sample from the time point '5 minutes post CPB' in patients who had been preconditioned with two episodes of 30 minutes of 100% oxygen at 2.4 ATA, which was completed approximately 2 hours prior to on CPB CABG.

7.3 Methods

7.3.1 Selection of Primers for RT-PCR

The selection of primers and the primer sequences *(Table 7.1)* that were used for the reverse transcriptase polymerase chain reaction (RT-PCR) were based on previously published literature investigating the effects of ischaemia and reperfusion on the expression of myocardial eNOS (Valen et al., 2000), iNOS (Valen et al., 2000) and Hsp72 (Giannessi et al., 2003). The housekeeping gene used was β-Actin and its primer sequence too was obtained from the published literature (Giannessi et al., 2003).

7.3.1.1 Primers

Primers were purchased from Yorkshire Bioscience Ltd (Biocentre, York Science Park, Heslington, York, UK). The primers were reconstituted in RNase Free Water (Qiagen, UK) and had a concentration of

100μM. The primers were diluted 10 fold to a concentration of 10μM and stored as 10μl aliquots at -20ºC.

Table 7. 1: Primers used for RT-PCR

	Hsp72 Forward Primer	Hsp72 Reverse Primer	β-Actin Forward Primer	β-Actin Reverse Primer	iNOS Forward Primer	iNOS Reverse Primer	eNOS Forward Primer	eNOS Reverse Primer
Sequence (5'-3')	5'GCT-GAC-CAA-GAT-GAA-GGA-GAT-3'	5'-GAG-TCG-ATC-TCC-AGG-CTG-GC-3'	5'-GAG-ACC-TTC-AAC-ACC-CCA-GCC-3'	5'-GCC-CAT-CTC-TTG-CTC-GAA-GTC-3'	5'-AGT-TTC-TGG-CAG-CAA-CGG-3'	5'-TTA-AGT-TCT-GTC-CCG-GCA-G-3'	5'-ACC-TGC-AAA-GCA-GCA-AGT-CCA-GC-3'	5'-CCG-AAC-ACC-AAA-GTC-ATG-GGA-GT-3'
Product Size (bp)	467		457		532		737	
Melting Temperature (Tm)	60.3	63.4	63.7	61.8	56	56.7	64.2	62.4
No. Bases	22	20	21	21	18	19	23	23
GC%	50	65	61.9	67.1	55.6	52.6	56.5	52.2

bp = base pairs

7.3.2 RNA Extraction from Myocardial Biopsy

To determine the expression of iNOS, eNOS and Hsp72 mRNA in the right atrial biopsies, the myocardial samples were first removed from the -80ºC freezer. For the purpose of establishing a method, all the samples used were samples obtained at the time point '5 minutes post CPB' from 20 patients who were preconditioned with HBO_2.

All steps for RNA extraction were done in a Class 2 Biological Safety Cabinet (Faster, SLS, Nottingham). Prior to use, the working surface of this cabinet was cleaned with RNaseZap (Sigma, UK) to ensure the surface was free of RNase. Before the samples could thaw, a small piece of tissue from the sample was quickly excised and the remaining portion of the right atrial biopsy sample was returned to the -80ºC freezer. The excised right atrial tissue specimen was allowed to completely thaw and then weighed. Trizol (Invitrogen, UK) was used, in accordance with the manufacturer's protocol, to extract RNA from the right atrial tissue specimens.

Myocardial Specimen Disruption & Homogenisation

In a petri dish, the right atrial tissue specimen was teased apart and disrupted in 800µl of Trizol using two size 11 scalpels prior to homogenisation by passing it 5 times through a 20G needle and syringe. The homogenate was incubated for 5 minutes at room temperature before being centrifuged at 12,000*g* for 10 minutes at 4ºC. The supernatant (lysate) was then used for subsequent analysis.

Phase Separation

160µl of chloroform was added to the lysate, shaken vigorously for 15 seconds and then incubated for 2 minutes at room temperature before centrifugation at 10,000*g* for 15 minutes at 4ºC. The colourless upper aqueous phase containing RNA was decanted for subsequent use.

RNA Precipitation

At room temperature, 400µl of isopropyl alcohol was mixed with the aqueous phase to precipitate RNA. This was subsequently incubated for 10 minutes at room temperature before centrifugation at 10,000*g* for 10 minutes at 4ºC. The RNA precipitate formed a gel-like pellet on the bottom of the propylene tube.

RNA Wash

The RNA pellet was washed once with 800µl of 75% Ethanol [in Diethylpyrocarbonate (DEPC)-treated water] and then centrifuged at 7,500*g* for 5 minutes at 4 ºC.

Redissolving the RNA

The RNA gel pellet was allowed to dry at room temperature for 5 minutes and then dissolved in 50µl RNase Free Water by incubation in a water bath for 10 minutes at 55ºC. Typically, the volume of RNA solution obtained following this incubation was between 60 to 64µl.

Quantification of RNA

In order to quantify the concentration of the RNA that was extracted, the absorbance of the RNA at 260nm (A_{260}) was determined by using a spectrophotometer (GeneQuant [Pharmacia, UK]). The quantity of protein contaminants in the volume of extracted RNA was then determined by measuring the absorbances at 280nm (A_{280}) using the same spectrophotometer (GeneQuant [Pharmacia, UK]). By calculating the $A_{260/280}$ ratio, the purity of the extracted RNA was determined. 10mM of tris(hydroxymethyl)aminomethane choride (Tris.Cl) (pH=7.5) was used as the buffer during the process of measuring the A_{260} and A_{280} RNA absorbance. As per manufacturer requirements, when using the Trizol RNA extraction reagent, the RNA extracted was only used for RT-PCR when the $A_{260/280}$ ratio (i.e. purity of extracted RNA) was >1.65. Below is an example of how the A_{260} and A_{280} absorbance was measured and how the eventual quantity of extracted RNA was determined.

1. Control calibration of the spectrophotometer was done by pippeting 200µl of 10mM Tris.Cl (pH= 7.5) into a cuvette which was then placed in the spectrophotometer for spectorphotometric measurement.
2. The Tris.Cl was then removed from the cuvette and the cuvette was cleaned with distilled water.
3. 2µl of RNA (for example, from a total volume of 64µl) was then mixed with 198µl 10mM Tris.Cl (pH=7.5) in a propylene tube to make a 100 fold RNA dilution (200µl).
4. The diluted RNA was pipetted into the cuvette and placed into the calibrated spectrophotometer for RNA absorbance measurement.
5. The following is an example of the reading provided by the spectrophotometer:
    a. $A_{260} = 0.043$
    b. $A_{280} = 0.024$
    c. $A_{260/280} = 1.8$
    d. RNA Concentration = 2.1µg/ml
6. Using the RNA concentration measurement provided by the spectrophotometer, a corrected RNA concentration was then calculated to take into account the previous 100 fold dilution of the RNA. This correction was done as such:

$$2.1 \times 100 = 210\mu g/ml$$

7. Using the corrected RNA concentration, the quantity of RNA in the 64µl of RNA that was obtained following the redissolving of the RNA, was calculated as such:

$$64/1000 \times 210 = 13.44 \mu g \text{ of RNA}$$

8. Therefore,

    a. 1µg of RNA is contained in:

    $1/13.44 \times 64 = 4.8 \mu l$ of RNA

    b. 1µl of RNA contains:

    $1/64 \times 13.44 = 0.21 \mu g$ of RNA

7.3.3 Two Step RT-PCR

In order to detect β-actin, eNOS, iNOS and Hsp72 DNA in the right atrial tissue specimen, initially, a Two Step RT-PCR technique was used. The 2 steps of this technique are:

a) synthesis of cDNA, from the extracted RNA, via reverse transcription.
b) amplification of the respective DNA sequences via polymerase chain reaction (PCR) using primers for β-actin, eNOS, iNOS and Hsp72 DNA.

7.3.3.1 cDNA Synthesis

All the reagents required for this, apart from the Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT), were thawed and kept on ice. The M-MLV RT was only removed from the -20ºC when it was required for use. The cDNA synthesis process consisted of the following:

1. In a 0.5ml thin wall plastic tube, the following were added:
    a. 1μl oligo-dT Primer (0.5μg/μl) [Invitrogen, UK]
    b. an appropriate volume of RNA containing 1μg of RNA
    c. 1μl 10mM dNTP Mix [10mM each of dATP, dGTP, dCTP, dTTP in a solution of 0.6mM Tris-HCl (pH=7.5)] (Invitrogen, UK)
    d. 12μl DEPC treated distilled sterile water
2. The reaction mixture was heated in a thermal cycler (Techne Genius-Techne, Sone, UK) for 5 minutes at 65ºC to denature the dNTP probe and then chilled quickly in ice.
3. The following was then added to the reaction mixture:
    a. 4μl 5 x First Strand Buffer (Invitrogen, UK)
    b. 2μl Dithiothreitol (DTT) Buffer (Invitrogen, UK)
4. The contents of the tube was mixed and incubated in a thermal cycler (Techne Genius-Techne, Stone, UK) for 2 minutes at 37ºC.
5. 1μl of M-MLV RT (200U/μl) (Invitrogen, UK) was then added and mixed.
6. This reaction mixture was incubated in a thermal cycler (Techne Genius-Techne, Stone, UK) for 50 minutes at 37ºC to allow cDNA synthesis.
7. The reaction was inactivated by heating the sample in a thermal cycler (Techne Genius-Techne, Stone, UK) at 70ºC for 15 minutes.
8. The cDNA synthesised was stored at 4ºC for further analysis.

7.3.3.2 Quantification of cDNA

The cDNA was quantified using a spectrophotometer (GeneQuant [Pharmacia, Kent, UK]) in a manner that similar to RNA quantification as described in *section 7.3.2*.

7.3.3.3 PCR Of cDNA

All the primers and PCR Master Mix (Promega, UK), were removed from the -20ºC freezer, thawed and kept cool on ice. The cDNA that was required was removed from the 4ºC refrigerator and also kept cool on ice. The primers used had a final concentration of 10µM.

The PCR Master Mix consisted of

a) 50units/ml of *Taq* DNA Polymerase (in a propriety reaction buffer with pH=8.5)
b) 400µM each of dATP, dGTP, dCTP, dTTP
c) 3µM of $MgCl_2$

Preparation of Components for PCR of cDNA

1. 5 separate thin-walled tubes were set up, in duplicates, containing the components as described in *Table 7.2*.
2. All tubes were then place in a thermal cycler (Techne Genius-Techne, Stone, UK). The PCR cycling conditions used are given in *Table 7.3*.

Table 7. 2: Reaction Mixture for PCR of cDNA

	Tube 1	**Tube 2**	**Tube 3**	**Tube 4**	**Tube 5**
	β-Actin (Positive Control)	iNOS	eNOS	Hsp72	Negative Control
Forward β-Actin Primer (10µM)	1µl				1µl
Reverse β-Actin Primer (10µM)	1µl				1µl
Forward iNOS Primer (10µM)		1µl			
Reverse iNOS Primer (10µM)		1µl			
Forward eNOS Primer (10µM)			1µl		
Reverse eNOS Primer (10µM)			1µl		
Forward Hsp72 Primer (10µM)				1µl	
Reverse Hsp72 Primer (10µM)				1µl	
Template cDNA (1µg)	1µl	1µl	1µl	1µl	
PCR Master Mix	12.5µl	12.5µl	12.5µl	12.5µl	12.5µl
Nuclease Free H_2O	9.5µl	9.5µl	9.5µl	9.5µl	10.5µl
Total Reaction Volume	25µl	25µl	25µl	25µl	25µl

Table 7. 3: PCR Cycling Conditions

Cycles	Temperature	Time
1 x	Denaturation at 95ºC	5 minutes
28x	Denaturation at 94ºC	1 minute
	Annealing at 59ºC	1 minute
	Extension at 72ºC	1 minute
1 x	Prolonged extension at 60ºC	45 minutes
	4ºC	Holding post PCR

7.3.3.4 Gel Electrophoresis

In order to identify the size of the RT-PCR products, agarose gel electrophoresis was performed using a 2% gel.

7.3.3.4.1 2% Agarose Gel Preparation

3gm of Agarose 1000 gel powder (Invitrogen, UK) was dissolved in 150ml of 1x Tris/Borate/Ethylene Diamine Tetraacetate (EDTA) [TBE] by heating this mixture in a 500W microwave for 8 minutes. The gel was then allowed to cool for 5 minutes before 3µl of Ethidium Bromide (10 mg/ml stock) was mixed into the gel. The gel was then poured into a horizontal gel tank with a comb and left to set for approximately 2 hours.

7.3.3.4.2 ***Electrophoresis***

Once the gel wells had been loaded with the appropriate contents *(details of the content of each of the gel wells during the electrophoresis procedure, will be discussed later in the relevant sections),* the gel was then placed in an electrophoresis tank containing TBE. In a 4ºC room, a 100V current was then run through the tank for approximately 1 hour or until the blue dye front had moved through 80% of the gel. The gel was then removed from the tank and DNA bands were visualised under UV light using a gel documentation system (UVItec, Cambridge, UK).

7.3.4 One Step RT-PCR

In a One Step RT-PCR, cDNA synthesis and PCR are performed using an optimized buffer system in a single reaction tube without the requirement to add further reagents between cDNA synthesis and PCR. This simplified procedure reduced the potential for pipetting errors.

For this procedure, the Titan One Step RT-PCR kit was used (Roche Diagnostics GmbH, Germany). The components of the kit are shown in *Table 7.4.*

Table 7. 4: Titan One Step RT-PCR Kit Components

Component	Content
Titan Enzyme Mix	50µl of Titan enzyme mix [Avian Myeloblastosis Virus (AMV) and Expand High Fidelity] in storage buffer (1 reaction/µl)
dNTP mix	200µl; 10mM total (2.5mM each dNTP)
RNase Inhibitor	50µl (5U/µl)
Human Control RNA	50µl (2pg/µl); K562 total RNA with MS2 carrier RNA
Housekeeping (Control) Primer Mix	20µl; 20µM *(See Table 7.5)*
PCR H_2O	2 x 1ml
RT-PCR reaction buffer	1ml of 5 fold concentration with 7.5mM $MgCl_2$ and Dimethyl sulfoxide (DMSO)
DTT solution	1ml (100mM)
$MgCl_2$ stock solution	1ml (25mM)

Table 7. 5: Roche Titan One Step RT-PCR Control Primer

Control Primer	**Sequence (5'-3')**	**Size** (bp)
β-Actin (f)	5'-CCA-AGG-CCA-ACC-GCG-AGA-AGA-TGA-C-3'	587
β-Actin (r)	5'-AGG-GTA-CAT-GGT-GGT-GCC-GCC-AGA-C-3'	

f = forward primer *r = reverse primer* *bp = base pairs*

The preparation of the required reagents and the PCR cycling conditions for this analysis were performed according to the manufacturer's instructions.

Preparation of Master Mix 1

All the components for Master Mix 1 were thawed and placed on ice. All reagents were vortexed before setting up reactions. The initial concentration of the Yorkshire Bioscience primers that were used prior to making up Master Mix 1 was 10µM. A Nuclease Free thin wall plastic tube was used to set up each of the reaction mixture (Master Mix 1) as in *Table 7.6.* In total there were 7 thin wall plastic tubes, in duplicates, containing the necessary components for Master Mix 1. Following the addition of each of the components for Master Mix 1 into each of the thin wall plastic tubes, the tubes were then pulsed for 30 seconds at 10,000*g*.

Table 7. 6: Reaction Mixture for Master Mix 1

	Tube 1	**Tube 2**	**Tube 3**	**Tube 4**	**Tube 5**	**Tube 6**	**Tube 7**	Final Concentration
	Negative Control-1	β-Actin (Positive Control 1)	β-Actin (Positive Control 2)	β-Actin (Positive Control 3)	iNOS	eNOS	Hsp72	
H_2O	15.5μl	10.5μl	Make up volume to 25μl	Make up volume to 25μl	Make up volume to 25μl	Make up volume to 25μl	Make up volume to 25μl	
dNTP	4μl	4μl	4μl	4μl	4μl	4μl	4μl	0.2mM (each)
DTT	2.5μl	2.5μl	2.5μl	2.5μl	2.5μl	2.5μl	2.5μl	5mM
RNase Inhibitor	1μl	1μl	1μl	1μl	1μl	1μl	1μl	
Control RNA		5μl	5μl					10pg
Sample RNA				Appropriate Volume Containing 1μg RNA	Appropriate Volume Containing 1μg RNA	Appropriate Volume Containing 1μg RNA	Appropriate Volume Containing 1μg RNA	1μg
β-Actin Primer Mix (Roche) (f + r)	2μl	2μl						0.4μM
β-Actin Primer (YB) (f)			1μl	1μl				0.4μM
β-Actin Primer (YB)(r)			1μl	1μl				0.4μM
iNOS Primer (YB) (f)					1μl			0.4μM
iNOS Primer (YB)(r)					1μl			0.4μM
eNOS Primer (YB) (f)						1μl		0.4μM
eNOS Primer (YB)(r)						1μl		0.4μM
Hsp72 Primer (YB) (f)							1μl	0.4μM
Hsp72 Primer (YB)(r)							1μl	0.4μM
Total Volume (μl) per thin wall plastic tube	25	25	25	25	25	25	25	

f=forward; *r=reverse*

Preparation of Master Mix 2

All the components for Master Mix 2 were thawed and vortexed before setting up the reactions. A Nuclease Free thin wall plastic tube was used to set up each of the reaction mixture (Master Mix 2) as in *Table 7.7.* In total there were 7 thin wall plastic tubes, in duplicates, containing the necessary components for Master Mix 2. Each of the Master Mix 2 thin wall plastic tubes were then pulsed for 30 seconds at 10,000*g*. In this study, Master Mix 1 & 2 were prepared separately, as per manufacturer's instructions. The separate preparation of Master Mix 1 & 2, allows, if necessary, for a further step involving only Master Mix 2, to reduce DNA contamination with RNA or DNA from previous amplification procedures. This step involves heating Master Mix 2 for 2 minutes at 94℃ to inactivate reverse transcriptase AVM. This step was not done in this set of experiments as all other precautions to reduce error from DNA contamination were taken and this included having appropriate positive and negative controls, setting up a control reaction without RNA template and using RNase during the RNA extraction procedure.

Table 7. 7: Reaction Mixture for Master Mix 2

	Tube 1	**Tube 2**	**Tube 3**	**Tube 4**	**Tube 5**	**Tube 6**	**Tube 7**	Final Concentration
	Negative Control-1	β-Actin (Positive Control 1)	β-Actin (Positive Control 2)	β-Actin (Positive Control 3)	iNOS	eNOS	Hsp72	
H_2O	14µl	14µl	14µl	14µl	14µl	14µl	14µl	
5 x RT-PCR Buffer	10µl	10µl	10µl	10µl	10µl	10µl	10µl	1 x concentration with 1.5mM $MgCl_2$ and DMSO
Titan Enzyme Mix	1µl	1µl	1µl	1µl	1µl	1µl	1µl	
Total Volume (µl) per thin wall plastic tube	25	25	25	25	25	25	25	

RT-PCR Protocol

Master Mix 2 was mixed into Master Mix 1. The 50µl mixture of Master Mix 1 and 2 was then pulsed for 30 seconds at 10,000*g* before being placed in a thermal cycler (Techne Genius-Techne, Stone, UK) for 30 minutes at 60ºC to allow for reverse transcription. Following this, PCR was performed using the manufacturer recommended cycling conditions as in *Table 7.8.*

Table 7. 8: One Step RT-PCR Cycling Conditions

Cycles	Temperature	Time
1 x	Denaturation at 94ºC	2 minutes
35 x	Denaturation at 94ºC	30 seconds
	Annealing at 55ºC	30 seconds
	Elongation at 68ºC	30 seconds
1 x	Prolonged elongation at 68ºC	7 minutes
	4ºC	Holding post PCR

7.3.4.1 Gel Electrophoresis

A 2% agarose was made as described in *section 7.3.3.4.1*. 8µl of each of the RT-PCR product was mixed with 2µl of 1x PCR Loading Buffer before being loaded into the wells of the gel as shown in *Table 7.9*.

Table 7. 9: Gel Well Content for Electrophoresis of One Step RT-PCR Products

Well	Content
1	10µl Mixture of Negative Control* 1 + Loading Buffer
2	1µl 1000bp DNA Ladder (Promega, UK)
3	10µl Mixture of Positive Control 1* + Loading Buffer
4	10µl Mixture of Positive Control 2* + Loading Buffer
5	10µl Mixture of Positive Control 3* + Loading Buffer
6	10µl Mixture of iNOS RT-PCR product + Loading Buffer
7	10µl Mixture of eNOS RT-PCR product + Loading Buffer
8	10µl Mixture of Hsp72 RT-PCR product + Loading Buffer
9	1µl 100bp DNA Ladder

**(For the constituents of the positive and negative controls, see Table 7.6)*

Once the gel wells were loaded, electrophoresis was carried out as in *section 7.3.3.4.2*. Following the completion of the gel electrophoresis, DNA bands were visualised under UV light as in *section 7.3.3.4.2*.

7.4 Results

7.4.1 RNA Quantification Results

In this thesis, as per manufacturer's recommendations *(see section 7.3.2)*, the RNA extracted from each of the 20 post CPB right atrial specimens were only used for RT-PCR if the $A_{260/280}$ was >1.65. The $A_{260/}$ A_{280} in this study ranged from 1.7 to 1.9.

7.4.2 cDNA Quantification Results

All the 20 post CPB right atrial specimens that were used for cDNA synthesis, via the 2 Step RT-PCR, had their cDNA quantified. In this study, the cDNA that was synthesised had an $A_{260/280}$ ratio that ranged from between 1.56 to 1.62.

7.4.3 Demonstrating Effective Function of Purchased Primers

7.4.3.1 Amplification of β-Actin and Hsp72 in human buccal mucosa DNA

The aim of this experiment was to determine if the primers that were purchased from Yorkshire Bioscience functioned effectively when using the PCR cycling conditions described in *Table 7.3*. For this experiment, only the purchased β-Actin and Hsp72 primers were used. Human buccal mucosa DNA was used as the sample DNA and D16S539 was used as the positive control gene. The human buccal mucosal DNA was used as the sample DNA as it was readily available in the lab at the time of this experiment. It was obtained following a swab of the buccal mucosal surface of a volunteer in the lab. The DNA was extracted using a Qiagen QIAamp DNA Micro Kit. The D16S539 gene is a polymorphic microsatellite found on chromosome 16 at location q24.1 and is composed of the tetrameric repeat sequence GATA. It is a commonly used positive control gene during forensic testing. It was used as the positive control gene in this experiment as the primers (purchased from Eurofins MWG Operon, Germany) for this gene were readily available in the lab at the time.

In this experiment, 3 thin wall plastic tubes, in duplicates, were made up to a 10µl volume containing the PCR components listed in *Table 7.10*.

Table 7. 10: PCR MixtureTo Demonstrate Effective Function of Purchased Primers (β-Actin & Hsp72)

Components	**Volume**
1 x NH_4 Buffer [contains 16mM $(NH_4)_2SO_4$ and 67mM of Tris.HCL) (Bioline, UK)	1.0µl
$MgCl_2$ (3mM) (Bioline, UK)	0.3µl
dNTP (10µM) (Bioline, UK)	0.5µl
Forward primer (either β-Actin, Hsp72 or D16S539; 10µM)	1.0µl
Reverse primer (either β-Actin, Hsp72 or D16S539; 10µM)	1.0µl
Bovine Serum A (New England Biolabs, UK)	1.0µl
PCR H_2O (Bioline, UK)	4.0µl
Taq DNA Polymerase (50units/ml) (Bioline, UK)	0.2µl
Human buccal mucosa DNA (0.5µg)	1.0µl
Total Volume	10µl

Each tube was then place in a thermal cycler (Techne Genius-Techne, Stone, UK). The thermal cycling conditions used are listed in *Table 7.3*.

Table 7. 11: Gel Well Content for Electrophoresis to Demonstrate Effective Function of Purchased Primers (β-Actin & Hsp72)

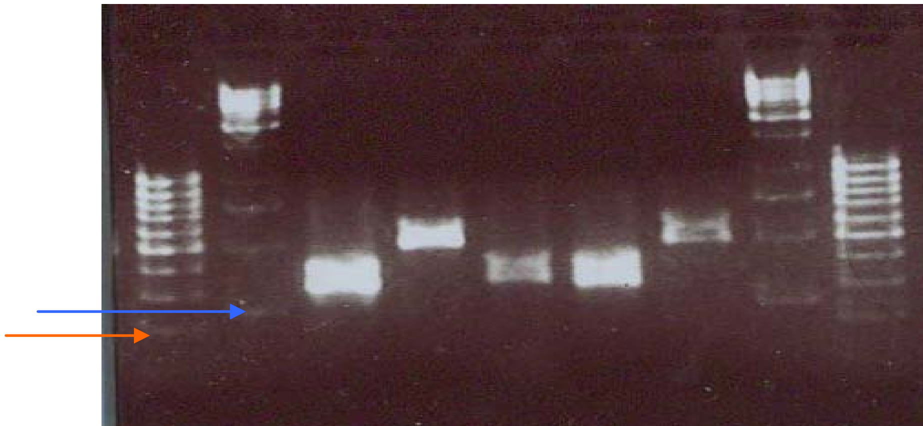
Well	**Content**
1	1μl 100bp DNA Ladder (Promega, UK)
2	1μl 1000bp DNA Ladder (Promega, UK)
3	The 10μl mixture of β-Actin RT-PCR product + 1x PCR Loading Buffer
4	The 10μl mixture of Hsp72 RT-PCR product + 1x PCR Loading Buffer
5	The 10μl mixture of D16S539 RT-PCR product (Positive Control) + 1x PCR Loading Buffer
6	The 10μl mixture of β-Actin RT-PCR product + 1x PCR Loading Buffer
7	The 10μl mixture of Hsp72 RT-PCR product + 1x PCR Loading Buffer
8	1μl 1000bp DNA Ladder (Promega, UK)
9	1μl 100bp DNA Ladder (Promega, UK)

Following PCR, the RT-PCR products were loaded into a 2% agarose gel that was made as described in *section 7.3.3.4.1*. 8μl of each of the RT-PCR product was mixed with 2μl of 1x PCR Loading Buffer before being loaded into the gel wells. The contents of the gel well are shown in *Table 7.11*.

Once the gel wells were loaded satisfactorily, electrophoresis was then conducted as in *section 7.3.3.4.2*. Following the completion of the gel electrophoresis, DNA bands were visualised under UV light as in *section 7.3.3.4.3*. *Figure 7.1* shows the image of the gel electrophoresis viewed under UV light.

Figure 7. 1: UV image of gel showing the product bands for β-Actin & Hsp72 following PCR using the purchased primers & human buccal mucosal DNA and, showing the product band for the positive control gene, D16S539.

Lane: 1 2 3 4 5 6 7 8 9



Yellow arrow indicates 100bp band (100-1000bp ladder)

Blue arrow indicate 1000bp band (1000-10000bp ladder)

Legend 7. 1: Legend for *Figure 7.1*

Lane	**Content**
1	100bp ladder
2	1000bp ladder
3	β-Actin
4	Hsp72
5	D16S539
6	β-Actin
7	Hsp72
8	1000bp ladder
9	100bp ladder

In *Figure 7.1*, RT-PCR products were visible under UV light for β-Actin (457bp), Hsp72 (467bp) and D16S539 (330bp). A 100bp and 1000bp DNA ladder were used to enable easier identification of the RT-PCR products base pair sizes. This experiment demonstrated that the purchased β-Actin and Hsp72 primers functioned optimally using the PCR conditions listed in *Table 7.3*.

This experiment was repeated twice and gave the similar results. Optimisation experiments for the purchased eNOS and iNOS primers, using the PCR conditions listed in *Table 7.3,* were not performed as the success of this experiment, using the purchased primers for β-Actin and Hsp72, provided sufficient evidence that primers purchased from Yorkshire Bioscience were reliable and would functioned appropriately.

7.4.4 Assessment of Myocardial Specimen DNA Expression using Purchased Primers.

7.4.4.1 Detection of β-Actin, Hsp72, eNOS and iNOS in sample myocardial DNA using a Two Step RT-PCR

These experiments were conducted to assess the expression of mRNA for β-Actin, Hsp72, eNOS and iNOS in the 20 post CPB right atrial myocardial specimens, following the RNA extraction procedure, as in *section 7.3.2* and, the Two Step RT-PCR procedure, as in *section 7.3.3*. In this series of experiments, in the second step of the Two Step RT-PCR procedure, the Promega PCR Master Mix was used rather than individual PCR components as in *section 7.4.3*. This was done due to the simplicity and ease of use of a PCR Master Mix.

In this series of experiments, following the Two Step RT-PCR procedure *(see section 7.3.3)* and 2% agarose gel preparation *(see section 7.3.3.4.1)*, 8μl of each of the RT-PCR product was mixed with 2μl of 1x PCR Loading Buffer. The 10μl mixture was then loaded into the gel wells. The content of each gel well is described in *Table 7.12*.

Table 7. 12: Gel Well Content for Electrophoresis to Demonstrate Presence of β-Actin, eNOS, iNOS and Hsp72 in Sample Myocardial cDNA following Two Step RT-PCR.

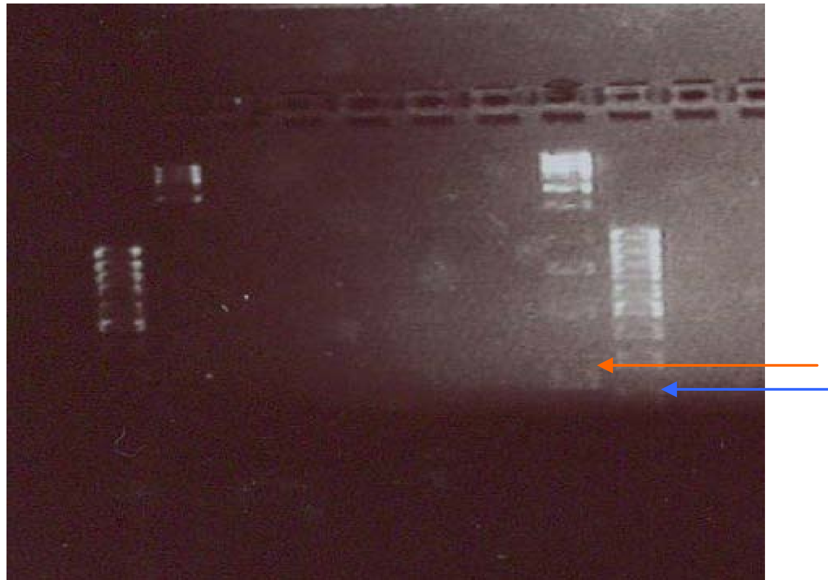
Well	**Content**
1	1µl 100bp DNA Ladder (Promega, UK)
2	1µl 1000bp DNA Ladder (Promega, UK)
3	The 10µl mixture of Hsp72 RT-PCR product + 1x PCR Loading Buffer
4	The 10µl mixture of eNOS RT-PCR product + 1x PCR Loading Buffer
5	The 10µl mixture of iNOS RT-PCR product + 1x PCR Loading Buffer
6	The 10µl mixture of β-Actin RT-PCR product (Positive Control) + 1x PCR Loading Buffer
7	The 10µl mixture of Negative Control (β-Actin RT-PCR without sample myocardial DNA) product + 1x PCR Loading Buffer
8	1µl 1000bp DNA Ladder (Promega, UK)
9	1µl 100bp DNA Ladder (Promega, UK)

Once the gel wells were loaded satisfactorily, electrophoresis was then conducted as in *section 7.3.3.4.2*. Following the completion of the gel electrophoresis, DNA bands were visualised under UV light as in *section 7.3.3.4.3*.

For each of the 20 post CPB right atrial myocardial specimen used, a similar experiment was performed using 4 increasing amounts of RNA, 0.03µg, 0.05µg, 0.3µg and 1µg, respectively. To optimise conditions, a range of denaturation (94ºC-98ºC) and annealing (58ºC-62ºC) temperatures were also used during the PCR procedure. However, in all the optimisation experiments, none of the β-Actin, Hsp72, eNOS or iNOS RT-PCR product bands were visible, in the gel, under UV light. *Figure 7.2* is an example of one of those gels. A 100bp and 1000bp DNA ladder was used to enable easier identification of the base pair product band of interest.

Figure 7. 2: UV image of gel showing no product bands bands following electrophoresis of the Two Step RT-PCR products (β-Actin, Hsp72, eNOS and iNOS) that was obtained from 0.03µg of sample myocardial RNA.

Lane: 1 2 3 4 5 6 7 8 9



Yellow arrow indicates 100bp band (100-1000bp ladder)

Blue arrow indicates 1000bp band (1000-10000bp ladder)

Legend 7. 2: Legend for *Figure 7.2*

Lane	Content
1	100bp ladder
2	1000bp ladder
3	Hsp72
4	eNOS
5	iNOS
6	Positive Control
7	Negative Control
8	1000bp ladder
9	100bp ladder

7.4.4.2 Two Step RT-PCR involving human buccal mucosa DNA and sample myocardial DNA

As the RT-PCR experiments in *section 7.4.4.1*, where sample myocardial DNA and a PCR Master Mix were used during the second step of the Two Step RT-PCR procedure, did not yielded any bands, one possible reason for this may have been the Two Step RT-PCR process itself. The problem could arise either in the process of synthesising cDNA from the extracted myocardial RNA (first step of the Two Step RT-PCR process) or during the PCR step (second step of the Two Step RT-PCR) of the process.

In order to establish if there was a problem in synthesising cDNA from the myocardial RNA *(section 7.3.3.1)* during the first step of the Two Step RT-PCR process, an experiment was set up using post CPB right atrial myocardial sample DNA (obtained following RNA extraction as in *section 7.3.2* and cDNA production as in *section 7.3.3.1*) and human buccal mucosa DNA (as used in the experiment in *section 7.4.3.1*).

In this experiment, 10 thin wall plastic tubes, in duplicates, were made up to a 25µl volume containing the PCR components listed in *Table 7.13*.

Table 7. 13: PCR Mixture to produce the required PCR products from Human Buccal Mucosa DNA & Myocardial Sample cDNA (obtained follwing Two Step RT-PCR)

Components	**Volume**
Promega PCR Master Mix	12.5µl
Forward Primer (β-Actin, D16S539, Hsp72, eNOS or iNOS; 10µM)	1µl
Reverse Primer (β-Actin, D16S539, Hsp72, eNOS or iNOS; 10µM)	1µl
Sample myocardial (0.7µg/µl) or human buccal mucosa DNA (0.5µg/µl)	1µl
Nuclease Free H_2O	9.5µl
Total Volume	25µl

Each tube was then place in a thermal cycler (Techne Genius-Techne, Stone, UK). The thermal cycling conditions listed in *Table 7.3* were used.

RT-PCR products were then loaded into a 2% agarose that was made as described in *section 7.3.3.4.1.* 8µl of each of the RT-PCR product was mixed with 2µl of 1x PCR Loading Buffer before being loaded into the gel wells. The contents of each gel well is described in *Table 7.14*.

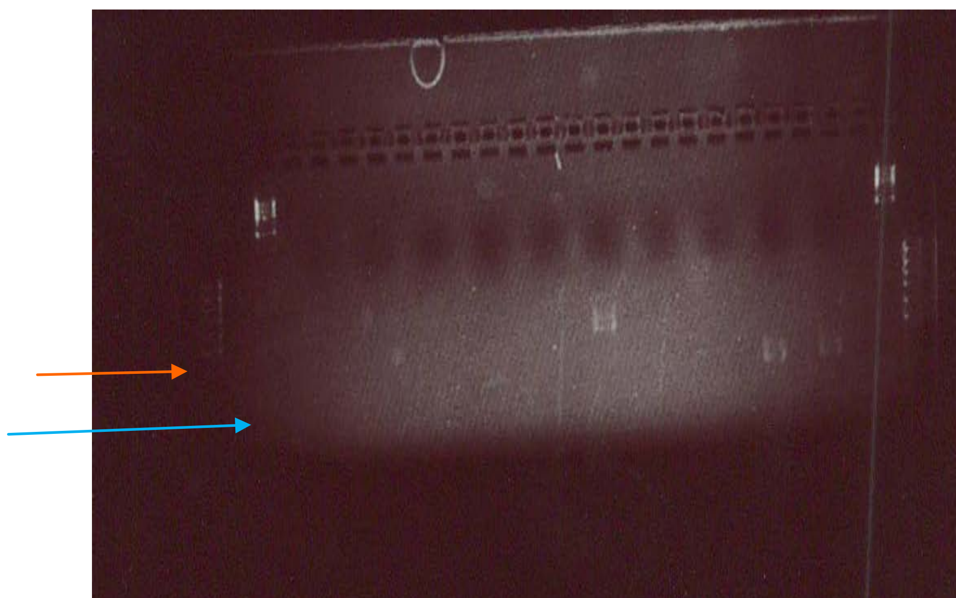
Table 7. 14: Gel Well Content for Electrophoresis using Human Buccal Mucosa DNA & Sample Myocardial cDNA (obtained following Two Step RT-PCR).

Well	**Content**
1	1µl 100bp DNA Ladder (Promega, UK)
2	1µl 1000bp DNA Ladder (Promega, UK)
3	10µl mixture of sample myocardial Hsp72 RT-PCR product + 1x PCR Loading Buffer
4	10µl mixture of sample myocardial eNOS RT-PCR product + 1x PCR Loading Buffer
5	10µl mixture of sample myocardial iNOS RT-PCR product + 1x PCR Loading Buffer
6	10µl mixture of sample myocardial β-Actin RT-PCR product + 1x PCR Loading Buffer
7	10µl mixture of sample myocardial D16S539 RT-PCR product + 1x PCR Loading Buffer
8	10µl mixture of buccal mucosa Hsp72 RT-PCR product + 1x PCR Loading Buffer
9	10µl mixture of buccal mucosa eNOS RT-PCR product + 1x PCR Loading Buffer
10	10µl mixture of buccal mucosa iNOS RT-PCR product + 1x PCR Loading Buffer
11	10µl mixture of buccal mucosa β-Actin RT-PCR product + 1x PCR Loading Buffer
12	10µl mixture of buccal mucosa D16S539 RT-PCR product + 1x PCR Loading Buffer
13	1µl 1000bp DNA Ladder (Promega, UK)
14	1µl 100bp DNA Ladder (Promega, UK)

Once the gel wells were loaded satisfactorily, electrophoresis was then carried out as in *section 7.3.3.4.2*. Following the completion of the gel electrophoresis, DNA bands were visualised under UV light as in *section 7.3.3.4.3.* The image of the gel as viewed under UV light are shown in *Figures 7.3.*

Figure 7. 3: UV image gel showing no products bands from the of sample myocardial cDNA (obtained following the Two Step RT-PCR) but showing product bands for Human Buccal Mucosal Hsp72 & D16S539.

Lane: 1 2 3 4 5 6 7 8 9 10 11 12 13 14



Arrow indicating 100bp band (100-1000bp ladder)

Arrow indicating 1000bp band (1000-10000bp ladder).

Legend 7. 3: Legend for *Figure 7.3*

Lane	Content
1	100bp ladder
2	1000bp ladder
3	Myocardial Hsp72
4	Myocardial eNOS
5	Myocardial iNOS
6	Myocardial β-Actin
7	Myocardial D16S539
8	Buccal Mucosa Hsp72
9	Buccal Mucosa eNOS
10	Buccal Mucosa iNOS
11	Buccal Mucosa β-Actin
12	Buccal Mucosa D16S539
13	100bp ladder
14	1000bp ladder

The above experiment was repeated twice with similar results. None of the sample myocardial RT-PCR products i.e. β-Actin (expected at 457bp), D16S539 (expected at 330bp), Hsp72 (expected at 467bp), eNOS (expected at 737bp) or iNOS (expected at 532bp) were visible under UV light. However, from the buccal mucosa RT-PCR products, apart from eNOS and iNOS, the RT-PCR products for β-Actin, D16S539 and Hsp72 (Lanes 10, 11 and 12) were visible under UV light at the appropriate band scale. It is possible that eNOS and iNOS RT-PCR products from the buccal mucosa DNA were not visualised because the purchased primers for eNOS and iNOS required further optimisation before being used this study. This may be the case as, while in *section 7.4.3,* it was demonstrated that the purchased primers for β-Actin and the Hsp72 functioned appropriately, this was not demonstrated for the purchased primers of eNOS and iNOS. An assumption was made then that as the purchased primers for β-Actin and the Hsp72 functioned appropriately, the same would be the case for the purchased eNOS and iNOS primers. However, the detection of β-Actin and Hsp72 RT-PCR product using the buccal mucosa DNA, also suggest that the failure to produce any RT-PCR product from the sample myocardial DNA may also lie in the process of synthesising cDNA from the sample myocardial RNA. One possible reason that may have led to this, when using the right atrial myocardial spample RNA and the Two Step RT-PCR process, may have been contamination of the cDNA that was synthesised. In this study, the cDNA that was synthesised had a $A_{260/280}$ ratio which was between 1.56-1.62. Conventionally, a cDNA $A_{260/280}$ ratio which is less than 1.8 is suggestive of some cDNA contamination (Clark

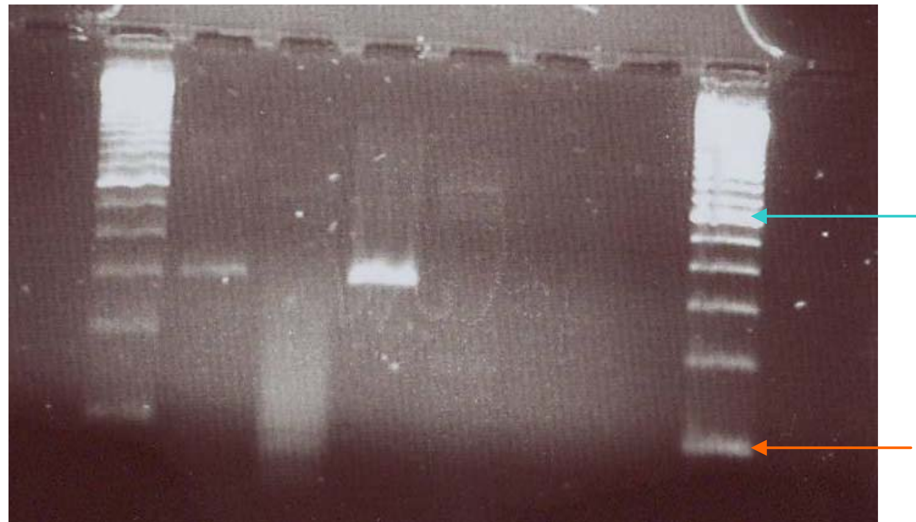
W, 2001) and this may have affected the outcome of the eNOS and iNOS PCR results. Another factor which may have affected the PCR results of the Two Step RT-PCR process, may be the possibility that there was an excess of reverse transcriptase enzyme (M-MLV RT) that was being used during the cDNA synthesis *(see section 7.3.3.1)* and this was inhibiting the PCR step of this two step process. This problem has been published in the literature (Suslov and Steindler, 2005). As a means of reducing cDNA contamination and limiting possible inhibition of the PCR process during the Two Step RT-PCR, due to excessive reverse transcriptase enzymes, a One Step RT-PCR was subsequently used to directly synthesise target myocardial DNA from the extracted RNA.

7.4.4.3 Results of the One Step RT-PCR using sample myocardial DNA

Following the One Step RT-PCR process *(section 7.3.4)*, a 2% agarose gel was prepared as in *section 7.3.3.4.1*. Gel loading and electrophoresis was done as in *section 7.3.4.2*. Following electrophoresis, the gel was viewed under UV light as in *section 7.3.3.4.3*. The image of the gel as viewed under UV light is shown in *Figures 7.4*.

Figure 7. 4: UV image of gel showing product bands for myocardial β-Actin and iNOS &, product band for Control β-Actin following One Step RT-PCR.

Lane 1 2 3 4 5 6 7 8 9



Blue arrow indicates 1000bp band (1000-10000bp ladder)
Yellow arrow indicates 100bp band (100-1000bp ladder)
(For the constituents of the positive and negative controls, see Table 7.5)

Legend 7. 4: Legend for *Figure 7.4*

Lane	Content
1	Negative Control* 1
2	1000bp ladder
3	Positive Control 1*
4	Positive Control 2*
5	Positive Control 3*
6	iNOS
7	eNOS
8	Hsp72
9	1000bp ladder

(For the constituents of the positive and negative controls, see Table 7.6)

From the *Figure 7.4* it can be seen that the Negative Control, in Lane 1, has no product band but Positive Control 1 (β-Actin RT-PCR product using control RNA) and Positive Control 3 (β-Actin RT-PCR product using sample RNA), in Lanes 3 and 5, respectively, have a product band each that have been illuminated by UV light at about the 500bp mark. The iNOS RT-PCR product in Lane 6 produced a faint band at about the 500bp mark. The RT-PCR products for eNOS (Lane 7) and Hsp72 (Lane 8) did not produce any bands. The above experiment was repeated for each of the 20 post CPB myocardial samples with similar results.

7.5 Discussion

The objective of this part of this study was to develop a RT-PCR method for analysing the expression of eNOS, iNOS and Hsp72 mRNA in the collected right atrial biopsies. Based on the results from this study, it is not possible to deduce any conclusion with regards to the effects of HBO_2 preconditioning on myocardial eNOS, iNOS and Hsp72 mRNA in the patients recruited to this study.

The results from the One Step RT-PCR experiment in *section 7.4.4.3* showed that in the 20 analysed post CPB right atrial biopsies from patients who were preconditioned with HBO_2 prior to CABG and IRI, in addition to β-Actin [from Positive Control 1 (using the Control RNA and β-Actin primers provided by the One Step RT-PCR kit) and Positive Control 3 (using sample myocardial RNA and purchased β-Actin primers)], only a faint iNOS DNA band was detectable. It suggests that, of the 3 genes (eNOS, iNOS and Hsp72) examined for mRNA expression at the time point '5 minutes post CPB' in these 20 patients, there was only low level expression of myocardial iNOS mRNA with no mRNA for eNOS or Hsp72. From the series of experiments involving the methods used in *section 7.4.4.3*, it is unclear why no DNA band was observed for the Positive Control 2 (using Control RNA from the One Step RT-PCR kit and the purchased β-Actin primers).

In this study, the iNOS protein was not measured in the myocardial samples that were obtained. This was because it had been demonstrated, in an experimental model of HBO_2 and IRI, that HBO_2 had no

effect on iNOS protein expression (Buras et al., 2000). iNOS is not a constitutive protein and its expressions needs to be induced by a stress event, such as an oxidative stress or ischaemia and reperfusion, which initiates its transcription. As such, instead of attempting to determine the expression of iNOS protein, this study attempted to determine if the oxidative stress of HBO_2 preconditioning in patients with CAD disease, administered prior to CABG and IRI, had any effect on the mRNA expression of iNOS. The results of this study appear to indicate that in patients who were exposed to the oxidative stress of HBO_2 preconditioning prior to on CPB CABG and IRI, by the end of the period of ischaemia and reperfusion and, CPB (i.e. at the time point '5 minutes post CPB'), there was some expression of iNOS mRNA.

With respect to eNOS and Hsp72 expression, in this study it was observed that, in the HBO_2 Group, following the termination of CPB, while there was no eNOS and Hsp72 mRNA detected, there was an abundance of eNOS *(see section 6.4.2)* and Hsp72 *(see section 6.4.3)* protein present. Could this suggest that by the time point '5 minutes post CPB' in this study, the RNA turnover process leading to eNOS and Hsp72 protein synthesis was complete, leaving only the final products of this process i.e. eNOS and Hsp72 protein, with no detectable mRNA for these proteins? To answer this questions, the expression of eNOS and Hsp72 mRNA in myocardial samples from earlier time points in this study need to be determined and, comparisons made with other patients in the HBO_2 and Control Groups.

7.6 Future Molecular Work

To further develop a functional method for the molecular analysis of myocardial eNOS, iNOS and Hsp72 mRNA, using the samples obtained from study, the following future work is proposed:

a. As optimisation of the conditions for use of the purchased eNOS and iNOS primers were not done, a set of experiments will be required to confirm the optimal conditions required for the function of these purchased primers, using an appropriate polymerase enzyme and PCR cycling conditions.

b. Reducing cDNA contamination during the reverse transcriptase process by preparing cDNA in a room that is separate from the room where PCR products are analysed. In addition to this, it would be important to verify that the purity of the cDNA is >1.65 before using in the PCR process.

c. Further optimisation of the RT-PCR process is required. As the best PCR result in this study were obtained when individual PCR components were used, as was done in *section 7.4.3.1*, as part of the second step of the Two Step RT-PCR, perhaps a return to this method of RT-PCR, using purer cDNA, may lead to more positive outcomes following gel electrophoresis. The use of individual PCR components, rather than a commercially available master mixes, will allow for further individualisation and optimisation of each of the PCR

components in particular, the magnesium and primer concentration used.

d. mRNA analysis in myocardial samples from time points earlier than the time point '5 minutes post CPB' in the HBO_2 Group. This will allow comparisons to be made with other time points in this group and hence enable deductions to be drawn on the effects of HBO_2 preconditioning on mRNA expression and mRNA turnover in this group of patients.

e. mRNA analysis in myocardial samples from patients in the Control Group. This will enable a comparison to be made with the HBO_2 Group and conclusions to be made on the possible molecular cardioprotective effects of HBO_2 preconditioning in this study.

8. Discussion

8.1 Cardioprotection Via Systemic HBO_2 Remote Preconditioning?

This small, pilot, proof of concept, phase 2, randomised controlled clinical study provides some level 2a evidence (Phillips B, March 2009) that remotely preconditioning CAD patients with systemic HBO_2, prior to first time elective on CPB CABG, with the use of intermittent ischaemic fibrillatory arrest, may be capable of leading to beneficial clinical, health economic and biological outcomes in a low risk group of patients. It met its primary objective and demonstrated that this modality of treatment was capable of improving myocardial LVSW following CABG. In addition to this, it was also observed that it may potentially improve peri-operative SV, pulmonary vascular flow prior to CABG and may also be a safe modality of treatment as it had the capacity to limit myocardial injury and post operative AEs. Furthermore, it was also shown that it may even be a cost effective treatment as it reduced the post operative length of stay in ICU.

Biologically, where the serum biomarkers were concerned, this study indirectly provided some evidence to suggest that HBO_2 preconditioning of this group of patients may be capable of facilitating endothelial protection by possibly causing the shedding of endothelial and/or platelet and, neutrophilic adhesion molecules into the circulation, hence leading to an increased post operative circulating load of soluble adhesion molecule. This

may lead to less neutrophil-endothelial adhesion and thus limit the ensuing deleterious consequences of this interaction. It was also observed that preconditioning with HBO_2 prior to ischaemia and reperfusion leads to a reduction in the amount of eNOS and Hsp72. This indirectly suggests *(see section 6.5)* that NO from the catalytic action of eNOS and Hsp72 are functioning to protect the myocardium via their individual mechanisms of action, hence accounting for the limited detection of free eNOS and Hsp72 in the myocardium. Furthermore, it was also observed that HBO_2 preconditioning may be able to further induce the endogenous expression of these biomarkers thus providing additional protection to the myocardium during the period of ischaemia and reperfusion.

All these observations, appear to suggest that preconditioning CAD patients with HBO_2, prior to on CPB CABG and IRI, may have the ability to enhance the cardiovascular protection available to this group of patients to allow for the better tolerance of the stress of IRI during CABG.

8.2 Study Limitations

Despite encouraging results from this study, the strength of the evidence is weak, particularly with respect to the secondary objectives. This is a consequence of the major limitation of this study, which was that the sample size was not large enough to assess multiple secondary endpoints. The 90% probability (power of the study) of detecting a difference of only 7.5% between the groups, and hence the sample size determination, was only estimated to be sufficient to assess the primary endpoint between the groups. As the pre-

defined statistical plan did not include a statistical analysis for the correction for multiple statistical testing when comparing the differences in the secondary endpoints between the groups, it was not possible to draw any statistical conclusions from the secondary endpoints. Hence, firm statistical conclusions could only be drawn from the primary endpoint. In keeping with this, from the analysis of the secondary endpoints, only comparative descriptive estimates between the groups were possible. The statistical results which accompanied any of the comparisons between those descriptive estimates, only provided a sensitivity analysis for the observed difference in estimates of the secondary endpoints between the groups. While no firm conclusions could be made from the results of the secondary endpoints, where possible and reasonable, an appropriate explanation was offered for the observed differences.

The patients recruited to this study had a relatively low operative risk. As such, the actual impact of HBO_2 preconditioning may not have been easy to appreciate. This low risk group of patients was selected for this study as the safety of HBO_2 in this group of patients is not well documented. It is quite to assume that more encouraging results may have been observed if the patients recruited to this study had a higher operative risk. In the case report by Yacoub *et.al (Yacoub and Zeitlin, 1965)*, a clinically significant improvement was observed in a post operative, cardiac patient with low cardiac output following treatment with HBO_2. If a high risk post operative patient, following IRI, responded well to post operative adjunctive treatment involving HBO_2 (i.e. HBO_2 post conditioning), it is also possible that high risk pre-operative patients may be preconditioned with HBO_2, prior to

IRI, to enable better tolerance to the stress of IRI. Furthermore, in the clinical study by Thurston *et.al (Thurston, 1971)*, it was observed that AMI patients with more severe MIs, cardiogenic shock or heart failure, performed better following treatment with HBO_2 compared to those patients who were less unwell. This further re-inforces the suggestion that this modality of treatment may be more beneficial for high risk patients.

In this study, the Control Group of patients were, pre-operatively, slightly more unwell compared to those in the HBO_2 Group. The former had a higher mean EUROSCORE and, consisted of more patients with a history of unstable angina, previous MIs, hypertension and diabetes mellitus. While all these are chance occurance despite randomisation, it may have led to a selection bias in the Control Group, which resulted in the slightly poorer outcomes of this group. Furthermore, in this group, the mean ischaemic and CPB times were also slightly longer. An attempt to minimise the confounding effects of ischaemic and CPB time were done by performing linear multivariate analysis that adjusted for longest cross-clamp time and CPB time. Moreover, as the number of coronary bupass to be performed has an impact on the duration of ischaemia and reperfusion and, hence the degree of IRI during CABG, the number of coronary bypasses can also be regarded as another potential confounder. In retrospect, the confounding effect of this could have been limited by startifying patients according to the number of coronary artety bypasses that were plan for them pre-operatively.

Finally, there was also no attempt to measure ROS, NO, the expression of adhesion molecules on the neutrophilic and endothelial surfaces,

neutrophil-endothelial interaction or an assessment of the long term effects of HBO_2 preconditioning on quality of life. As such, it was not possible to correlate the changes in ROS and NO generation with the changes in the expression of surface adhesion molecules or the expression of serum soluble adhesion molecules and, the long term effects this may have had on quality of life.

8.3 HBO$_2$ Preconditioning: A Method For ROS Preconditioning

Essentially, HBO_2 preconditioning prior to ischaemia and reperfusion is a mode of pharmacological preconditioning involving ROS. Various studies have demonstrated that low doses of ROS (Hegstad et al., 1997, Valen et al., 1996, Ytrehus et al., 1995) and ROS preconditioning (Sun et al., 1996, Takeshima et al., 1997, Valen et al., 1998) leads to myocardial protection. In fact, it has also been demonstrated that ROS preconditioning leads to better myocardial protection than just IPC alone (Yaguchi et al., 2003). Both HBO_2 therapy (Conconi et al., 2003) and, ischaemia and reperfusion (Robin et al., 2007) generate ROS. By preconditioning patients with HBO_2, which indirectly means exposing them to a non-lethal, controlled dose of ROS, a complex set of yet to be fully understood, biological pathways are initiated. As suggested by this study, the two possible pathways that maybe induced by HBO_2 are the peri-operative increase in circulating soluble adhesion molecules and the increased expression of cardioprotective proteins

(e.g. eNOS and Hsp72) during ischaemia and reperfusion. As such, when patients are later exposed to a further, possibly lethal, burst of ROS during ischaemia and reperfusion, which normally leads to cellular injury and clinical dysfunction (Moens et al., 2005), not only is there a protective mechanism (as a result of myocardial eNOS and Hsp72 expression) already in place to enable better tolerance to this insult but, there is possibly also a diminished ability to propagate the insult of IRI as there may be a reduced capacity for neutrophilic attachment to the endothelium (due to the increase in circulating soluble adhesion molecules).

8.4 The Future For HBO_2 Preconditioning

The area of HBO_2 preconditioning is intriguing as it opens up the concept that prophylactic treatment with HBO_2 may be capable of inducing cellular protection to enable better tolerance to a subsequent stressful event. Despite interesting, the future of HBO_2 preconditioning remains unclear. This is primarily because the field of HBO_2 medicine is still not widely appreciated as a possible treatment option in any particular area of unmet medical need. As such, it is not an area of high priority academic or pharmaceutical research. Moreover, as a modality of treatment without a specific disease area to treat, interest in using HBO_2 to treat patients is further impeded by the lack of it’s availability in major tertiatry hospitals. Where it is available, its feasibility for providing treatment to patients, particularly the critically ill, is challenged by chamber design and size issues which may make it unsuitable for routine clinical use. This particular obstacle has recently been addressed by a number

of centres around the world (USA, Sweden, Norway and Australia), by customising the design of HBO_2 chambers so that they resemble and function more like hospital wards and ICUs. This has enabled these centres to address the other challenge to HBO_2 medicine, which is the shortage of well-conducted randomised control clinical trials to demonstrate its efficacy and safety across therapeutic areas. While there are ample experimental studies and case reports demonstrating the biological and potential clinical benefits of HBO_2 treatment, there has been a shortage of clinical trials which support its beneficial use in a patient populations.

HBO_2 treatment is after all a therapy which involves the use of a drug, oxygen, at a dose of 100%, that is delivered at a pressure above atmospheric pressure, in a pressurised chamber. Just like any other drug, for HBO_2 to become a drug that is confidently used, its characteristics as a drug in clinical practice needs to be better understood. This can only be done by appropriately designed randomised control clinical trials to assess its clinical efficacy, safety and longer term side effects in a wide variety of clinical indications, other than CAD. Of particular interest would be the area of oncology and stem cell therapy. Early clinical trials appear to indicate that there may be a role for HBO_2, as a chemotherapeutic drug to adjunct other oncological agents, in the treatment of cancer (Ohguri et al., 2009). Where stem cells are concerned, HBO_2 treatment has been shown to enhance stem cell growth (Milovanova et al., 2009), mobilisation (Thom et al., 2006) and survival (Khan et al., 2009) and, thus may be utilised to adjunct to therapies which rely on stem cells.

With respect to HBO_2 preconditioning, CABG, IRI and myocardial protection, larger randomised control clinical studies are required to determine the not only the early effects of HBO_2 on myocardial function but also its delayed effects and, its durability over a longer period of time. Studies should be designed to compare HBO_2 to the current standard of care (SOC) treatment for inducing myocardial protection during CABG and IRI (e.g. anaesthetic agents). Instead of attempting to prove that HBO_2 is better than or superior to the current SOC treatment, studies should be designed to demonstrate the equivalence or non-inferiority of HBO_2 treatment compared to the SOC treatment. This is because demonstrating equivalence or non-inferiority, provides physicians with an alternative means, which is just as good or no worse than the current SOC treatment but, possibly safer and more tolerable, for optimising their patient's clinical condition prior to a planned clinical oxidative stress such as IRI as a result of vascular interventions. This message is not conveyed in a superiority trial which only demonstrates one drug as being better than the other instead of demonstrating one drug as being just as good as the other. As echoed by the Cochrane Collaborative (Bennett et al., 2005), methodological rigour is crucial in the assessment of the effectiveness of HBO_2 as a treatment option. As the awareness of the potential therapeutic benefits of HBO_2 preconditioning and, the commericial interest in this modality of treatment grows, so will more robust clinical studies. While the momentum in this area of research builds up, HBO_2 therapy will be highly reliant on on small focus groups that continue to pasionately investigate the its benefits.

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