INVESTIGATING THE ROLE OF NITRIC OXIDE CLONES IN CARDIOVASCULAR CONDITIONS

by

Sayqa Arif

A thesis submitted to the University of Birmingham for the degree of Doctor of Philosophy

School of Clinical and Experimental Medicine

College of Medical and Dental Sciences

University of Birmingham, UK

August 2014

UNIVERSITY OF BIRMINGHAM

RESEARCH THESIS

Author's Declaration

Full name (block capitals, surname first): ARIF, SAYQA

Full title of thesis/dissertation (block capitals): INVESTIGATING THE ROLE OF NITRIC OXIDE CLONES IN CARDIOVASCULAR CONDITIONS

College/School/Department (block capitals): SCHOOL OF CLINICAL AND EXPERIMENTAL MEDICINE, COLLEGE OF MEDICAL AND DENTAL SCIENCES

Date of award of degree (leave blank):

1. I understand that one printed and one electronic copy of my thesis/dissertation (the Work) will be deposited in the University Library (the Library) and in a suitable electronic repository, for permanent retention.
2. Without changing the content, the Library or any third party with whom it has an agreement to do so, may convert either copy into a format or medium for the purpose of long-term retention, accessibility and preservation.
3. The Library will publish, and/or arrange with appropriate third parties for the non-exclusive publication of, a bibliographic description of the thesis/dissertation, and the author's abstract.
4. Unless arrangements are made to the contrary, (see paragraph 6. below), the Library is authorised to make the Work available for consultation in the Library, and via a recognised inter library loans system. The Library is authorised to make the electronic copy of the Work freely accessible to individuals and institutions - including automated agents - via the Internet.
5. Rights granted to the University of Birmingham through this agreement are entirely non-exclusive. I retain all my rights in the Work in its present version or future derivative works.
6. I understand that I may apply to the University to retain the right to withhold access to the content of my thesis/dissertation. Access to the paper version may be withheld for a period which shall not normally exceed four calendar years from the congregation at which the degree is conferred. The actual length of the period will be specified in the application, together with the precise reasons for making that application. The electronic copy may be withheld from dissemination via the web or other networks for any period.
7. I have obtained permission for any use made of substantial amounts of published or unpublished copyright material (text, illustrations, etc) where the rights are owned by a third party, other than as permitted under either The Copyright Designs and Patents Act 1988 (as modified by any related or successor legislation) or the Terms and Conditions of any Licence governing its use.
8. The content of the copies I shall deposit with the Library will be the final version of my thesis, as approved by the Examiners.
9. I understand that the Library and administrators of any electronic theses repository do not hold any obligation to take legal action on behalf of myself, or other rights holders, in the event of a breach of intellectual property rights, or any other right, in the material deposited.
10. I understand that, in the event of my thesis/dissertation being not approved by the Examiners, this declaration will become null and void.

Signature: 

Date: …22 AUGUST 2014……

For Library use (please leave blank):

Classmark: **Accession number:** **Control number:**

eTheses Repository url:

Abstract

Previously thought to be inert, nitrite is now deemed to be an important endogenous source of nitric oxide (NO), particularly during hypoxia and ischaemia. Largely animal and some limited human studies propose a role for nitrite in 'hypoxic vasodilatation' and ischaemia-reperfusion injury.

One study explores the effect of systemic nitrite infusion on cardiac and peripheral haemodynamic parameters in healthy subjects, during normoxia and hypoxia. Despite a lack of observed effects in healthy subjects, a study performed in patients with heart failure during normoxia demonstrated favourable haemodynamic effects on cardiac output, albeit at high doses of nitrite. In patients undergoing coronary artery bypass surgery, low dose nitrite infusion afforded protection against ischaemia-reperfusion injury. Several mechanisms of nitrite reduction to NO have been described and remain to be fully elucidated. The role of one of these putative mechanisms, namely mitochondrial aldehyde dehydrogenase (ALDH2) was explored during normoxia and hypoxia. *Ex vivo* human vascular ring studies confirmed ALDH2 as an important nitrite reductase, in contrast to *in vivo* observations in the forearm vasculature, suggesting that numerous mechanisms are involved *in vivo* which are harder to isolate. Furthermore, this hypoxia vasodilatory role was not replicated in with GTN in the human forearm vasculature.

Acknowledgements

The research and preparation of this thesis would not have been possible without the help of several people.

Firstly, I would like to thank Professor Michael Frenneaux, for giving me the opportunity to undertake research under his guidance, and for his continued support and patience throughout. I am indebted to Dr Melanie Madhani for her invaluable guidance and assistance with the laboratory analyses, and for her generous support in helping me to obtain a British Heart Foundation junior fellowship to complete my research. I would also like to express my gratitude to the late professor Robert Bonser, for his direction and support in the undertaking of the surgical study. The studies would not have been completed without assistance from my peers - Dr Roger Beadle, Dr Ashvini Menon, Dr Julian Ormerod, Ulasini Naidoo and Rebekah Weaver for their assistance with the conduct of these studies. I also owe gratitude to Dr Martin Feelisch and Bernadette Fernandez (Southampton University) for plasma analysis of nitric oxide metabolites.

I would like to thank the British Heart Foundation who kindly funded this research.

I would like to thank my parents for their vision and support in understanding the importance of education contrary to cultural traditions. Finally, I would like to thank my husband, Jahangir and my children, Aisha and Ismail, for their overwhelming support and patience during the time taken in the preparation of this thesis.

Statement of Contribution to Research

The studies were conceived and designed following discussion with my supervisor, Professor Frenneaux. The laboratory analyses and the ALDH2 study (Chapter 6) was conceived and designed with further assistance from Dr Melanie Madhani.

Execution

I obtained all regulatory/ethical approvals for the studies. I undertook all subject recruitment for the studies. The systemic vascular study described in Chapter 2 was undertaken by myself. The study described in chapter 3 was conducted jointly with Dr Julian Ormerod, with myself undertaking the invasive cardiac and haemodynamic measurement with Swan-Ganz catheterisation. I performed the forearm venous occlusion plethysmography studies, described in Chapter 4 and Chapter 6. The vascular myography studies were performed with assistance from Dr Melanie Madhani. Dr Madhani and her assistance Aine O'Sullivan performed the Western Blot, plasma isoprostane and plasma cyclic GMP analyses. Plasma nitrite/nitrate analysis was performed by Bernadette Fernandez (Southampton University).

Data analysis

I collated and analysed all the data myself.

Published abstracts/conference presentations

1. **Sayqa Arif**, Abdul R Maher, Julian OM Ormerod, Sindu Mohan, Melanie Madhani, Michael P Frenneaux. The Acute Haemodynamic Effects of Intravenous Sodium Nitrite in Healthy Volunteers during normoxia and Hypoxia

Abstract [EHJ Sept 2010; 31(Suppl 1):704] and poster presentation

ESC Congress, Stockholm September 2010

2. **Sayqa Arif**, Bilal Ladak, Shrusti Tripathy, Melanie Madhani, Michael P Frenneaux

Nitrates do not exhibit a hypoxia-specific vasodilatory effect similar to nitrite

Poster presentation

Physiology Society UK Meeting, Manchester June 2010

3. **Sayqa Arif**, Abdul R Maher, Julian OM Ormerod, Sindu Mohan, Melanie Madhani, Michael P Frenneaux. The Acute Haemodynamic Effects of Intravenous Sodium Nitrite in Healthy Volunteers during normoxia and Hypoxia

Abstract [Heart June 2010; 96(Suppl 1):A36] and poster presentation

BSC Conference, Manchester June 2010

Table of Contents

List of Tables

List of Figures

Chapter 1: General Introduction

1.1 Overview

A significant breakthrough in Medicine was the finding that the gaseous free radical, nitric oxide (NO) is an important bioactive vasodilator and signaling molecule in the cardiovascular system. NO is integral to a vast array of functions *in vivo* which include smooth muscle relaxation and vasodilatation; modulation of cardiac contractility; protection against ischaemia-reperfusion injury; central nervous system neurotransmission; peripheral nervous system neurotransmission affecting gastrointestinal, respiratory and genitourinary function; platelet aggregation and host and defence mechanisms (1). In 1980, Furchgott and colleagues demonstrated that endothelial cells release an 'endothelium-derived relaxing factor' (EDRF) which contributes to vascular dilatation (2). Years later, Moncada and Ignarro independently reported that this EDRF was NO, a substance more commonly recognised as a toxic pollutant and lung irritant (3, 4). Prior to these discoveries, Murad et al. had established that NO relaxes vascular smooth muscle through the activation of soluble guanylyl cyclase (sGC) raising the possibility of NO as an endogenous molecule (5). The significance of these discoveries was reflected in the award of a Nobel Prize for the key researchers concerned. Over the last three decades, a great deal of attention has focused on the mechanisms of NO release/production both *in vitro* and *in vivo*, and its role in numerous physiological and pathophysiological processes.

1.2 Endogenous NO production

The main mechanisms of *in vivo* NO production now appears to be well established. These are outlined below.

1.2.1 Enzymatic production of NO

The majority of endogenous NO is generated using L-arginine as a substrate, resulting in formation of NO and L-citrulline (6). In the vascular endothelium this occurs in response to shear stress or a host of vasoactive substances present in the blood stream such as acetylcholine (Ach), bradykinin, substance P and adenosine (1), as depicted in **figure 1.1**.

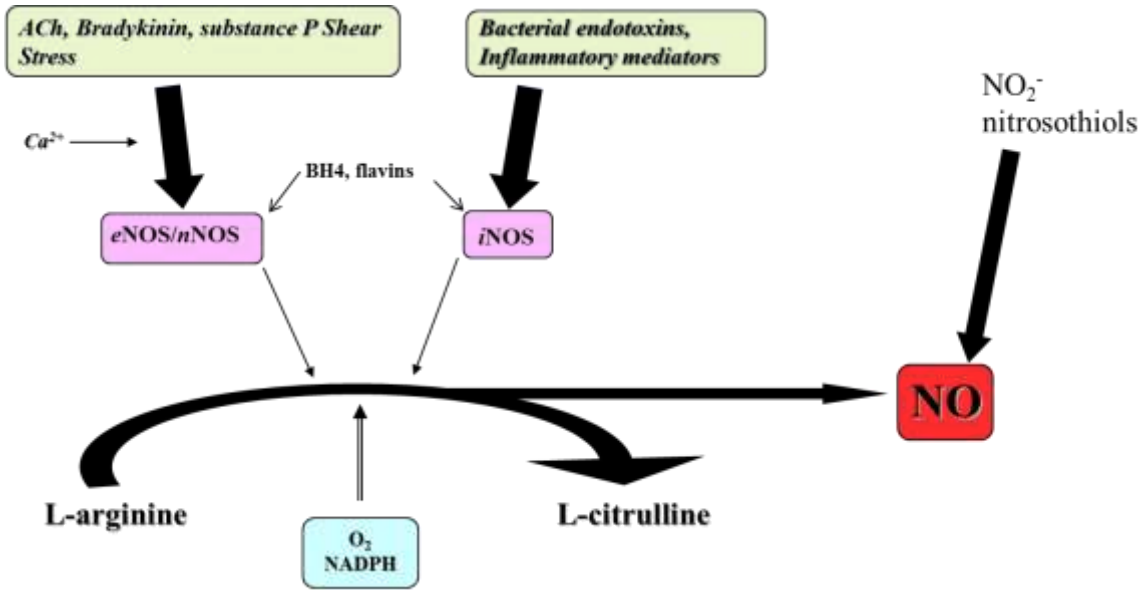


Figure 1.1: Simplified summary of NO production *in vivo*.

In addition to the availability of L-arginine, several key components are required for this process to occur. Firstly this reaction is catalysed by a group of enzymes called NO synthases (NOS) which are found throughout the human body (4, 6). Three well recognised isoforms of this enzyme exist, namely neuronal NOS (nNOS or NOS-1), inducible NOS (iNOS or NOS-2) and endothelial NOS (eNOS or NOS-3) (7). They are found in abundance in a variety of mammalian tissues and all three are expressed in the heart in myocytes and/or

endocardial and coronary artery endothelial cells (8, 9). nNOS and eNOS are constitutive enzymes that are activated by intracellular calcium/calmodulin. iNOS is a calcium-independent, inducible enzyme that is regulated at the level of gene transcription, in response to pro-inflammatory cytokines and bacterial endotoxins and is thought to be responsible for the vasodilatation seen in septic shock. It is present in low levels normally but is capable of generating large quantities of NO when activated (10). A fourth NOS enzyme, mitochondrial NOS (mtNOS) has also been discovered, and is thought to be important in modulation of mitochondrial respiration (11, 12). However there is some controversy surrounding the presence of a separate mtNOS. Whether this is a distinct NOS enzyme or a variant of the other NOS enzymes, which has undergone modification within the mitochondria, remains uncertain (13, 14).

Although the expression may vary in different tissues and conditions, all NOS enzymes ultimately lead to NO production through a series of redox reactions and contribute to the majority of *in vivo* NO generation (15). Endothelium-derived NO production via eNOS, is thought to increase basal human blood flow by 25-30%, but when stimulated this effect is much greater (16). Secondly, essential co-factors for NOS function (particularly eNOS and nNOS) include nicotinamide adenine dinculeotide phosphate (NADPH), tetrahydrobiopterin (BH_4), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and haem proteins (1, 17). A third integral component for adequate function of NOS enzymes is the availability of oxygen. The usual L-arginine-NOS pathway is attenuated as oxygen levels decrease with a consequent reduction in NO bioavailability (18). A reduction in any of these substrates/co-factors results in reduced NOS activity and reduced NO generation, leading to endothelial dysfunction which has been described in many disease states, e.g. diabetes, atherosclerotic disease and most cardiovascular conditions (19). One important such process of NOS

dysfunction is NOS uncoupling, whereby NOS is unable to convert L-arginine to L-citrulline. In this process, an electron is removed from from NADPH and donated to molecular oxygen to generate superoxide (O_2^-) instead of NO. This O_2^- production ultimately leads to oxidative stress which has well recognised, deleterious effects on cardiovascular health (20). Depletion of BH4 is thought to be the most essential mediator of NOS uncoupling (21).

1.2.2 Non-enzymatic production of NO

The requirement of oxygen for the production of NO via the L-arginine-NO pathway has raised the question of NO bioavailability when oxygen tension falls – is NO manufactured by any other mechanisms? NO is a highly reactive gas and any that does not diffuse into surrounding tissue immediately is converted to stable products e.g. nitrite, nitrates, nitrosothiols (22-24). In ischaemic/hypoxic conditions, release of NO from these stable molecules via a NOS-independent pathway may play an important role in NO bioavailability (23, 25-27). Recent evidence suggests that the nitrite anion is not an inert molecule as originally thought, and may be recycled to generate NO in hypoxic/ischaemic conditions (26). The basis of this thesis is the nitrite anion (NO_2^-) and an investigation into its role in biological processes. Nitrite will be discussed in more detail from section 1.5 onwards.

1.3 Fate of NO within the vasculature

1.3.1 cGMP dependent processes

As depicted in **figure 1.2**, following production NO can undergo several processes to effect signalling and downstream changes to alter biological function. NO generated by endothelial cells through activation of eNOS, diffuses into the vascular smooth muscle where it activates

the haem-containing NO receptor, sGC (5). This in turn stimulates production of the second messenger cyclic guanosine monophosphate (cGMP) from intracellular guanosine-5'-triphosphate (GTP). cGMP acts on downstream effectors e.g. protein kinases (PKG), initiating a cascade of phosphorylation reactions to facilitate cGMP-dependent relaxation of vascular smooth muscle and inhibition of platelet aggregation (28-30). Alternative intracellular downstream targets of cGMP include cGMP-gated cation channels and cyclic nucleotide phosphodiesterases (30).

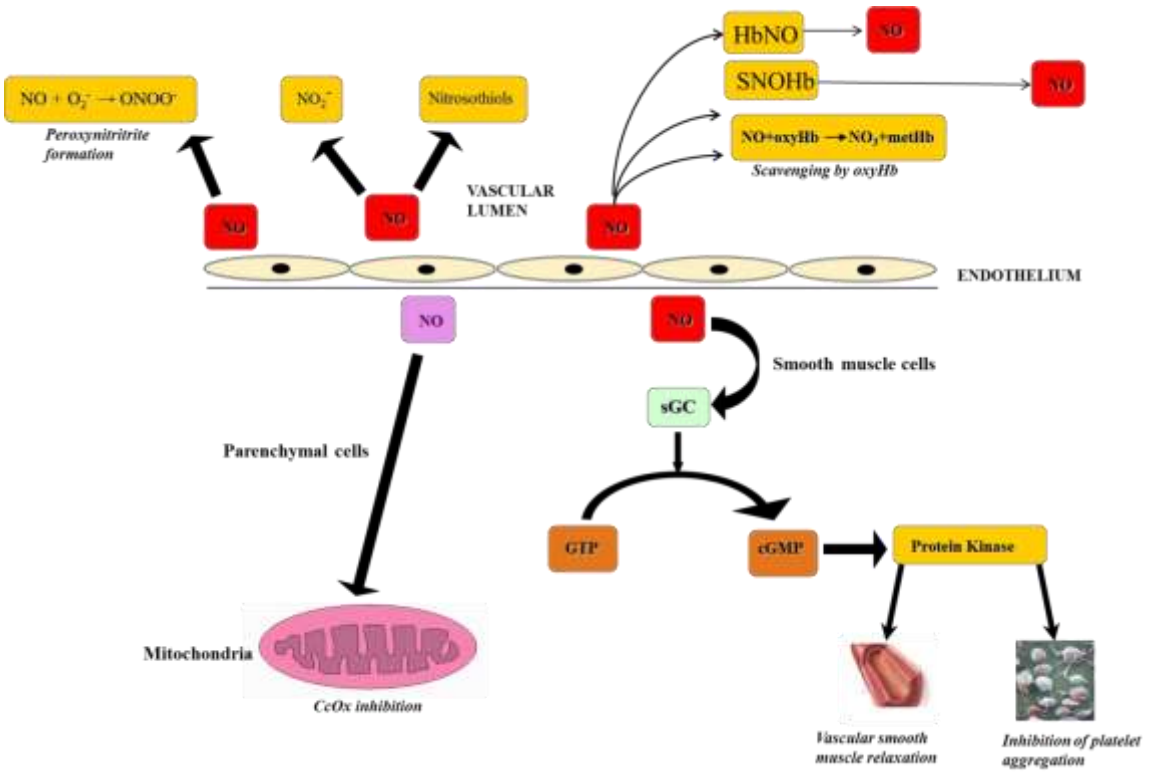


Figure 1.2: The fate of NO within the vasculature.

1.3.2 cGMP independent processes

Within mitochondria, cytochrome c oxidase (C*c*Ox) is a key component of the mitochondrial respiratory chain and hence oxidative phosphorylation and ATP generation. At normal physiological concentrations, NO competes with oxygen to reversibly bind and inactivate C*c*Ox, thus reducing mitochondrial oxygen consumption (31). This process is thought to exert beneficial effects by increasing the bioavailability of oxygen and allowing deeper penetration of oxygen into tissues (32). Furthermore it is thought that this pathway may be important in regulation of hypoxic vasodilatation(33).

NO is a highly reactive molecule and any that remains within the vascular lumen is rapidly converted to other, more stable metabolites. Its half life in the vasculature is in the range of 2milliseconds to less than 2 seconds (34). The majority of NO diffuses into erthyrocytes and is scavenged heavily by oxyhaemoglobin (oxyHb) to form methaemoglobin (metHb) and nitrate through the following reaction:-

$$NO + Hb(Fe^{2+})O_2 \rightarrow NO_3^- + Hb(Fe^{3+}) \quad (35)$$

The small amount of NO that remains within the plasma reacts with thiol (SH) containing amino acids, peptides and proteins to form S-nitrosothiols, a process termed S-nitrosylation (36, 37). These S-nitrosothiols include S-nitroso-albumin, S-nitroso-haemoglobin, S-nitroso-L-cysteine and S-nitroso-L-glutathione amongst others and they preserve NO bioactivity within blood/tissue (24, 38, 39). They are considered to be stable storage forms of NO, with

the ability to release NO in conditions of hypoxia/ischaemia when NOS enzyme activity is inhibited (40, 41).

Experimental studies suggest that an alternative method of stabilizing NO stores with the vasculature is through the formation of nitrite (NO_2^-), via either auto-oxidation to nitrite or an oxidation reaction catalysed by caeruloplasmin (42). Historically, nitrite was considered to be an inert biological by-product of NO metabolism, but studies in recent years suggest it is a stable endocrine store of NO which is able to release NO in conditions where the classical L-arginine-NOS pathway is inhibited, i.e. during hypoxia/ischaemia (26, 43-46).

Both the 'S-nitrosylation' and 'nitrite' theories of NO formation and release, particularly in conditions of low oxygen concentration are the subject of intense debate and the evidence to date is unclear as to which predominates. These theories are discussed in section 1.7.1 on hypoxic vasodilatation.

Unscavenged superoxide radicals (O_2^-) react with great affinity with NO to form the harmful peroxynitrite ($ONOO^-$):-

$$NO + O_2^- \rightarrow ONOO^- \qquad (47)$$

Peroxynitrite is a harmful substance which oxidizes the NOS co-factor BH_4, resulting in eNOS uncoupling and reduced NO synthesis, which in turn leads to further production of superoxide radicals (48). Generation of superoxide radicals and peroxynitrite has been implicated in a variety of pathological disorders, namely atherosclerosis, myocardial

infarction, myocarditis, heart failure, shock, diabetic complications and neurodegenerative and inflammatory disorders (49).

1.4 Biological functions of NO within the cardiovascular system

NO is a ubiquitous molecule involved in a variety of biological functions including regulation of vascular tone and smooth muscle cell proliferation, inhibition of platelet aggregation, anti-inflammatory effects and signalling within neural networks (50). NO derived from the endothelium accounts for a vast proportion of the biological functions exhibited by NO and some of these pertaining to the cardiovascular system are described herein.

1.4.1 Regulation of vascular tone

NO is critical in the regulation of vascular relaxation and tone, and hence blood flow. Shear stress, produced by blood flowing past the endothelium is the predominant stimulus for release of NO from the endothelium, which in turn alters vascular tone to meet the body's demands (50-52). Other stimuli include agonists such as acetylcholine or bradykinin which can also effect a change in vascular tone through release of NO from the endothelium (53). Endothelial NO diffuses into adjacent vascular smooth muscle cells and functions as a paracrine signalling molecule, where it activates sGC and through a cGMP-dependent mechanism results in the relaxation of vascular smooth muscle. As described in section 1.3.1, activation of cGMP results in activation of protein kinases. Protein kinase G (PKG) activates myosin light chain phosphatase, the enzyme that dephosphorylates myosin light chains, leading to smooth muscle relaxation. There are two main types of PKG – PKG I and PKG II. PKG I is primarily implicated in smooth muscle relaxation and platelet aggregation (54).

PKG I also causes a decrease in intracellular Ca^{2+} due to inhibition of Ca^{2+} entry into the cells (51).

Several animal and human studies have demonstrated the importance of NO generation from eNOS in vascular relaxation and tone (52, 55, 56). Lack of NO bioavailability either due to attenuated production or accelerated breakdown, is considered a hallmark of endothelial dysfunction. Endothelial dysfunction is an independent predictor of cardiovascular mortality and morbidity and, is associated with a range of cardiovascular conditions including atherosclerosis, hypertension and coronary artery disease (57).

NO is also important in the control of smooth cell proliferation, and studies have shown that endogenously produced NO limits proliferation in numerous cell types, not just vascular smooth muscle cells (58).

1.4.2 Inhibition of platelet aggregation

Platelets serve as another target of NO-mediated activity. Endothelial cells release NO which can diffuse into platelets to prevent platelet adhesion and aggregation (59, 60). Additionally, NO produced by eNOS can act in an autocrine manner to regulate platelet function and inhibit platelet aggregation (61-63). This process is effected through the activation of sGC and cGMP to release PKG. Several mechanisms of PKG have been identified as the route via which platelet aggregation is inhibited by NO, and these include recruitment of sarcoplasmic reticlulum, ATPase – dependent refilling of intra-platelet Ca^{2+} stores, phosphorylation of thromboxane A_2 receptors, direct cGMP activation of cyclic adenosine monophosphate (cAMP) and inhibition of phosphatidylinositide 3-kinase (PI3-K) activation (64). Further

evidence suggests that NO can regulate platelet function via cGMP independent methods (65). Although the exact mechanisms still remain to be fully decribed, it is well established that NO serves a critical function in inhibiting platelet aggregation and controlling platelet function *in vivo*.

1.4.3 Modulation of myocardial function

A wealth of studies with differing results exist, regarding the role of NO in regulation of myocardial function, some of which are covered very briefly here (66). The mammalian heart expresses all three forms of NO synthases and NO can be derived from the endothelium of the coronary blood vessels, cardiomyocytes and nerve terminals (54). *n*Nos and *e*NOS are constitutively expressed whereas *i*NOS is expressed primarily during pathological states such as in heart failure (67) or particularly following septic shock with its associated cardio-depression (66). Contractility can be affected by NO indirectly through regulation of coronary vascular tone or directly through NO release from cardiomyocytes (68). Animal models have demonstrated that NO can, at low concentration (<0.05µM) causes an increase in myocardial contractility and at high concentration (>10µM) can exert a negatively inotropic effect (69, 70). These effects can be mediated through both cGMP-dependent and independent pathways (68). The heart rate can also be affected by NO. In a canine model, inhibition of NO was shown to reduce heart rate independently of an increase in baroreceptor stimulation (71). A direct effect on cardiac contraction was observed particularly from *n*NOS-derived NO (72). Deleterious effects of NO have been observed, whereby in a canine model of pacing-induced heart failure, Chen and colleagues noted an increase in NO synthesis in the failing heart primarily from *i*NOS. *i*NOS inhibition resulted in an increase in cardiac contractility (67). However, Shinke et al. reported that release of endogenous NO was

associated with a reduction in oxygen consumption through attenuation of the myocardial contractile response (73). In contrast, Recchia et al. noted a fall in basal cardiac NO production in heart failure (74). Other studies have demonstrated a role for the NO-sGC-cGMP cascade in the prevention of adverse remodeling post-myocardial infarct (75-77). These studies compromise a small number of experimental studies investigating NO and merely reflect the vast array of interactions/processes involving NO within biological systems, many of which remain to be fully discovered.

1.4.4 Modulation of mitochondrial biogenesis and function

Some studies suggests that NO plays an essential role in mitochondrial biogenesis and modulation of mitochondrial function. NO-mediated mitochondrial biogenesis is to some extent contributed to by eNOS-related regulation of blood flow to and therefore, oxygen supply to mitochondria (41) Other data suggest that NO generated by eNOS plays a role in mitochondrial biogenesis in a cGMP-dependent manner. Nissoli and colleagues observed an increase in mitochondrial DNA in a variety of cell types such as brown adipocytes. They demonstrated a reduction in mitochondrial biogenesis in the brown fat of eNOS knock-out mice. Moreover, these mice had a reduced metabolic rate and accelerated weight gain as compared to wild-type mice (78). The same group later reported that this effect is not restricted to specific cell/tissue types (79).

The role of NO in modulating mitochondrial function is more complex. Mitochondria are considered the ‘workhouse’ of cells in terms of energy production and respiration. NO has an impact on cell physiology and cell death through its effects on the mitochondria. This NO may be produced within the mitochondria or be transported into the organelle from outside

(80). Briefly, within mitochondria, the electron transport system and the oxidative phosphorylation system comprise 'the mitochondrial respiratory chain.' This respiratory chain is located on the inner mitochondrial membrane and consist of 5 protein complexes (I to V) and two electron carriers (coenzyme Q and cytochrome *c* oxidase) which generate energy in the form of adenosine triphosphate (ATP) (81-83). NO at physiological concentrations, can inhibit mitochondrial respiration by competing with oxygen and reversibly binding to C*c*Ox. This has first demonstrated in *in vitro* studies with isolated mitochondria by adding NO donors to mitochondrial preparations (84), and later *in vivo* in animal models (85, 86). However, C*c*Ox has to be severely inhibited for this to translate into a significant reduction in total body oxygen consumption. This is thought to be a protective mechanism to prevent severe inhibition of C*c*Ox by NO under normal physiological conditions (87). The interaction between NO and C*c*Ox also results in generation of particularly high levels of reactive oxygen species (ROS), regulation of mitochondrial membrane potential, activation of AMP-kinase, regulation of glycolysis, modulation of mitochondrial Ca^{2+} release, modulation of transcription factor activity and induction of cytoprotection (88).

Furthermore, it is well recognised that NO inhibits autophagy through several mechanisms including S-nitrosylation (89). NO causes irreversible inhibition of several mitochondrial enzymes, either directly or indirectly. NO-mediated nitrosation of thiol residues on creatinine phosphokinase irreversibly disrupt ATP production (90). Peroxynitrite (which diffuses into the mitochondria from the cytosol or that generated through the reaction of NO with reactive oxygen species) can react with NO to irreversibly damage enzymes involved in the citric acid cycle and inhibit glycolysis, leading to irreversible mitochondrial damage (91).

Paradoxically, NO can both protect against ischaemia-reperfusion injury as well as causing cell apoptosis and/or necrosis, through its actions on the mitochondria. (88), (92, 93). The effect of NO on the membrane permeability transition pore (MPTP) and its role in cytoprotection is discussed in further details in section 1.7.2.2.

1.4.5 Role of NO in myocardial ischaemia-reperfusion injury

1.4.5.1 Ischaemia-reperfusion injury

Ischaemia is a pathophysiological process which occurs in a variety of clinical conditions such as acute myocardial infarction, stroke, cardiac surgery and peripheral vascular disease. If left untreated, an ischaemic insult will lead to irreversible cellular injury and ultimately cell death. The obvious treatment is well-timed reperfusion (restoration of blood flow) to the affected organ/tissue. However reperfusion in itself can add further to the ischaemic injury that has occurred. This phenomenon is known as 'ischaemia-reperfusion injury' (IRI) and can limit the benefits seen from restoration of blood flow, enhancing end-organ damage. It was first described in 1960 by Jennings and colleagues (94). The authors described the histological features of canine myocardium, which had undergone temporary ischaemia, followed by reperfusion. These features included cellular swelling, myofibril contracture, and disruption of the sarcolemma and intra-mitochondrial calcium phosphate particles. Although the majority of cellular necrosis occurs during ischaemia and/or early after reperfusion, the process of injury can be seen days after reperfusion (95). These mechanisms are described in detail in section 1.7.7.2.

1.4.5.2 NO and myocardial protection

Numerous studies have implied a protective role for NO in limiting the damage caused by IRI. Whereas other studies have raised controversy by failing to identify a clear protective role of NO and suggest that NO in the setting of IRI may actually be harmful (96). One theory to account for these contradictory findings is that cytoprotection afforded by NO may be a function of NO concentration. Low, nanomolar, levels of NO may stimulate cytoprotection whereas pharmacological (micromolar and millimolar) levels may mediate cell death (97).

Current opinion attributes NO-mediated protection against IRI to occur via both eNOS and/or non-enzymatic-mediated NO production. Inhibition of bradykinin (and therefore NO release) was associated with diminished myocardial protection (98). Administration of adenosine (through activation of eNOS and subsequent NO release) has also been shown to be cardioprotective against IRI (99). Opening of mitochondrial-ATP potassium channels (100), inhibition of MPTP (101) and activation of the reperfusion-injury salvage kinase pathway (102) are important mechanisms in protection against IRI. NO release has been shown to be an important step in these processes (103, 104). NO-mediated myocardial protection against IRI has been demonstrated to occur in both cardiac pre- and post-conditioning (68).

1.5 Nitrite

As described previously, NO is a highly reactive molecule which is readily scavenged in plasma to form alternative, more stable stores of NO (34). One such stable storage product is nitrite. Nitrite was originally thought to be an inert by-product of NO metabolism however, it is now regarded as a significant source of endogenous NO under certain conditions (26).

Nitrite can be generated in humans either endogenously or obtained exogenously from dietary sources or the environment.

1.5.1 Endogenous nitrite production

In the human body, nitrite is generated endogenously by two main methods:-

(1) Endogenous L-arginine-NO-pathway

NO is readily oxidised to NO_2^- and nitrate (NO_3) in blood and tissue, and accounts for the majority of nitrite production (105, 106). Approximately 70% of nitrite in mammals is considered to be derived from oxidation of NO (43, 107). Historically, NO auto-oxidation was thought to be the main method of nitrite generation from NO (108). However, an integral discovery regarding nitrite production was that caeruloplasmin (a multi copper oxidase) converted NO to NO^+ with further hydration to nitrite (109). Shiva and colleagues demonstrated substantially lower plasma nitrite levels in caeruloplasmin knockout mice and in humans with congenital acaeruloplasminaemia. This process is proposed to offer an alternative to auto-oxidation of NO, especially in view of the rapid scavenging properties of oxyhaemoglobin which compete with auto-oxidation. During pathological states, such as sepsis where *i*NOS expression increases, plasma nitrite and nitrate concentrations are markedly elevated also (110, 111).

(2) Endogenous entero-salivary circulation of nitrates

An alternative source in humans is dietary nitrate which is recycled to nitrite via the entero-salivary circulation, as depicted in **figure 1.3**. Firstly, dietary nitrates are absorbed into the blood stream, with up to 25% being taken up and concentrated in the salivary glands (112).

This uptake into the salivary glands is thought to be facilitated by the anion exchange channel, Sialin (113). Thereafter, these salivary nitrates are reduced to nitrite by salivary reductase enzymes in the oral cavity, with NO_2^- reduction to NO occurring within the stomach. The remaining nitrite is absorbed by the stomach to be converted to NO in blood and tissue (114-117). It is estimated that about two-thirds of nitrite, of dietary origin is produced in this way with one-third being obtained from the ingestion of cured meat products (117).

The concentration of nitrite found to occur in human plasma is reported to be between 100 – 600nM (43, 118, 119). Furthermore, endogenous nitrite is found primarily within tissue rather than in plasma (38, 120).

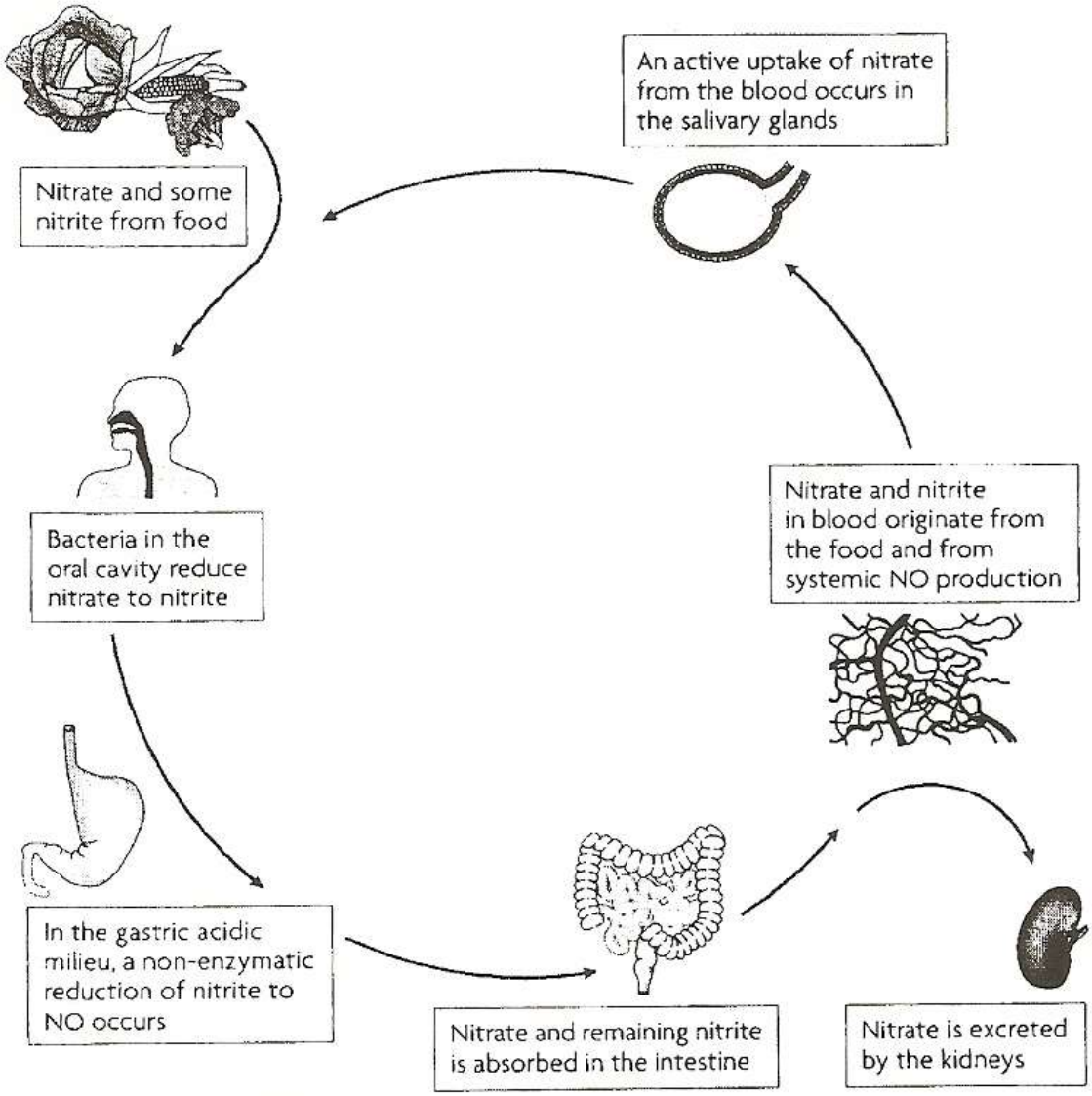


Figure 1.3: The entero-salivary circulation of nitrates in humans

(117)

1.5.2 Exogenous sources of nitrite

Dietary sources account for the majority of exogenous nitrate and to a lesser degree nitrite (116). Nitrate-rich foods (particularly leafy green vegetables) are associated with an increase

in plasma levels of both nitrates and nitrite (121). Nitrite has been used for centuries as a food preservative, and cured meat products represent a direct source of nitrite (122). Environmental sources of nitrite also include pollutant nitrogen oxides from cigarette smoke and car exhaust fumes, contaminated groundwater commonly due to fertilizers, livestock, and human excreta (123).

1.6 NO production from nitrite

The generation of NO from nitrite has been the subject of intense debate and research over the last two decades. Oxygen is crucial for the synthesis of NO via the conventional arginine-NOS-NO pathway (1). In conditions where there is a lack of oxygen e.g. during hypoxia and/or ischaemia, the ability of NOS to generate NO is thus limited. Nitrite has emerged as a source of NO in these situations (117). Numerous mechanisms for nitrite-mediated NO reduction, independent of the oxygen-dependent eNOS pathway have been investigated.

1.6.1 Acidification of nitrite

Acidification (or acid disproportionation) of nitrite has been shown to generate NO from nitrite independent of enzymes, as follows:-

$$NO_2^- + H+ \rightarrow HNO2$$

$$2HNO_2 \rightarrow 2N_2O + H_20$$

$$N_20_3 \rightarrow NO + NO_2$$

(124)

This reaction occurs primarily in the acidic stomach. In the presence of ascorbic acid or polyphenols (sourced from the diet), there is greater NO production with less generation of other nitrogen oxides such as nitrous oxide and di-nitrogen trioxide (125, 126). The resultant NO is thought to play a role in gastric defence mechanisms against microbial organisms and in regulation of gastric blood flow (124, 127). In theory, this mechanism can occur in any tissue where pH has been significantly reduced. Zweier and co-workers demonstrated this in cardiac ischaemic cardiac tissue where NO production significantly increased following administration of nitrite (25). The pH of ischaemic cardiac tissue can decrease below 6 and it was proposed that in this environment there is nitrite-mediated reduction to NO via acid disproportionation of nitrite (128). This mechanism has also been demonstrated in vascular tissue preparations and ischaemic cardiac tissue, *in vitro* (129-133).

1.6.2 Haemoglobin and deoxyhaemoglobin

The interaction between nitrite and haemoglobin was noted over 100 years ago when Haldane proposed that, in salted meat products nitrate was first reduced to nitrite which then underwent deoxyhaemoglobin-mediated reduction to NO (134). In 1937, Brooks expanded on this reaction further and suggested that nitrous acid reacts with deoxyhaemoglobin to generate NO and methaemoglobin (equation 1). The NO then binds a second deoxyhaemoglobin to produce iron−nitrosyl-haemoglobin (equation 2):-

$$NO^{2-} + HbFe^{2+} + H^{+} \rightarrow NO + HbFe^{3+} + OH^{-} \qquad \text{(equation 1)}$$

$$NO + HbFe^{2+} \rightarrow HbFe^{2+}\text{-}NO \qquad \text{(equation 2)}$$

In 1981, Doyle and colleagues, delineated the exact biochemistry of this reaction and reinforced that nitrite reacts with deoxyhemoglobin (in the presence of protons) to produce methaemoglobin and NO (136).

Haemoglobin-mediated nitrite-dependent NO synthesis has been proposed as an explanation for hypoxic vasodilation, but the evidence that haemoglobin (especially oxyhaemoglobin) acts as a potent NO scavenger has rendered this hypothesis the subject of intense controversy (45, 137-139). Haemoglobin de-oxygenation is closely associated with the physiological oxygen gradient, with the greatest rate of de-oxyhameoglobin-mediated nitrite reduction occurring when haemoglobin is 50% saturated with oxygen (Hb P_{50}) (140). This was reported by Cosby and co-workers who observed that the vasodilatory effects of nitrite at near physiological and supra-physiological concentrations correlated with an increase in NO production. NO prodyction was measured by the rate of formation of iron-nitrosylated haemoglobin, which increases as haemoglobin oxygen saturation decreases (26). Some of the controversy surrounding haem-protein mediated nitrite reduction concerns the mechanism of NO-escape (produced by nitrite reduction) from the scavenging properties of these haem proteins both within and outside of the red blood cells (RBC). It remains to be fully determined whether NO formed from the reaction of nitrite with deoxyhaemoglobin is directly exported from the red blood cell or is transformed into another NO adduct (RXNO) to mediate nitrite-induced vasodilation elsewhere (123). Grubina et al. proposed that at low oxygen saturations the reductive deoxyhaemoglobin-nitrite reaction dominates whereas at high oxygen saturations the oxidative oxyhaemoglobin-nitrite reaction dominates. The two reactions are deemed to occur to a similar degree at Hb P_{50} (138). At physiologically low

haemoglobin oxygen saturations, nitrite will thus preferentially react with deoxyhaemoglobin to produce NO, rather than be incapacitated by oxyhaemoglobin to the physiologically inactive nitrate. An alternative theory suggests that the NO is converted to more stable substances such as S-nitrosothiols which then effect NO-mediated signaling/processes (41). This will be discussed further in section 1.7.1 on hypoxic vasodilatation.

1.6.3 Myoglobin

With a wealth of studies investigating the role of haemoglobin as a nitrite reductase, the role of myoglobin has been explored also (141). High concentration of myoglobin is found in erythrocytes (20 mM) and cardiac/skeletal muscle (300–900 μM) respectively (142, 143) Oxymyoglobin has been shown to catalyse the conversion of nitrite to NO 60 times faster than deoxyhaemglobin (144), 144, 145). The levels of nitrite (0.5 - 10μM) in tissue e.g. heart, liver, brain, are greater than in blood (120, 145). In these tissues myoglobin may function as a hypoxia-specific NO donor. Myoglobin has been found to serve as a nitrite reductase at a very low oxygen tension because of its high oxygen affinity (139). Two recent studies have demonstrated the presence of myoglobin in the murine vascular wall where it contributes to nitrite-mediated vascular relaxation, particularly under hypoxic conditions (146, 147).

1.6.4 Neuroglobin

Neuroglobin is a haem protein that is of uncertain physiological significance, and may promote survival of neurones under hypoxic conditions (148). One recent *in vitro* study by Tiso and co-workers has postulated that neuroglobin serves as an important nitrite reductase

in conditions of oxidative stress (149). Further pre-clinical studies are required to investigate this mechanism.

1.6.5 Cytoglobin

In comparison to other haem proteins, cytoglobin is relatively newly discovered ubiquitous molecule, with the first literature report of its existence published in 2002 (150). Recently, Li and colleagues demonstrated cytoglobin-mediated NO generation from nitrite and sGC activation under hypoxic conditions in isolated smooth muscle cell preparations (151). Again, further studies are required to investigate its properties as a significant nitrite reductase.

1.6.6 Enzymatic reduction

Several enzymes are implicated in nitrite reduction to NO, which include xanthine oxidoreductase, aldehyde oxidase, eNOS and mitochondrial aldehyde dehydrogenase (ALDH2).

1) **Xanthine oxidoreductase** (XOR) is an enzyme involved in purine metabolism and catalyses the conversion of hypoxanthine to xanthine which is further converted to uric acid. XOR has been found in endothelial, epithelial and connective tissue cells (152). The role of XOR in the conversion of nitrate to nitrite has been described previously (153-155). Following on from this, published data has established its role in the reduction of nitrite to nitric oxide also (156-158). This conversion has been subsequently demonstrated in rat and human hearts and in rat kidney (27, 159). Recently, Webb and colleagues demonstrated that human blood vessels and red blood

cells reduce nitrite to NO through the enzymatic conversion of XOR (160). One commonality to these observations is that nitrite reduction to nitric oxide occurs preferentially in very hypoxic/anoxic and/or ischaemic conditions. Dejam and colleagues did not observe any diminution of nitrite-mediated forearm vascular response following inhibition of XOR during normoxia in humans (161). Recently, Ghosh et al. demonstrated a nitrite-mediated reduction in blood pressure in humans. This was attributed largely to the nitrite reductase activity of erythrocyte-XOR (162).

2) **Aldehyde oxidase** is a cytosolic enzyme, similar in morphology to XOR (163). A study performed in rat cardiac and hepatic tissue under anaerobic conditions measured NO generation in the presence of aldehyde oxidase (164). The investigators reported that inhibition of aldehyde oxidase in tissue resulted in attenuated NO production, but not in blood, with oxygen tension, and pH levels being reported as important regulators of aldehyde oxidase-mediated NO production. These findings were confirmed in a later study by the same group which sought to characterise aldehyde oxidase-mediated nitrite reduction further. (165).

3) **eNOS** was shown to catalyse the reduction of nitrite to NO during anoxia in a recent study using cultured endothelial cells (166). Webb and colleagues have provided further *in vitro* support of this mechanism when they demonstrated that during hypoxia alone (without acidosis) eNOS facilitates the conversion of nitrite to NO in red blood cell supernatants obtained from human blood samples and rat blood vessels (160). In a study of renal IRI utilising eNOS-knockout mice, the benefits of nitrite-mediated protection against renal IRI was attenuated/absent in eNOS-knockout mice compared to wild-type animals (167). These are interesting observations as eNOS has

conventionally been thought to be an oxygen-dependent NO generator from L-arginine with nitrite as a by-product of this conversion.

4) **Mitochondrial aldehyde dehydrogenase** (ALDH2) was identified by Chen and colleagues as an important enzyme involved in the bioconversion of GTN to 1,2-GDN in animals (168). They suggested that nitrate tolerance may be caused by ALDH2 inhibition. Furthermore, they proposed that nitrite may undergo reduction to NO via ALDH2. The role of ALDH2 in the bio-activation of organic nitrates has been confirmed in subsequent *in vitro* and *in vivo* animal studies (168-170). This was corroborated in healthy volunteers when it was shown that the forearm vascular response to GTN was attenuated when the ALDH2 enzyme was inhibited by disulfiram (171). Evidence suggests that nitrite is a major product of GTN bio-activation (172). Additionally, other experimental studies suggest that mitochondria may be involved in the reduction of NO_2^- to NO (173, 174). Therefore, it is feasible to hypothesise that ALDH2 may be important in nitrtite reduction to NO. Contrary to these studies, Philpott and colleagues demonstrated that administration of GTN in patients undergoing coronary artery bypass grafting surgery, rapidly inactivates vascular ALDH, and that this inactivation occurs prior to the development of significant nitrate tolerance, and prior to detectable impairment of GTN bioconversion, arguing against this as the central mechanism responsible for tolerance of organic nitrates (175). Perlman and colleagues recently reported a dose-response relationship for the cardio-protective effect of nitrite in a rat model (176). Using a proteomic approach they showed that the phosphorylation of ALDH2 consistently correlated with cardio-protection. As NO_2^- has been discovered to be an important source of NO during hypoxia and/or ischaemia, ALDH2 may be an important nitrite

reductase during these conditions and whether ALDH2 is of prime importance in nitrite-mediated vasodilatation also remains unknown. This mechanism needs elucidating further and how this translates into physiological effects is as yet unknown, and will provide the basis of some of the experiments for the purposes of this thesis.

1.6.7 Mitcohondrial enzymes

Some components of the mitochondrial respiratory chain have been identified as possessing nitrite reductase activity e.g. complex III (177) and Complex IV (178). These studies have demonstrated that under conditions of hypoxia or even anoxia and reduced pH, nitrite is reduced to NO at a cellular level *in vitro*. These findings have yet to be confirmed *in vivo*.

1.7 Downstream effects of nitrite

The role of nitrite in modulating biological function has concentrated predominantly on two focal concepts – (1) hypoxic vasodilatation and (2) protection against IRI.

1.7.1 Nitrite and hypoxic vasodilatation

It is well recognised that as haemoglobin oxygen concentrations drop, there is an increase in local blood flow to tissues to try and restore oxygen supply to meet metabolic demand (179). This occurs in the local vasculature of organs and is termed 'hypoxic vasodilatation.' Important steps of hypoxic vasodilatation, as for most physiological mechanisms include a stimulus, a sensor, a signal and an effector. In hypoxic vasodilatation, the stimulus is hypoxia and the effector is vascular smooth muscle (41). To date, the underlying hypoxic sensing and

effector signaling mechanisms remain to be fully elucidated. NO has been proposed as an effector of hypoxic vasodilatation (180, 181). However, a considerable amount of controversy surrounds the role of NO (and therefore nitrite-derived NO) in effecting hypoxic vasodilatation. It has been demonstrated that haemoglobin within erythrocytes play an important role in hypoxic vasodilatation by acting as the sensor that transduces the hypoxic stimulus into a signal via allosteric transformation from the R-state (relaxed) to the T-state (tense), with the formation of NO (144, 182, 183). The resulting interactions of and mechanisms of departure of NO from the red blood cell (RBC) into the vasculature, to effect hypoxic vasodilatation without being scavenged by haemoglobin first, remain unclear. It has therefore, been suggested that nitrite-derived NO cannot contribute significantly to hypoxic vasodilatation. The resulting NO, particularly in erythrocytes, would be heavily scavenged by haemoglobin before it can exert its vasodilatory effect (184).

An alternative theory proposes that NO reacts with nitrosyl-adducts (e.g. thiols or albumin) to form more stable, products such as nitroso-haemoglobin (Hb-SNO) which can then effect hypoxic vasodilatation (41, 182). How this Hb-SNO exerts its effect remains to be fully determined. One theory is that the SNO-Hb is facilitated by Anion Exchanger 1, a protein found in abundance in the RBC membrane where it facilitates anion exchange (185). SNO-Hb may exit the RBC using other transporters such as ATP-binding cassette protein or Km transporters (186). However, conflicting studies have failed to show sufficient circulating SNO-Hb concentrations to effect hypoxic vasodilatation (187-189). Other substances including ATP, adenosine, vasoactive peptides, carbon dioxide, protons and potassium ions have also been proposed as effectors of hypoxic vasodilatation (190).

Despite this controversy, the hypoxic vasodilatory effect of nitrite itself has been observed in several studies. Interestingly, this was demonstrated as far back as the 19th Century when Brunton reported in the *Lancet* that amyl nitrite reduced both angina and blood pressure (191). This was reported again in 1937 and subsequently confirmed in 1953 (192, 193). However, high concentrations of nitrite (100 - 1000μmol/L) were required to induce vaso-relaxation in isolated aortic rings (193). Some interest was re-ignited when arterio-venous gradients of nitrite were observed in the human forearm vasculature with an augmentation of these gradients during exercise and regional NOS inhibition (194). However, others demonstrated nitrite to be a relatively ineffective *in vivo* vasodilator in the forearms of healthy volunteers even with concentrations of up to 200μmol/L (195). This discrepancy between the micromolar concentrations required to demonstrate an effect *in vitro* and the nanomolar concentrations present *in vivo* led to the conclusion that nitrite was an insignificant physiological vasodilator. Later studies challenged Lauer and colleagues' findings signifying that nitrite may be important in physiological regulation of blood flow. Nagababu and colleagues observed that nitrite reduction at reduced oxygen pressures is a major source of erythrocyte NO (196). Cosby and co-workers observed nitrite-mediated vasodilatation in the human forearm vasculature at near physiological concentrations and relaxation of rat aorta with nitrite during hypoxia (26). A further study in isolated rodent aortic rings demonstrated attenuation of nitrite-dependent vasodilation at high haemoglobin oxygen saturation, with stimulation of vasodilation as haemoglobin oxygen saturation decreased to 50% (197). In a separate study using isolated rabbit vascular rings, Ingram and co-workers reported a greater dilatation of pulmonary artery as compared to aorta, with an enhanced hypoxic vasodilatation in all vessels (198). Maher and colleagues demonstrated exogenous intra-arterial nitrite administration only modestly dilates forearm resistance vessels, but potently dilates capacitance vessels under normoxic conditions (46).

Additionally, under hypoxic conditions, nitrite markedly dilated forearm resistance vessels. In a separate study, Ingram and colleagues demonstrated nitrite-mediated arterial and pulmonary vasodilatation following administration of systemic nitrite infusion in healthy volunteers(199). The clinical potential of these and further studies are reviewed in section 1.8.

There are limited explanations as to how nitrite causes hypoxic vasodilatation. One potential theory is that nitrite-reacted haemoglobin has a high affinity for the RBC membrane and that this high affinity contributes to RBC-induced vasodilatation by two pathways. Firstly the increased membrane binding activates glycolysis and the synthesis of ATP. This newly synthesized ATP is released from the RBC under hypoxic conditions. The released ATP interacts with purinergic receptors on the endothelium that stimulate the synthesis of NO by endothelial NO synthase. This reaction will induce vasodilation without requiring that NO be released from the RBC. Secondly, the interaction with the membrane, of intermediates formed during the reaction of nitrite with deoxygenated haemoglobin, stimulates the release of NO from these intermediates. NO released on the membrane can escape the large pool of intracellular haemoglobin and be released into the vasculature resulting in vasodilatation (200). Other theories of how nitrite exits the RBC, propose the use of transporters such as the Anion Exchanger 1 or through protonation to nitrous acid (201, 202). With the paucity of data in this area, the role of nitrite in hypoxic vasodilatation therefore remains controversial.

1.7.2 Nitrite and ischaemia-reperfusion injury

Several molecular processes are implicated in the development of ischaemia and IRI. An appreciation of these allows better understanding of methods of protection against IRI and, therefore are summarised herein.

1.7.2.1 Ischaemia

Ischaemia itself results in a cascade of changes which ultimately lead to cellular necrosis/death. The myocardium in particular is a highly efficient ATP generating organ, which is achieved through an abundance of mitochondria and largely aerobic metabolism. An ischaemic insult results in a reduction in rapid ATP depletion and high-energy intracellular phosphate due to lack of myocardial anaerobic metabolic pathways (203). A 90% reduction in myocardial ATP is thought to result in irreversible cellular damage (204, 205). Ischaemia is also associated with a decrease in intra-cellular pH, which is counteracted by proton (H^+) efflux through the sodium/proton (Na^+/H^+) exchanger but at the expense of intra-cellular Na^+ accumulation (206, 207). This coupled with an inactivation of ATPases, e.g. ATP-dependent Ca^{2+} re-uptake results in calcium overload. In combination with an initiation of the apoptotic cascade through various intra-celluar proteases and opening of the MPTP, cellular and ultimately tissue/organ death occurs (208).

1.7.2.2 Mechanisms of IRI

Timely reperfusion and hence re-oxygenation is known to prevent irreversible ischaemia-related cellular damage. However, it is well recognised that despite prompt restoration of blood flow to ischaemic tissue/organs, reperfusion itself can cause further injury (94). This

paradox is termed ‘ischaemia-reperfusion injury’ (IRI). Methods to inhibit the deleterious effect of IRI has been the ‘holy grail’ of many a research study due to its potential to improve clinical outcomes in several circumstances e.g. following an acute myocardial infarction (AMI), in cardiac surgery with cardio-pulmonary bypass, following organ transplantation and following cardiac arrest and have been extensively reviewed by others (209-211).

Several inter-related mechanisms are thought to be implicated in the development of IRI, and are summarised as follows:-

(1) Re-oxygenation and oxygen free radicals

During reperfusion, re-oxygenation of the ischaemic myocardium, although providing essential oxygen to the affected area, also adds to the injury through release of further oxygen free radicals (212, 213). Hearse and colleagues first reported this in an animal model, where they observed that severe myocardial injury developed with the onset of re-oxygenation in rats (214). During reperfusion, oxygen undergoes reduction leading to the formation of ROS such as superoxide anion (O_2^-), hydroxyl radical ($OH^\cdot$) and reactive nitrogen species such as peroxynitrite ($ONOO^-$) (215). Other sources of ROS include xanthine oxidase and NADPH oxidase (216). ROS are highly reactive molecules which are generated in both the endothelial cells and the myocytes and interact with the cell membrane resulting in oxidative stress and myocardial cell damage/necrosis (217). It has been postulated that ROS-mediated damage in the setting of IR involves the MPTP (218).

The contribution of oxygen free radicals to IRI has been reported in a variety of clinical settings of myocardial IRI (219, 220). In addition to the direct effects of oxygen free radicals, they also reduce the availability of NO and therefore the cardio-protective effects that arise

from it (216). The role of these oxygen free radicals, however, has been debated in the literature with inconclusive evidence in both animal and clinical studies protection afforded by antioxidant therapy (215).

(2) Changes in intracellular pH

As described, during ischaemia, intracellular and interstitial pH decreases as a result of anaerobic metabolism with lactate accumulation and ATP breakdown, resulting in excessive levels of protons (206, 207). Upon reperfusion, interstitial H^+ are washed out creating a proton gradient across the cellular membrane and resulting in activation of the Na^+/H^+ exchanger. This leads to extrusion of H^+ at the expense of an increase in intracellular Na^+ (75). This reduces intracellular acidosis, a protective element against hyper-contracture of the myofibrils, and loss of its protective abilities (221, 222). Additionally, consequent rise in Na^+ leads to activation of the Na^+/Ca^{2+} exchanger, further increasing the intracellular levels of Ca^{2+} (223).

(3) Calcium overload and hypercontracture

Elevated levels of intracellular Ca^{2+} are observed during reperfusion, which is significant in IRI (224). This is partly thought to be due to a sequence of downstream effects of rapid normalisation of extra-cellular pH following reperfusion, followed by activation of the Na^+/H^+ exchanger, resulting in stimulation of the Na^+/Ca^{2+} exchanger with the end result being substantial intracellular Ca^{2+} overload (225). Furthermore, it is suggested that ischaemia induces defects in the cell's viability to regulate Ca^{2+} such that upon reperfusion the cell accumulates toxic levels of Ca^{2+}. This leads to myocyte death by hyper-contraction of the myocardial cells, which may also result in cellular disruption and necrosis of adjacent cells (226-228). Development of hyper-contracture causes mechanical stiffness and tissue

necrosis. The contracture can affect the entire heart as may occur after global ischemia, e.g. during prolonged cardiac surgery (226).

(4) Mitochondrial Permeability Transition Pore

The Mitochondrial Permeability Transition Pore (MPTP) is a non-selective channel located on the inner mitochondrial membrane (229). Normally, the inner mitochondrial membrane is impermeable to the majority of metabolites/substances. Opening of the MPTP depletes the mitochondrial energy stores and initiates a cascade leading to cell apoptosis and necrosis (230). During the ischaemic phase of the insult, the MPTP remains closed and opens within the first few minutes of the reperfusion phase (231). Factors which promote MPTP opening include Ca^{2+} overload, raised phosphate concentration, restoration of pH and oxidative stress (with production of 0_2^-), all of which are critical mediators of IRI (232-234). It has been postulated that the MPTP opens within minutes after onset of reperfusion, when pH has started to recover (234). Opening of the MPTP is thought to signify the onset of irreversible cellular necrosis. The significance of this structure as a possible target in providing protection against IRI has been demonstrated in numerous pre-clinical studies, where inhibition of the MPTP confers protection against IRI (235, 236).

(5) Other mechanisms

Other important contributory factors in myocardial IRI include neutrophil activation and accumulation, platelet-mediated injury, augmentation of the complement and of the renin-angiotensin systems. Excessive neutrophils can produce oxygen free radicals, proteases and pro-inflammatory mediators and can contribute to leucocyte entrapment in the capillaries, leading to micro-vascular plugging (237, 238). Platelets also contribute to micro-vascular dysfunction and complement activation results in myocardial necrosis through direct cell

injury by increasing cell permeability and release of histamine and platelet activating factor and stimulation of neutrophil adherence and superoxide production (239, 240). Stimulation of the renin-angiotensin system has been associated with myocardial necrosis particularly following AMI (241). Blockade of the renin-angiotensin system is readily achieved through the use of angiotensin converting enzyme inhibitors or angiotensin-II receptor blockers which are widely available for treatment of cardiovascular disease. The use of these agents in studies, has demonstrated a reduction in reperfusion arrhythmias and myocardial infarct size with improved functional recovery following myocardial IRI (242).

1.7.2.3 Clinical manifestations of myocardial IRI

The clinical manifestations of the molecular processes which underpin myocardial IRI include myocardial stunning, reperfusion arrhythmias, endothelial dysfunction, myocyte necrosis and microvascular dysfunction including the 'no re-flow' phenomenon (226). These have been noted in several settings e.g. following an AMI treated by thrombolysis therapy/primary percutaneous coronary intervention (PCI), during cardiac surgery with the use of cardio-pulmonary bypass (CPB), during heart transplantation and following a cardiac arrest (209-211).

(1) Myocardial stunning

Myocardial stunning is a commonly encountered manifestation of IRI. It is defined as 'prolonged post-ischaemic mechanical dysfunction that persists after reperfusion of previously ischaemic tissue in the absence of irreversible damage, including myocardial necrosis' (243). This suggests that it is transient and reversible. Myocardial stunning is encountered in a variety of clinical scenarios where ischaemia and reperfusion are present -

restoration of blood flow following AMI through thrombolysis or PCI, in unstable or exercise-induced angina, following cardiac transplantation/ 'on-pump' cardiac surgery and following cardiopulmonary resuscitation (244-248). In the setting of cardiac surgery, the incidence of stunning has been reported to be as high as 60% and usually manifests as a low cardiac output state and a requirement for inotropic support (249). Length of complete recovery can be variable, ranging from a few days to upto 6 months (247, 250-252). Myocardial stunning is deemed a marker of poor prognosis, particularly after cardiac ischaemic events (253). Additionally, there is some evidence to suggest that myocardial stunning may lead to heart failure due to repeated episodes of myocardial ischaemia and reperfusion (254).

(2) Reperfusion arrhythmias

Reperfusion arrhythmias such as accelerated idio-ventricular arrhythmias, ventricular tachycardia or ventricular fibrillation typically occur after AMI and although easily treated if recognised, can be lethal (255). They often occur following the onset of reperfusion (256). It has been suggested that these arrhythmias are related to the sudden ionic changes or generation of oxygen free radicals (257, 258).

(3) Endothelial dysfunction

The first evidence of coronary endothelial dysfunction following IRI originated from Ku and colleagues who measured coronary vascular reactivity following 90 minutes of myocardial ischaemia and up to 120 minutes after onset of reperfusion(259). They observed that coronary endothelial dysfunction was a prominent feature of myocardial IRI. In a recent study, remote endothelial dysfunction has also been observed in rat mesenteric arteries following 60 minutes of myocardial ischaemia followed by 120 minutes of reperfusion (260). Interestingly,

there is some data which states that ischaemia alone is not associated with significant endothelial dysfunction (261, 262). It is during reperfusion that this injury predominantly occurs and is mediated through NO-dependent mechanisms, while endothelium-independent responses and smooth muscle responsiveness are maintained (263). Oxygen-derived free radicals appear to also play a significant role in IRI-associated coronary endothelial dysfunction (264, 265). Response to vasoconstrictors such as endothelin I, is enhanced and this coupled with the response to oxygen-derived free radicals leads to coronary vasoconstriction and reduced coronary blood flow (266). This endothelial dysfunction intensifies platelet and neutrophil activation further compounding IRI (267). In a canine study, coronary endothelial dysfunction was still present 12 weeks after the ischaemic event (268). In a further human study, coronary endothelial dysfunction was evident 6 months after AMI treated by early reperfusion (269).

(4) Myocyte necrosis and death

The myocardium can tolerate up to 15 minutes of severe and even global myocardial ischaemia without cardiomyocyte necrosis and death (270). Prolonged ischaemia resulting in irreversible injury followed by reperfusion leads to the development of 'contraction band necrosis' whereby the affected tissue swells and develops contraction bands (271). This signifies lethal irreversible injury which cannot be undone. In animal models, contraction band necrosis has been associated with sudden death (272).

(5) Micro-vascular dysfunction and the 'no re-flow' phenomenon

Micro-vascular dysfunction encompasses several processes including endothelial dysfunction, micro-vascular obstruction (downstream micro-embolism of platelets), de novo thrombosis, and neutrophil capillary plugging, oedema and oxidative stress (226, 273). It can

cause the ‘no-reflow’ phenomenon whereby restoration of blood flow to an ischaemic organ is often not fully restored after release of a vascular occlusion – a situation commonly encountered by Cardiologists in patients undergoing PCI (273). It can be associated with AMI, myocardial rupture and death (274, 275). No-reflow has been shown to be associated with a poor prognosis in several clinical trials and correlates with micro-vascular obstruction on cardiac magnetic resonance imaging (276-280).

1.7.2.4 Protection against IRI

(1) Ischaemic Conditioning

The clinical importance of IRI has resulted in decades of research investigating methods to protect against it. This is termed ‘conditioning’. Anecdotal evidence for this came from reports of reduced myocardial injury following AMI in patients with pre-infarct angina (281). In 1986, Murry et al. demonstrated that a period of sub-lethal ischaemia prior to an AMI reduced subsequent myocardial injury by 75% (282). This was termed ‘ischaemic pre-conditioning’ whereby the heart was primed for a lethal ischaemic insult with brief periods of preceding sub-lethal ischaemia. Two discrete time windows of ischaemic pre-conditioning (IPC) have been reported. The first (‘immediate’ or ‘early’) window of protection is seen immediately after the protective stimulus is administered and can last 2-3 hours (282). The the second (or ‘late’) window of conditioning occurs 12-24 hours following the first window of protection (283-285). This delayed protection although less powerful, appears to last 2-3 days (286). This biphasic response has been observed in human studies also (287, 288). Since Murry’s seminal paper describing this ‘classical’ method of IPC, subsequent experimental studies have investigated a variety of both pharmacological and non-pharmacological interventions which can effect conditioning. Furthermore, differences have been discovered

in the molecular processes underlying these two time windows of protection. Early IPC is achieved through rapid reactions which are complete in a short period of time e.g. activation of existing ion channels, receptors, existing enzymes etc. (289). Late IPC is dependent on changes in gene expression and new protein synthesis e.g. NOS, heat shock proteins and anti-oxidant enzymes (290, 291). Some of these reactions will be discussed in section (2) '*Mechanisms underlying ischaemic conditioning*, below.

Protection has also been observed when the conditioning stimulus is delivered following onset of reperfusion. This was first identified by Na and colleagues in felines whereby delivering a pre-lethal ischaemic insult prior to AMI reduced subsequent arrhythmias (292). In a subsequent canine study, a 44% reduction in myocardial infarct size was noted with improvement of post-ischaemic endothelial dysfunction following delivery of sub-lethal ischaemia in early reperfusion (293). The protective stimulus needs to be delivered early in reperfusion. Post-conditioning offers an attractive therapeutic option in the setting of conditions such as an acute AMI, where the timing of index ischaemia is unpredictable. Several animal and small-sized human studies have demonstrated significant reductions in AMI-related myocardial injury leading to publication of a Position Paper from the Working Group of Cellular Biology of the Heart of the European Society of Cardiology (294). Although, this paper does not advocate the widespread use of post-conditioning stimuli in clinical practice, it recognises its potential as an important adjunct to current AMI therapy and recommends further large-scale clinical trials to determine prognostic benefit.

(2) Mechanisms underlying myocardial ischaemic conditioning

In their entirety, both IRI and myocardial conditioning encompass a highly complex set of molecular and biochemical processes. Intense research into this area in recent years has

yielded an abundance of reactions and mechanisms integral to myocardial protection, in a variety of species and preparations. It is now accepted that myocardial conditioning comprises 3 broad components – triggers, mediator cascades and effectors. Crucial ingredients of this complex process include NO, protein kinase activation and mitochondria (295). Moreover, activation of these protective pathways appears to occur immediately after onset of reperfusion (296).

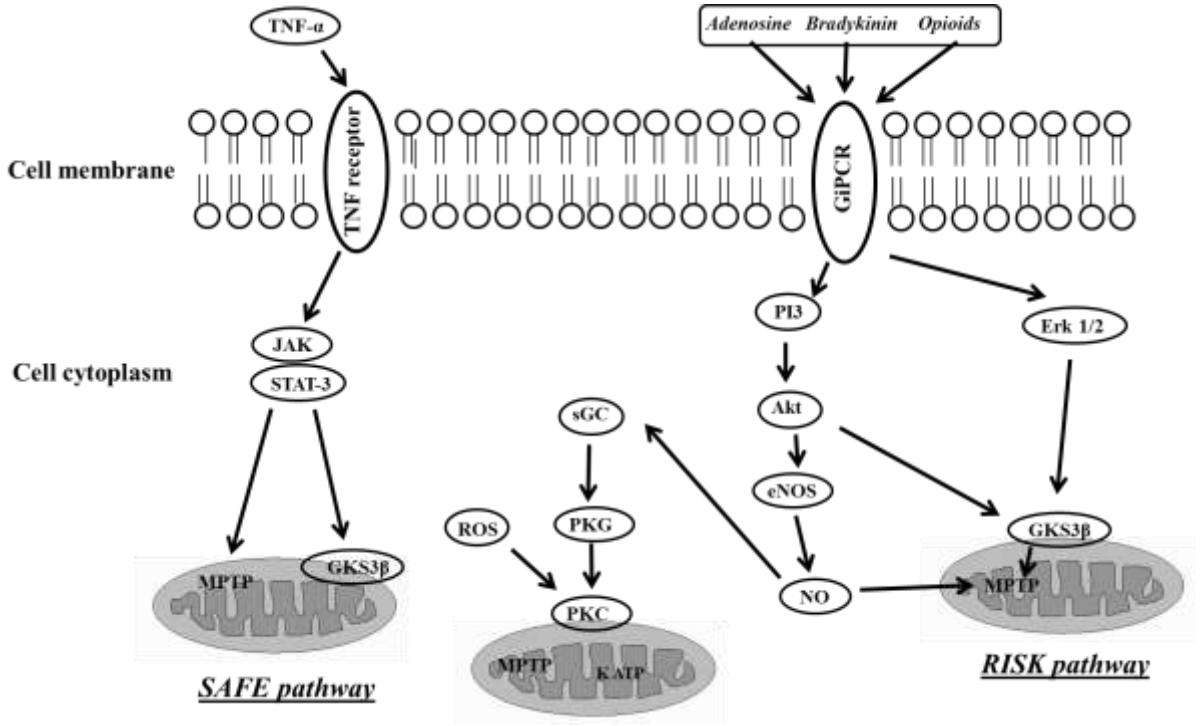


Figure 1.4: Molecular mechanisms involved in cyto-protection against IRI. The SAFE, RISK and PKC_NO pathways.

Firstly, triggers of conditioning include substances such as adenosine, bradykinin, or opioids (297-299). These are released during ischaemia and act on cell-surface receptors such as G_i

protein-coupled receptors (G_iPCR), initiating a cascade of signaling (mediator) pathways which include the 'Reperfusion Injury Salvage Kinase' (RISK) pathway and the 'Survivor Activating Factor Enhancement (SAFE) pathway, as depicted in **figure 1.4** (296, 300, 301). These in turn converge on a final common effector - the MPTP. The MPTP opens approximately 3 minutes after onset of reperfusion and is thought to signify the onset of irreversible cellular necrosis (231). Stimulation of cardio-protective mechanisms abrogate opening of the MPTP, with consequent reduction in IRI during ischaemic pre- and post-conditioning (235, 236, 302).

(3) Reperfusion Injury Salvage Kinases (RISK) Pathway

The RISK pathway was first described by Hausenloy and colleagues in 2004 (102). Research performed by this group provided two important insights into ischaemic conditioning -(1) classification of the RISK pathway and (2) the onset of reperfusion as the crucial phase of ischaemic conditioning via the RISK pathway (296). The salient components of this pathway are depicted in **figure 1.4**. Stimulation of receptors e.g. G_iCPR, leads to activation of the prosurvival kinases - phosphatidylinositol-3-OH kinase (P13K–Akt) and extra-cellular signal-regulated kinases (Erk 1/2). The role of these substances in ischaemic protection had been investigated prior to classification of the RISK pathway. Early *in vitro* experiments first demonstrated P13K–Akt-mediated protection against IRI (303, 304). Tong and colleagues confirmed these findings in an Langendorff rodent heart model, when they observed that inhibition of P13K–Akt abrogated the protective effect of IPC through a decrease in recovery of left ventricular developed pressure (305). Similarly, both *in vi*tro and *in vivo* experimental studies have confirmed a role of Erk1/2 in cardioprotection (306-308).

A variety of studies reveal these pro-survival kinases confer cardio-protection through phosphorylation of downstream effectors which include eNOS (309), p70S6K (310), Bcl-2-associated death promoter or BAD (311), protein kinase C (312) and Glycogen Synthase Kinase-3β or GSK-3β (313). Inhibitory phosphorylation of GSK-3β occurs during the lethal ischaemic period (305). It is recognised that inhibitory phosphorylation of GSK-3β increases the threshold and therefore prevents MPTP opening during reperfusion, thus promoting protection against IRI (302, 314).

(4) Survivor Activating Factor Enhancement (SAFE) Pathway

In 2009, Lecour described the SAFE pathway (**figure 1.4**) as an alternative cardio-protective signaling (mediator) process (301). The cytokine tumour necrosis factor-alpha (TNF-α) has been implicated in affording protection in several models of IRI (315, 316). It is postulated that TNF- α binds to its cell surface receptors, found in abundance on cardiomyocytes, which in turn stimulate 'janus kinase' (JAK) and the 'signal transducer and activator of transcription' (STAT) pathways (300). Inhibition of JAK-STAT was shown to increase apoptosis in rat cardiomyocytes (317). Activation of this pathway has been observed in both immediate and delayed IPC and post conditioning. Xuan and colleagues demonstrated a protective role due to up-regulation of the JAK-STAT pathway in a mouse model of myocardial IRI (318). A recent human study demonstrated enhanced activation of STAT-5 during early reperfusion in myocardial cells of pre-conditioned patients compared to controls (319). One of the downstream effects of JAK-STAT stimulation is inhibitory phosphorylation of GSK3-β (320). Most studies have evaluated the role of the SAFE pathway in IPC. TNF- α and STAT-3 have been implicated in ischaemic post-conditioning also (321, 322). Increasingly, inhibition of MPTP opening is recognised to be the final common effector of

cardiac conditioning pathways. As yet, a direct association between the SAFE pathway and MPTP opening has yet to be established.

(5) PKC_NO mediated ischaemic protection

A further important cardio-protective pathway involves both NO and PKC as crucial ingredients for mediating ischaemic conditioning. PKC is activated by 2 main mechanisms – (1) directly by cell surface receptors, e.g. G_iCPR or (2) indirectly by P13-Akt via upregulation of eNOS with consequent NO production. This NO in turn activates PKC via PKG. The process is completed by activation of mitochondrial ATP-dependent potassium channels (K_{ATP}) and formation of ROS (323, 324). High levels of ROS are known to be detrimental to cardiomyocytes, whereas in low levels they effect cardio-protective signaling which can occur through stimulation of mitogen-activated kinase (MAPK) and inhibition of opening of MPTP (217, 323).

(6) Mitochondrial - the final common effector

IRI protective pathways are believed to converge on the mitochondria. The MPTP is proposed to be the final common effector of the cardio-protective signaling cascades described. The MPTP is a non-selective channel located on the inner mitochondrial membrane. It was first described in 1976 (325) with further work in the 1980s signifying its role in cardio-protection (232). Griffith and colleagues first observed that the MPTP remains closed during ischaemia and opens following onset of reperfusion (231). In 2002, Hausenloy and co-workers reported that ischaemic pre-conditioning was effected through inhibition of the MPTP following onset of reperfusion. Administration of cyclosporine A (an inhibitor of MPTP opening) reduced infarct size in an isolated rat heart model of IRI by 24% (235). In 2005, Argaud et al demonstrated that post-conditioning in rabbits subjected to cardiac IRI, is

afforded through inhibition of MPTP (236). These earlier animal studies paved the way for further human studies investigating the role of the MPTP in IRI. Piot et al undertook a small, pilot study investigating inhibition of MPTP opening during reperfusion in patients with AMI undergoing PCI. They observed a reduction in myocardial infarct size as assessed by plasma creatinine kinase levels and on cardiac magnetic resonance (CMR) studies (326). Inhibition of MPTP opening was conferred through administration of intravenous cyclosporine A. Currently, a double-blind randomised-control trial called the MITOCARE Study is underway (327). Patient with an AMI undergoing PCI will receive placebo or TRO40303 (inhibitor of MPTP opening). The primary outcome measure will be reduction in infarct size (assessed as plasma creatinine kinase and troponin I area under the curve over 3 days). The main secondary endpoint will be infarct size normalized to the myocardium at risk (expressed by the myocardial salvage index assessed by cardiac magnetic resonance). Hausenloy's initial study in 2002 prompted extensive further research into the role of the MPTP as the end-effector of IRI (235). However much still remains to be elucidated about this channel and its exact role in the complex pathophysiology of IRI.

1.7.2.5 Evidence for nitrite in protection against IRI

Decades of research has explored the role of both pharmacological and non-pharmacological methods of cardiac protection against IRI. As described previously, NO has a well-established role in cardio-protective pathways. More recently, nitrite has emerged as an important *in vivo* NO donor, principally in acidotic and hypoxic conditions (117). Mounting evidence suggests that nitrite may have an important role to play in protection against myocardial IRI (328). Interestingly, the cardioprotective effect of nitrite through release of NO, was observed as far back as 1990 in a feline model of myocardial IRI, before the

bioactivity of nitrite was fully appreciated (329). Additionally, an association between ischaemic pre-conditioning and increases in cardiac/tissue nitrite and nitrate levels has been observed previously (330, 331). Years later, following renewed interest in nitrite as a bioactive source of endogenous NO, Webb and colleagues, using a Lagendorff rat heart model of IRI demonstrated nitrite-mediated cardio-protection. A 60% reduction in myocardial infarct size was recorded following nitrite (both 10 and 100 μM), with no abrogation of myocardial injury following administration of a NO scavenger (27). This cardio-protection was thought to be mediated through nitrite reduction to NO via XOR, promoted by the hypoxic and acidotic environment that occurs during ischaemia. Similar findings were noted in murine models of IRI by Duranski et al (332)and Shiva et al (333). *In vivo* studies corroborated these findings by demonstrating reduction in myocardial injury in rat, murine and canine models of IRI (334, 335). Gonzalez and colleagues explored the effect of low-dose nitrite infusion administered to dogs during myocardial ischaemia (336). Two differing nitrite administration protocols were utilised (1) a 60-minute infusion of 0.20μmol/min/kg (20 minutes) followed by 0.17μmol/min/kg (40 minutes); (2) a 5-minute infusion of 0.20μmol/min/kg immediately prior to reperfusion. The primary endpoint of infarct as a proportion of area at risk (assessed using CMR) was significantly reduced with both the 60-minute and 5-minute infusions from 70±10% in the placebo group compared with 23±5% ($p<0.001$) and 36±8% ($p<0.05$) in the nitrite-treated animals, respectively. In a recent rat study, cardio-protection observed following administration of inhaled NO was associated with an increase in plasma nitrite levels. Furthermore, intravenous nitrite produced a similar cardio-protective effect to inhaled NO, suggesting that nitrite is a potential biocarrier of NO activity (337). In a human study, the role of nitrite-mediated protection in a forearm model of IRI was explored. Administration of dietary nitrate in the form of beetroot juice (with a resultant increase in plasma nitrite) to 10 healthy subjects was associated with diminution of

ischaemia-induced endothelial dysfunction (338). In a murine cardiac arrest model, intravenous nitrite therapy improved cardiac function, survival and neurological outcomes (339). This study also provided important mechanistic data regarding nitrite molecular activity e.g. nitrite treatment restored intra-cardiac nitrite (depleted as a result of cardiac arrest), increased S-nitrosothiol levels, and positively modulated mitochondrial function. Recently, the same group have published preliminary data from an on-going phase I human trial of intravenous nitrite therapy in human cardiac arrest survivors exploring the safety and tolerability of intravenous nitrite administration, suggesting nitrite is safe to administer to humans in doses that confer cardio-protection in animals (340).

1.7.2.5 Mechanisms of nitrite-mediated cyto-protection

Despite an increasing number of studies detailing nitrite-mediated protection against IRI, there is a relative paucity of data outlining the exact mechanisms of this protection. The likelihood is that nitrite-mediated cardio-protection occurs primarily via generation of NO in the ischaemic tissue. NO-mediated cyto-protection occurs as described in section 1.4.5. However, NO-independent protective activity may also occur. Mitochondria are established to be important in IRI protection (295). It has been previously shown that nitrite is cardio-protective though modulation of mitochondrial function (333). A recent study demonstrated that mitochondrial biogenesis also occurs, independent of an NO-mediated pathway (341). Perlman and colleagues provided some further important insights into this field (176). Their experiments demonstrated that nitrite exhibits a dose-response relationship in affording cardio-protection. Low (0.1mg/kg) and high (10mg/kg) doses of nitrite were cardio-protective whereas intermediate (1.0mg/kg) doses had no effect. Furthermore, nitrite directly and through NO-release alters tissue redox status, alters transcription factor signaling, protein

expression and cardiac metabolism. Perlman's experiments also provided a greater understanding into the mechanisms of NO release from nitrite. Singh and colleagues demonstrated an abrogation of oxidative stress in rat myocardium subject to hypobaric hypoxia following supplementation of dietary water with nitrite (342). They observed an increase in transcription factors, anti-oxidant genes and cardio-protective molecules. Dezfulian's study, although investigating mechanisms of neuro-protection, have again demonstrated protection via modulation of mitochondrial function, and S-nitrosation independent of sGC-signaling pathways (340). Other examples lend support to NO-independent nitrite-mediated cyto-protection, e.g. nitrated lipids (which can form through a reaction between unsaturated fatty acids and nitrite) have been demonstrated to confer cardio-protection (343, 344).

To date, there is extremely limited data on mechanisms of protection against IRI in the human myocardium undergoing cardiac surgery, with no studies investigating the mechanisms of nitrite-mediated cardio-protection specifically. There is one human study of remote ischaemic preconditioning demonstrating STAT-5 activation in the left ventricular myocardium at early reperfusion after cardioplegic ischaemic arrest (319). This deficit in knowledge within this subject forms the basis of some of the experiments outlined herein.

1.8 Potential clinical applications of nitrite in man

1.8.1 Protection against ischaemia-reperfusion injury

As described in the previous section, one of the main therapeutic applications of nitrite includes protection of the myocardium against IRI. Clinical situations where this may be important includes in the settings of AMI (336), cardiac surgery (no *in vivo* animal human

studies), cardiac transplantation (no *in vivo* animal or human studies) and cardiac arrest (339). Nitrite-mediated tissue/organ protection has been demonstrated in other clinical conditions such as cerebrovascular accidents and sub-arachnoid haemorrhage (345, 346). There are several on-going clinical trials investigating nitrite-mediated protection in humans (www.clinicaltrials.gov).

1.8.2 Pulmonary arterial hypertension

Pulmonary artery hypertension (PAH) is associated with a poor prognosis with limited treatment options demonstrating a mortality benefit. Treatment is largely aimed at improving morbidity. Lack of NO bioavailability appears to be an important feature in the development of PAH (347). The majority of available therapeutic options enhance NO bioavailability e.g. phosphodiesterase-5 inhibitors (348, 349). The pulmonary vasculature is relatively hypoxic compared to the systemic vasculature, with an oxygen saturation of between 60-80% (350). The ability of nitrite, either directly or through release of NO, to induce vascular relaxation during hypoxic conditions can potentially be exploited in treating PAH.

Hunter et al induced PAH in new-born lambs by rendering them hypoxic or inducing vasoconstriction with thromboxane (351). They noted a 60-70% reduction in pulmonary artery blood pressure following administration of nebulised sodium nitrite. No significant change in systemic blood pressure was observed. Comparable findings were noted in an analogous rat model of hypoxia and thromboxane-induced PAH with intravenous sodium nitrite (352). In humans, Ingram and colleagues noted vasodilatation of the hypoxic pulmonary vasculature following administration of intravenous sodium nitrite (199). Interestingly, this vasodilatory effect persisted even after plasma nitrite concentrations had

returned to baseline levels. Furthermore, increased NO bioavailability may result in alteration of the adverse structural changes seen in the pulmonary vasculature in PAH as demonstrated in several animal models (353-355). Further human data is required to determine the potential clinical benefit of nitrite therapy in PAH.

1.8.3 Heart failure

Heart failure (HF) poses a significant health burden in the Western World and it is therefore not unsurprising that it comprises a significant proportion of the workload for Physicians and Cardiologists (356, 357). Studies have demonstrated nitrite to be a selective vasodilator in the human vasculature (46, 199). Nossaman et al studied the cardiovascular and pulmonary haemodynamic effects of nitrite and nitrates (glyceryl trinitrate, GTN) in rats (358) They demonstrated a significant reduction in pulmonary and systemic arterial blood pressure, although GTN was 1000-fold more potent than nitrite.

With its profile of relatively selective dilatation of systemic capacitance and pulmonary resistance vessels, nitrite is a potentially attractive treatment for decompensated HF. It would likely increase cardiac output, and decrease ventricular diastolic pressures with less reduction in blood pressure than would be seen with a potent arterial vasodilator such as GTN (359). To date, no human studies investigating the role of nitrite in heart failure exist.

1.8.4 Systemic arterial hypertension

Hypertension remains a leading cause of cardiovascular morbidity and mortality (360). Although, several pharmacological options exist to treat hypertension, dietary methods of

arterial blood pressure reduction have been intensely studied. Diets rich in fruit and leafy vegetables have been shown to lower blood pressure and provide beneficial cardiovascular effects (361). Some vegetables such as spinach, beetroot and lettuce, contain high levels of nitrate (121), some of which is converted to nitrite when ingested, as described in section 1.5.1. Therefore, it would seem intuitive that nitrite with its vasodilatory properties is a reasonable candidate to lower blood pressure.

In 2005, Larsen and colleagues demonstrated the blood pressure lowering effects of oral nitrate supplementation in healthy volunteers (362). 72 hour administration of oral sodium nitrate resulted in a significant reduction in mean and diastolic blood pressure associated with an increase in plasma nitrite levels. Webb and colleagues provided oral nitrate supplementation via the ingestion of 500mL of beetroot juice to healthy subjects, and compared it to placebo (water). They noted a significant reduction ($p<0.01$ for all) in systolic (10.4±3.0mmHg), diastolic (8.1±2.1mmHg) and mean (8.0±2.1mmHg) arterial blood pressure 2-3 hours after ingestion. This was attributable to increased nitrite levels because interruption of the entero-salivary circulation resulted in an abrogation of the plasma nitrite increase and blood pressure lowering effects. Kapil and co-workers investigated this one-step further by demonstrating a dose-dependent increase in plasma nitrite levels and associated blood pressure lowering effects, following oral administration of beetroot juice and inorganic nitrate supplementation (in the form of potassium nitrate) in healthy volunteers (363). Additional studies have reported a significant reduction in blood pressure in hypertensive patients with no previous pharmacological therapy receiving oral nitrate supplementation (162). Interestingly, the rise in plasma nitrite observed in these hypertensive subjects had previously been shown to have no effect on lowering blood pressure in healthy volunteers. However, a recent study by the same group utilised this ‘beetroot juice’ model of nitrite

supplementation in patients with diabetes with conflicting results (364). Following a 2-week intervention period, there was no significant change in blood pressure, endothelial function or insulin sensitivity, despite an increase in plasma nitrate and nitrite levels. These contradictory effects between health and disease-states require further exploration.

1.8.5 Other clinical applications

NO plays a key role in vascular function and remodelling, and therefore nitrite as a NO-donor may have the potential to induce angiogenesis and promote recovery in chronic ischaemic conditions such as peripheral vascular disease (PVD) (365). Kumar and colleagues demonstrated that administration of intravenous sodium nitrite improves ischaemic hind-limb blood flow, stimulates endothelial cell proliferation and stimulates angiogenesis in an NO-dependent manner (366). Other studies have observed differences in plasma nitrite levels in different groups of patients with PVD suggesting that altered NO bioavailability is important in the disease process (367-369). Again, no human data exists as to the role of nitrite in angiogenesis.

Revascularisation in the form of coronary or peripheral angioplasty/stent insertion can be complicated by an exaggerated healing process, namely intimal hyperplasia resulting in re-stenosis of the affected artery (370). Alef and colleagues have demonstrated an abrogation of intimal hyperplasia seen in isolated rodent and murine vessels, when sodium nitrite is administered prior to balloon injury (371). Whether this can be translated into human models of percutaneous coronary/peripheral arterial revascularisation with balloon angioplasty/stenting remains to be seen.

Septic shock is a life-threatening condition, with evidence suggesting that failure of the micro-circulation with resulting vasoconstriction may be an important pathophysiological manifestation (372). Pre-clinical studies suggest that NO donors may improve microcirculatory blood flow and tissue oxygenation with favourable mortality outcomes, despite the concurrent macro-vascular hypotension (373). As such, nitrite with its limited effect on arterial responsiveness could confer a potential benefit in patients with septic shock by improving micro-vascular blood flow especially in acidotic/ischaemic tissues.

Platelet inhibition is an important adjunct to coronary artery stenting to reduce the risk of stent thrombosis and other athero-thrombotic events (374). NO is well known to attenuate platelet activity *in vivo* (59, 60). A recent study demonstrated an abrogation (although not statistically significant) of platelet activity in healthy, human subjects following oral administration of nitrate/nitrite via reduction to NO (375). This requires further exploration in humans and disease states to confirm the role of nitrite as an adjunct to anti-platelet therapy.

1.9 Objectives

Despite great advances in our understanding of nitrite biology, much remains to be elucidated about the importance of nitrite in health and disease states, at both a mechanistic and clinical level. The research presented herein aims to expand on the workings of nitrite further and to add to the relative paucity of existing data in humans. Several, small scale studies have formed the experimental procedures undertaken and are summarised below.

(1) Following on from studies undertaken by Maher and Colleagues (46), we sought to determine the systemic effect of nitrite in healthy volunteers, to establish dose-response relationships during normoxia and hypoxia, and its effect on peripheral and cardiovascular haemodynamics (CHAPTER 2). We sought to investigate the effect of intravenous sodium nitrite on acute hemodynamic parameters in healthy volunteers, during both normoxia and hypoxia. We hypothesised the following:-

i. **While subjects are breathing room air, there would be no significant vasodilatation of the resistance vessels as verified by an unchanged arterial blood pressure and systemic vascular resistance;**
ii. **When subjects inspire 12% oxygen, rendering arterial blood relatively hypoxic, a significant reduction in arterial blood pressure and systemic vascular resistance would be observed.**

(2) A profile of relatively selective dilatation of systemic capacitance and pulmonary resistance vessels would be potentially attractive in the treatment of decompensated heart failure (HF) since it would likely increase cardiac output (CO), and decrease ventricular diastolic pressures with less reduction in blood pressure than would be seen with an arterial vasodilator (359). Therefore, the second series of experiments explored the effect of systemic nitrite administration in patients with advanced heart failure undergoing assessment for cardiac transplantation (CHAPTER 3). **We hypothesised that in patients with advanced HF, intravenous sodium nitrite infusion would reduce pre-load (as measured by a reduction in mean right atrial pressure), which would be associated with an increase in cardiac output, but without a change in arterial blood pressure or systemic vascular resistance.**

(3) Nitrite as a NO donor with its relative venoselectivity and attribute of specific hypoxic vasodilatation, could potentially offer an alternative therapy to the well-established inorganic nitrates (e.g. GTN) which are widely used in clinical practice. Whether this characteristic is truly unique to nitrite or inorganic nitrates exhibits a similar hypoxic vasodilatation remains unknown. A small experiment performed in healthy volunteers aimed to establish whether GTN exhibits hypoxia-specific vasodilatation in the forearm vasculature (CHAPTER 4). **We hypothesised that hypoxia would not augment GTN-mediated increase in forearm blood flow (as measured by venous occlusion plethysmography) as compared to normoxia.**

(4) With a wealth of pre-clinical data suggesting a role for nitrite in protection against ischaemia-reperfusion injury, we carried out a 'proof of principle' study in patients undergoing coronary artery bypass grafting (CABG) surgery investigating molecular mechanisms of nitrite-mediated cardioprotection (CHAPTER 5). We sought to determine:-

i. **The optimal timing of nitrite-mediated cardioprotection against IRI e.g. early window and late window.**
ii. **The optimal dose of nitrite required, for cardioprotection.**
iii. **We hypothesised that nitrite-mediated cardioprotection will occur via activation of the RISK pathway, as evidenced by an upregulation of signalling molecules involved in this pathway i.e. pAKT and eNOS.**

(5) ALDH2 is one enzyme that has been implicated in nitrite-mediated nitrite reduction to NO (CHAPTER 6). Its role in nitre-mediated reduction to NO in humans has not been fully explored. We hypothesised that:-

i. **In patients with HF, nitrite-mediated increase in forearm blood flow would be attenuated by ALDH2 inhibition.**
ii. **This attenuation of nitrite-mediated vascular relaxation would occur preferentially during hypoxia, as compared to normoxia.**

These experiments are discussed in further detail, in their respective chapters.

Chapter 2: Systemic Nitrite Infusion in Healthy Volunteers during Normoxia and Hypoxia

2.1 Introduction

During the last decade, the importance of nitrite in biological processes has been increasingly appreciated. Nitrite can undergo reduction to NO by various potential mechanisms especially under hypoxic and/or acidotic conditions (45, 144, 146, 158, 160, 176, 328). Furthermore, nitrite has been shown to elicit NO-dependent vasodilatation (26, 161), angiogenesis (366) and cardioprotection (376). Nitrite itself may also potentially exert biological effects on the heart and vasculature directly via S – nitrosylation of proteins (377).

Interestingly, nitrite was shown to have vasodilatory properties as far back as 1937 and subsequently in 1953 (192, 193). In Furchgott and co-workers's experiments, high concentrations of nitrite (100 - 1000μmol/L) were required to induce vasorelaxation in isolated rabbit aortic rings. Decades later it was shown to be a relatively weak vasodilator in the forearms of healthy volunteers even with concentrations of up to 200μmol/L (195). This discrepancy between the micromolar doses required to see an effect *in vitro* and the nanomolar concentrations present *in vivo* rendered nitrite an insignificant physiological vasodilator. However, later studies challenged Lauer's conclusions, indicating that nitrite may be important in physiological regulation of blood flow, cell signalling and cytoprotection following ischaemia-reperfusion injury. In some of these studies, the vasodilatory properties of nitrite were observed at pharmacological concentrations including those not greatly above physiological concentrations. (26, 46, 131, 161, 328, 338, 351, 378, 379).

The observation that during intra-arterial infusion of nitrite, forearm vasodilation was enhanced following exercise raised the possibility that nitrite bioactivity was enhanced by hypoxia (26, 161). Findings consistent with this hypoxic vasodilatory effect have been

reported by others. Studies in animal models have demonstrated that nitrite administration augments pulmonary vasodilatation during hypoxia (351, 352, 380). Although the pulmonary vasculature is fundamentally different to the systemic vasculature, hypoxic augmentation of the pulmonary vasculature is a commonality to these studies.

Hypoxic vasodilatation has been observed in humans also. In the intact forearm vasculature of healthy subjects, intra-arterial sodium nitrite infusion only modestly dilates forearm resistance vessels, but potently dilates capacitance vessels (that are exposed to substantially lower ambient oxygen tension) under normoxic conditions (46). Moreover, under hypoxic conditions, nitrite markedly dilated forearm resistance vessels. Studies undertaken by Ingram and colleagues reported that systemic nitrite infusion abrogated the increase in pulmonary artery pressure induced by hypoxia in healthy volunteers and that this effect was not dependent on an increase in plasma nitrite levels (199). Recently, Pluta et al. explored the safety and toxicity of intravenous sodium nitrite which provided further insight into the potential of sodium nitrite as a clinical therapy (381). They infused healthy volunteers with escalating doses of sodium nitrite (from 0.07µg/kg/min up to a maximal dose 8.9µg/kg/min) and were able to ascertain that the maximal tolerated dose was 4.5µg/kg/min and that dose limited toxicity occurred at 7.4µg/kg/min. The results of these recent studies have been published after the study presented herein was undertaken. However, limited data still exist on the effects of systemic nitrite infusions on the cardiovascular system in man, particularly during hypoxia and this requires further exploration.

One of the potential side-effects of sodium nitrite administration is methaemoglobin accumulation. This side-effect is exploited in the treatment of cyanide poisoning with 300mg intravenous sodium nitrite. Cyanide ions combine with nitrite-generated

methaemoglobin to produce cyanmethaemoglobin, thus protecting cytochrome oxidase from the cyanide ions. In cyanide poisoning, levels of 30-40% are tolerated safely, with symptoms occurring above levels of 10-20%. Symptoms of methaemoglobinemia include dyspnoea, lethargy, tachycardia, cyanosis and decreasing sonsciousness as methemoglobin levels rise above 55%, with eventual death with levels > 70% (382). In one study, the highest dose of nitrite used (110µg/kg/min) in healthy adults induced methaemoglobinaemia of 3.2% (161). Furthermore, Pluta et al. who reported a modest increases in metHb (approx. 5%) during sustained infusion of the highest infusion rate of nitrite (>500 µg/kg/hour which translates into ~ 9µg/kg/min) used in their study (381). This is an important safety aspect of sodium nitrite administration that requires addressing in studies investigating the role of nitrite in humans, particularly with sustained infusions.

2.2 Objectives

This study sought to determine a dose of intravenous sodium nitrite in healthy volunteers that was safe, tolerable and efficacious. This was to be achieved by administering brief infusions of sodium nitrite at escalating doses and during prolonged infusions (several hours). In order to better understand the vascular and cardiac effects of systemic nitrite infusion we evaluated the haemodynamic effects of systemic nitrite infusion in healthy subjects (during both normoxia and hypoxia), evaluating the dose -response relationship for these effects during both brief and prolonged infusions. We hypothesised that:-

(1) Intravenous infusions of sodium nitrite would be efficacious, safe and well tolerated in man.

(2) Systemic nitrite infusion would have little effect on blood pressure or systemic vascular resistance in healthy subjects during normoxia, but would reduce both when subjects were made hypoxaemic.

2.3 Power calculation

This is a 20 subject study, with participants acting as their own control (measurements during normoxia and hypoxia). We anticipated a 10% reduction in MABP during hypoxia, with $\alpha = 0.05$ and $1-\beta = 0.85$ this required 16 subjects. 20 subjects in total were recruited to allow for subject drop-out.

2.4 Methods

2.4.1 Participants

Subjects were recruited from the University of Birmingham. The studies were granted full ethical approval (University of Birmingham ERN_10-0247). The investigation conforms to the principles outlined in the Declaration of Helsinki. All of the subjects gave written, informed consent after satisfying the inclusion and exclusion criteria (see below). The study was performed at the University of Birmingham Clinical Research Block in a quiet vascular laboratory that was temperature-controlled at 22 - 24ºC. All subjects were requested to abstain from alcohol and follow a low nitrate/nitrite diet for 24 hours (**table 2.1**) and to abstain from caffeine-containing drinks for 24 hours before the study.

Inclusion criteria included age over 18 years, history of no-smoking and no regular medication (except the oral contraceptive pill). All subjects underwent a cardiovascular

examination and had a normal electrocardiogram (ECG). Pregnant women or nursing mothers, those with BMI≥30kg/m^2 and migraine sufferers were excluded from the study. 20 subjects were recruited into this study.

Leafy vegetables e.g.	Cured meat	Pickled vegetables
Lettuce	Cured fish	Pickled meat
Beetroot		Pickled fish
Celery		
Radish		
Rhubarb		
Turnip		
Spinach		

Table 2.1: List of food items subjects were asked to abstain from, as part of a nitrate/nitrite poor diet.

2.4.2 Pilot study - effects of sodium nitrite infusion on methaemoglobin

At the time of this study being conducted, no human data existed on systemic sodium nitrite infusion in humans. Therefore, prior to the main study being conducted, a pilot safety study on 7 healthy volunteers was conducted to ascertain the safety/tolerability of intravenous (i.v.) sodium nitrite and to quantify the increase in methaemoglobin (metHb) associated with systemic sodium nitrite administration. Subjects received 10, 30 or 60µg/kg/min of intravenous sodium nitrite for up to 6 hours or until metHb levels reached 10% of total haemoglobin. A cut-off of 10% was chosen to allow a wide safety margin as symptoms are noted to occur above 10-20% (382).

Subjects rested in a semi recumbent position on a hospital bed and an intravenous cannula (Venflon, 20 Gauge) was placed in each antecubital fossa. One intravenous cannula was used to administer the infusion, with blood obtained from the contra-lateral arm cannula. Continuous cardiac monitoring and peripheral haemodynamic variables were measured using the Taskforce® Monitor (CN Systems, Graz, Austria) and oxygen saturations using pulse oximetry (Nellcor N-180, Nellcor, Pleasanton, California). Heart rate (HR) and blood pressure (BP) were recorded at baseline and every 30 minutes for 6 hours or until the infusion was terminated. Venous blood samples were taken at baseline at 30 minute intervals, and analysed using a blood gas analyser (Bayer Rapidlab 865, Siemens, Tarrytown, NY) for metHb levels. This pilot data allowed the determination of the sodium nitrite doses for this study in healthy volunteers and for a further study in heart failure patients (Chapter 3).

2.4.3 Haemodynamic effects of short term incremental infusion of sodium nitrite

20 healthy volunteers were recruited into a single-blind placebo-controlled study to evaluate the dose-response relationship of systemic sodium nitrite infusion during normoxia and during hypoxia. Each subject attended on two separate occasions a week apart, with random order allocation to hypoxia and normoxia. The study design (study A) is depicted in **figure 2.1A**. At each visit, subjects were studied twice (60 minutes apart) with systemic infusions of escalating doses of sodium nitrite 20minutes each or placebo, also in random order.

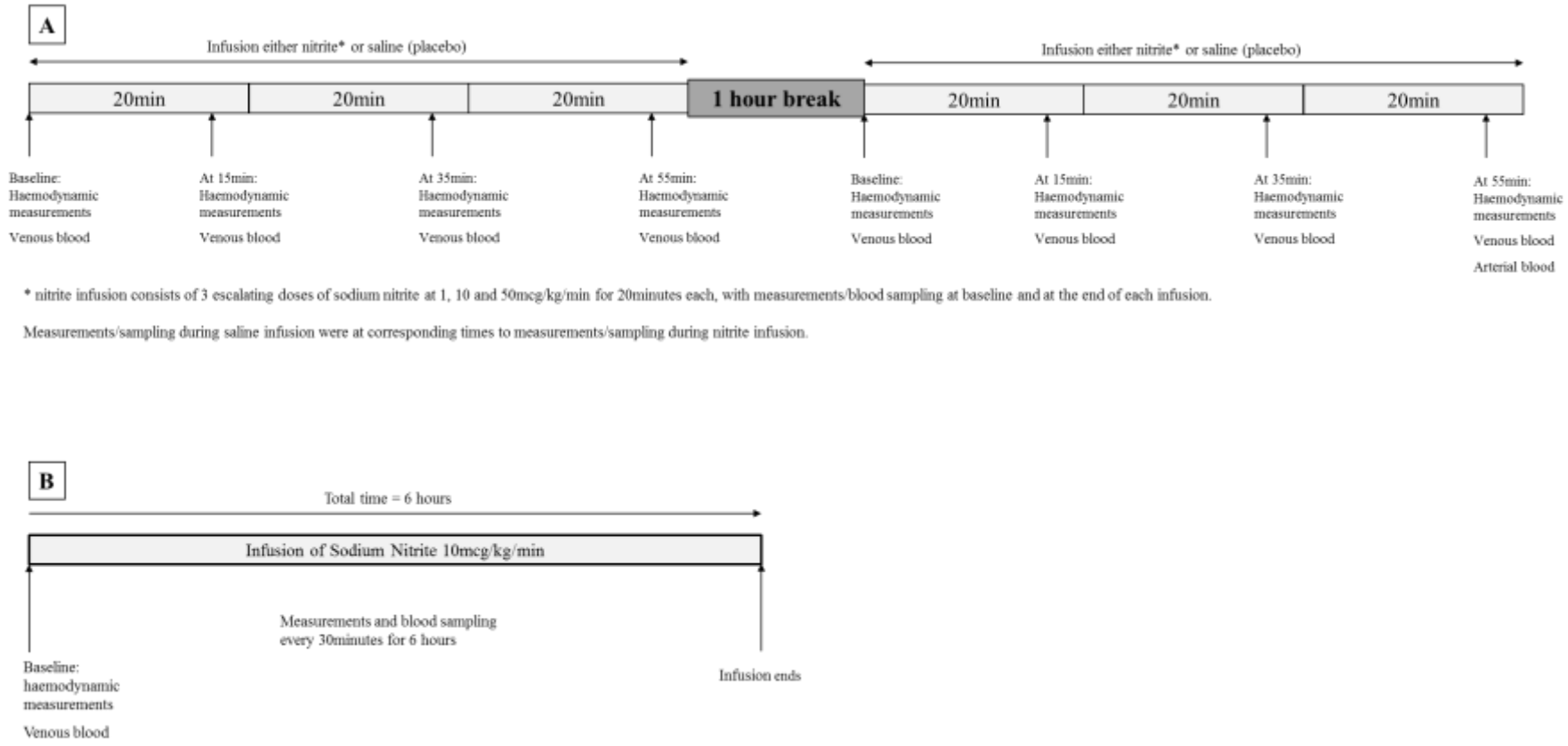


Figure 2.1: Study protocol. (A): Participants received escalating infusions of intravenous 0.9% saline and sodium nitrite for 20minutes each during both normoxia and hypoxia, in random order allocation; (B) Participants received a 6-hour infusion of intravenous sodium nitrite (10mcg/kg/min) over 6 hours.

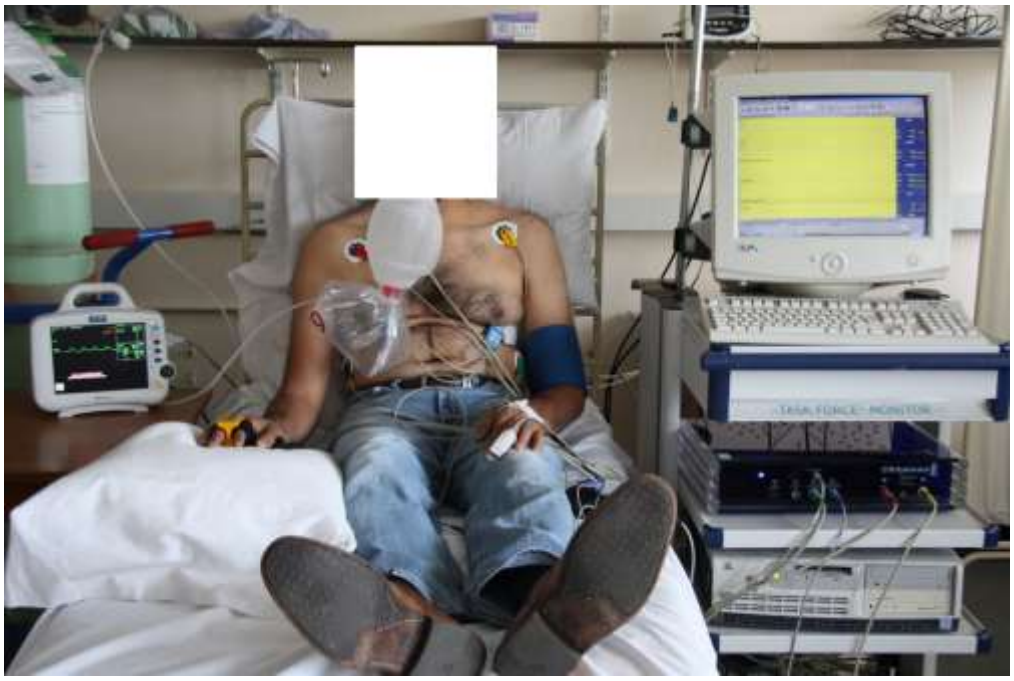


Figure 2.2: Photograph depicting typical experimental set-up with administration of hypoxia.

Subjects rested in a semi-recumbent position on a hospital bed. Intravenous cannulae (Venflon, 20 Gauge) were inserted into a vein in each ante-cubital fossa. Each subject underwent placement of a finger cuff for continuous blood pressure recording, an upper arm cuff for blood pressure measurement and electrocardiogram and band electrodes on the trunk for impedance cardiography (TaskForce Monitor®, CN Systems, Graz, Austria).

Following 10 minutes of rest, an i.v. infusion of either 0.9% sodium chloride (placebo) or sodium nitrite was administered for 60 minutes, in a single-blind randomised order. Nitrite infusion consisted of 3 escalating infusion rates of sodium nitrite: 1, 10 and 50µg/kg/min for 20minutes each. All infusions were administered at a rate of 1ml/min. Cardiac (cardiac output, CO; stroke volume, SV; systemic vascular resistance, SVR) and peripheral (HR and BP) haemodynamic variables were measured non-invasively by transthoracic electrical bio-

impedance (Taskforce® Monitor, CN Systems, Graz, Austria), as used previously (383). BP and HR were measured continuously. Changes in SV, CO and SVR were calculated and recorded using the Taskforce® monitoring system. Although absolute values of these parameters measured by the bio-impedance technique differ significantly from those measured using the invasive thermo-dilution technique it has been shown to be a valid way of assessing changes in these parameters (384). Haemodynamic variables were recorded at 15minutes, 35minutes and 55minutes into each infusion over a 5-minute period, to coincide with the final 5minutes of each nitrite infusion dose. Arterial oxygen saturation levels were monitored continuously using pulse oximetry (Nellcor N-180, Nellcor, Pleasanton, California). At the end of each recording period, venous blood samples were obtained (from the non-infused arm) and analysed as described later in section 2.3.5. After the 60-minute infusion, subjects were allowed a 60 minute rest following which they received a further 60 minute infusion of the opposite treatment allocation (i.e. sodium nitrite or placebo) as described above. Following the second infusion of each visit, an arterial blood sample was obtained from the right radial artery for immediate blood gas analysis using a blood gas analyser (Bayer Rapidlab 865, Siemens, Tarrytown, NY). The experimental set-up is depicted in **figure 2.2**.

During the hypoxia visit, hypoxia was induced by subjects inspiring 12% oxygen via a facemask connected to a two-way valve. The target oxygen saturation as measured by pulse oximetry was in the range 83% - 88%, aiming for an approximate pO2 6.32–7.32 KPa based on Severinghaus's calculations (385). Attainment of target oxygen saturation was followed by a 10minute stabilisation period, after which the study proceeded (as described above) with subjects receiving sodium nitrite and placebo infusions in a cross-over method.

2.4.4 Haemodynamic effects of sustained infusion of sodium nitrite

Following on from the main limb of the study as described above, 6 additional healthy volunteers (study B) were allocated to receive a 6-hour infusion of 10µg/kg/min of i.v. sodium nitrite (at a rate of 1ml/min) during normoxia to assess the sustained haemodynamic effects and metHb accumulation of this infusion rate (as depicted in **figure 2.1B**).

Subjects rested in a semi recumbent position on a hospital bed and an intravenous cannula (Venflon, 20 Gauge) was placed in each antecubital fossa. Peripheral haemodynamic variables (HR and MABP) were measured using the Taskforce® Monitor and oxygen saturations using pulse oximetry as described above, at baseline and every 30 minutes for 6 hours. Venous blood samples were taken, from the contra-lateral arm at baseline at 30 minute intervals, and analysed as described in section 2.3.5.

2.4.5 Blood sampling

Venous blood samples were obtained at the time points indicated in **figure 2.1**. 8mL of blood was transferred into two EDTA collection tubes (K3 EDTA, BD Vacutainers). 400µL of the thiol alkylator N-ethylmaleimide (Merck, Germany) was added to one of the tubes (1mM final concentration) and centrifuged at 2000rpm at 4ºC. Plasma samples were immediately snap-frozen in liquid nitrogen and stored at -80ºC for subsequent analysis of plasma nitrite/nitrates and protein-bound NO (RXNO). A further 2ml of venous blood was analysed immediately using a blood gas analyser (Bayer Rapidlab 865, Siemens, Tarrytown, NY) for pH, lactate and metHb quantification. In study A (**figure 2.1A**), an arterial blood sample was obtained from the subjects at the end of each visit to ascertain change in arterial oxygen content following administration of 12% oxygen.

Plasma nitrite/nitrate and RXNO analysis

Briefly, at the time of analysis, the NEM-treated samples were thawed and added to a solution containing potassium ferricyanide (NO-haem species) for nitrite/nitrate measurement and a tri-iodide-containing reaction mixture (for the release of RXNO species), which was continually washed with nitrogen. Group-specific reductive denitrosation by iodine–iodide was used to measure plasma nitrite and nitrate and RXNO concentrations (386). Subsequent detection of liberated NO was undertaken using by gas-phase chemiluminescence using a gas phase chemiluminescence detector (CLD 77am sp, EcoPhysics) under reduced light conditions, to avoid photolytic decomposition of NO and NO-species (387).

Plasma cGMP assay

Plasma cGMP was determined using an enzyme immunoassay (cGMP EIA Biotrak System, GE Healthcare UK Ltd) according to the manufacturer's instructions. Briefly, plasma was diluted 1:10 with assay buffer (0.05M sodium acetate, pH 6.0 containing 0.002% (w/v) bovine serum albumin and 0.01% (w/v) preservative). 50µL aliquots of sample per well were added to a 96-well plate in triplicate, containing 100µL of rabbit anti-cGMP antibody. The plate was covered and allowed to incubate at 4ºC for 2 hours. 100 µL of cGMP-peroxidase conjugate was added with further incubation of the plates at 4ºC for 1 hour. All wells were washed four times, followed by the addition of 200µL of peroxidase substrate. The plates were covered and gently agitated for 30minutes at room temperature using a micro-plate shaker. 100 µL of 1M Sulphuric acid was added each well to terminate the reaction. A spectrophotometric microplate reader (450nm) was used to measure the absorbance of each well. For study A, these analyses were performed at baseline and peak nitrite infusion rate of 50µg/kg/min only.

2.4.6 Study medication

Sodium nitrite was obtained from Martindale Pharmaceuticals UK (Brentwood, United Kingdom) and 0.9% sodium chloride from Baxter, UK. Sodium nitrite was diluted to the required concentration using 0.9% sodium chloride solution.

2.4.7 Statistical analysis

The data were analysed using SPSS version 21.0 software (SPSS Inc., Chicago, USA). The data are expressed as mean ± standard error of mean (mean±SEM), unless otherwise stated. Probability values of <0.05 were considered statistically significant. One-way repeated measures ANOVA with consecutive Bonferroni post-hoc test was used to test for changes from baseline in haemodynamic parameters, during study A. Non-normally distributed data (study B) was analysed with Friedman repeated measures test with the Wilcoxon signed-rank test for post-hoc analysis. Paired data were analysed using a paired Student's t-test.

2.5 Results

2.5.1 Preliminary studies

7 healthy subjects (mean age 25±4 years, 5 male) were recruited into the supplemental pilot study. A dose-dependent increase in metHb during sustained i.v. infusion of 10, 30 or 60µg/kg/min of sodium nitrite was observed (**figure 2.3A**). 60µg/kg/min of sodium nitrite induced a rapid increase in venous metHb up to 10%, 2.5hours into the infusion and the infusion was terminated at this point as pre-specified. The two subjects receiving

30µg/kg/min of i.v. nitrite also achieved metHb levels of 10% but this occurred over a longer time period (4.5 and 6 hours). 10µg/kg/min was well tolerated with a maximal metHb rise of 2.1%. All infusion rates were well tolerated with no adverse symptoms reported. The HR and mean arterial blood pressure (MABP) profile from these participants are shown in **figure 2.3B** and **figure 2.3C**.

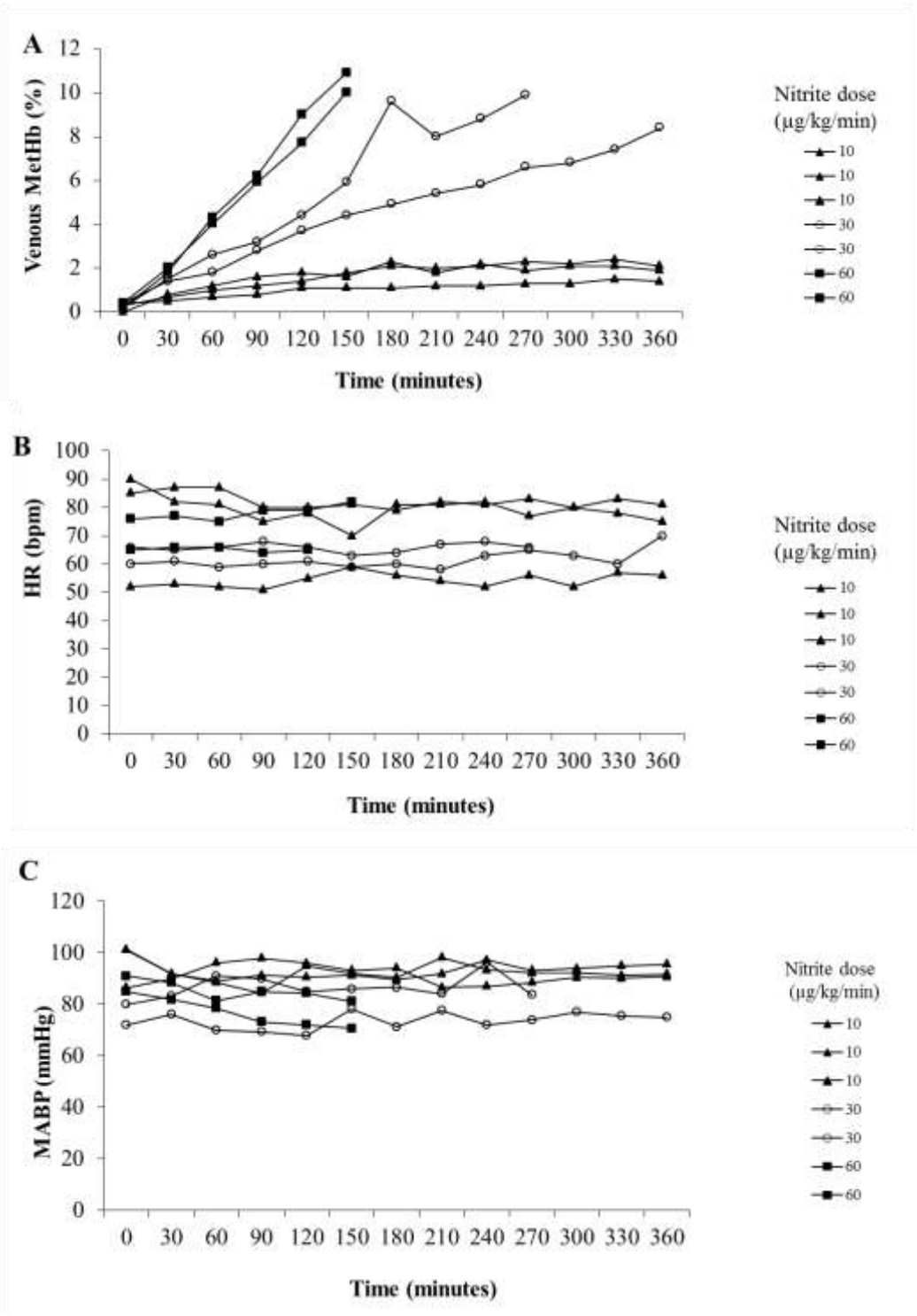


Figure 2.3: Individual subject data from the pilot study. (A) Venous metHb; (B) Heart rate profile; (C) Mean arterial blood pressure (MABP) during sustained infusions of 10, 30 or 60µg/kg/min of i.v. sodium nitrite. N=7.

2.5.2 Baseline characteristics of subjects

The table below (**table 2.2**) outlines the baseline characteristics of the subjects.

	Study A n=19	Study B n=6
Age, years (mean±SD)	23±3	25±5
Gender, m	16	3
Body mass index (kg/m^2, mean±SD)	23±3	24±2
HR (bpm)	66±2	69±6
MABP (mmHg)	83±3	93±4

HR, heart rate; MABP, mean arterial blood pressure.

Table 2.2: Baseline characteristics of study subjects

One subject who was recruited into study A was excluded from subsequent analysis as he did not tolerate hypoxia, therefore no data was acquired. As he withdrew from the whole study, no normoxia data was obtained on this participant either.

2.5.3 Haemodynamic effects of short term incremental nitrite infusion (study A) during normoxia and hypoxia in healthy volunteers

During the hypoxia phase, mean arterial oxygen saturations were significantly lower than during normoxia ($p<0.0001$ normoxia *vs* hypoxia readings) and remained consistently so throughout the study period (**table 2.3**). This was mirrored by changes in arterial oxygen content obtained from the arterial blood sample, as depicted in **figure 2.4** (15.1±0.9KPa during normoxia *vs* 6.5±0.2KPa during hypoxia, $p<0.0001$, n=12).

Table 2.3: Arterial oxygen saturations during study A, as measured by pulse oximetry.

	Arterial O_2 saturations (%)	
	Saline	
Time (minutes)	*Normoxia*	*Hypoxia*
0 (baseline)	98.1±0.2	85.8±0.8****
20	98.3±0.2	86.0±0.9****
40	98.5±0.2	85.8±1.0****
60	98.5±0.2	86.5±0.9****
	Nitrite	
Nitrite dose (µg/kg/min)	*Normoxia*	*Hypoxia*
0 (baseline)	98.4±0.3	85.1±0.9****
1	98.6±0.3	85.2±0.8****
10	98.9±0.2	85.6±0.8****
50	98.4±0.2	85.9±0.8****

*p-value <0.0001 compared to pre-infusion values at the corresponding time-point in the normoxia arm of the study. N=19, $p<0.05$ is statistically significant.

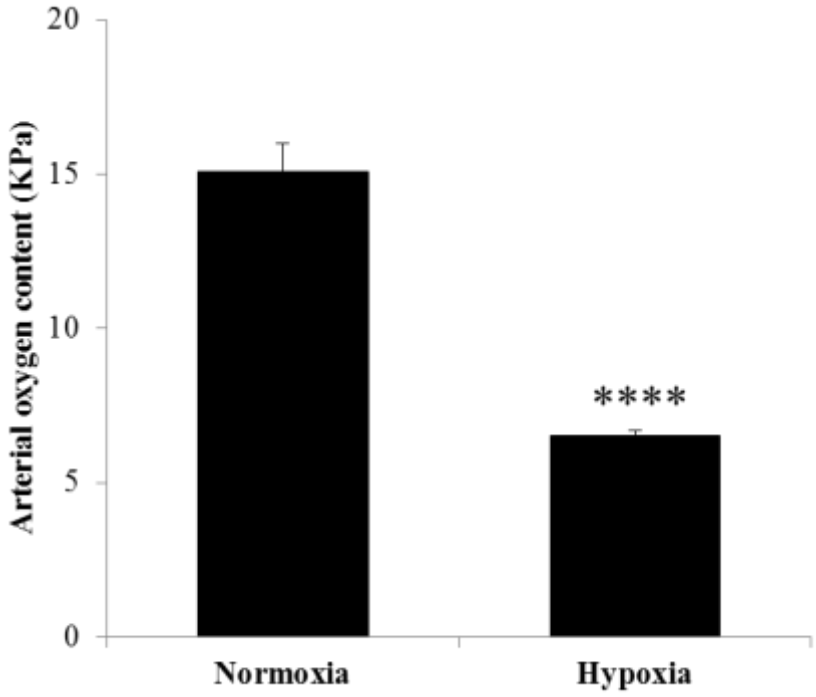


Figure 2.4: Arterial oxygen content obtained via an arterial blood sample at the end of the normoxia and the hypoxia protocols in study A. *p<0.0001; n=12.

As shown in **figure 2.5A**, HR increased following nitrite infusion during normoxia (from 64±2bpm at baseline to 71±2bpm at peak nitrite infusion rate, $p<0.001$) but during hypoxia the change in HR from baseline was not significant (77±3bpm to 81±3 respectively, $p>0.05$). Baseline HR was significantly higher during hypoxia (74±3bpm prior to saline infusion and 77±3bpm prior to nitrite infusion) compared to normoxia (66±3bpm prior to saline infusion and 64±2bpm prior to nitrite infusion, $p<0.001$). No change from baseline in HR was observed during saline infusion under either normoxic or hypoxic conditions.

MABP (**figure 2.5B**) did not alter with either infusion during both normoxia and hypoxia (normoxia: 84±2mmHg at baseline *vs* 83±2mmHg during peak nitrite infusion rate, $p>0.05$; hypoxia: 86±3mmHg at baseline *vs* 85±3mmHg during peak nitrite infusion rate, $p>0.05$). During both normoxic and hypoxic conditions, SVR (**figure 2.5C**) did not change significantly with nitrite administration (normoxia: 1217±46 dyne*s/cm^5 at baseline *vs* 1150±41 dyne*s/cm^5 at peak nitrite infusion rate, $p>0.05$; hypoxia: 1128±72 at baseline *vs* 1061±49 dyne*s/cm^5 at peak nitrite infusion rate, $p>0.05$).

Baseline CO (**figure 2.6A**) was marginally higher during hypoxia *vs* normoxia, (5.6±0.1L/min *vs* 5.4±0.1L/min $p=0.05$). Under normoxic conditions there was a significant increase in CO from baseline to peak nitrite infusion rate (from 5.4±0.2 L/min to 5.7±0.2L/min, $p<0.05$) but not under hypoxic conditions (6.2±0.3 L/min to 6.4±0.2L/min, $p>0.05$). However, the effect of nitrite infusion on CO did not significantly differ between normoxic *vs* hypoxic conditions ($p>0.05$). SV (**figure 2.6B**) did not alter with either infusion during both normoxia and hypoxia (normoxia: 86.6±3.4ml at baseline *vs* 81.6±3.1ml during peak nitrite infusion rate, $p>0.05$; hypoxia: 81.6±3.0ml at baseline *vs* 80.1±2.9ml during peak nitrite infusion rate, $p>0.05$).

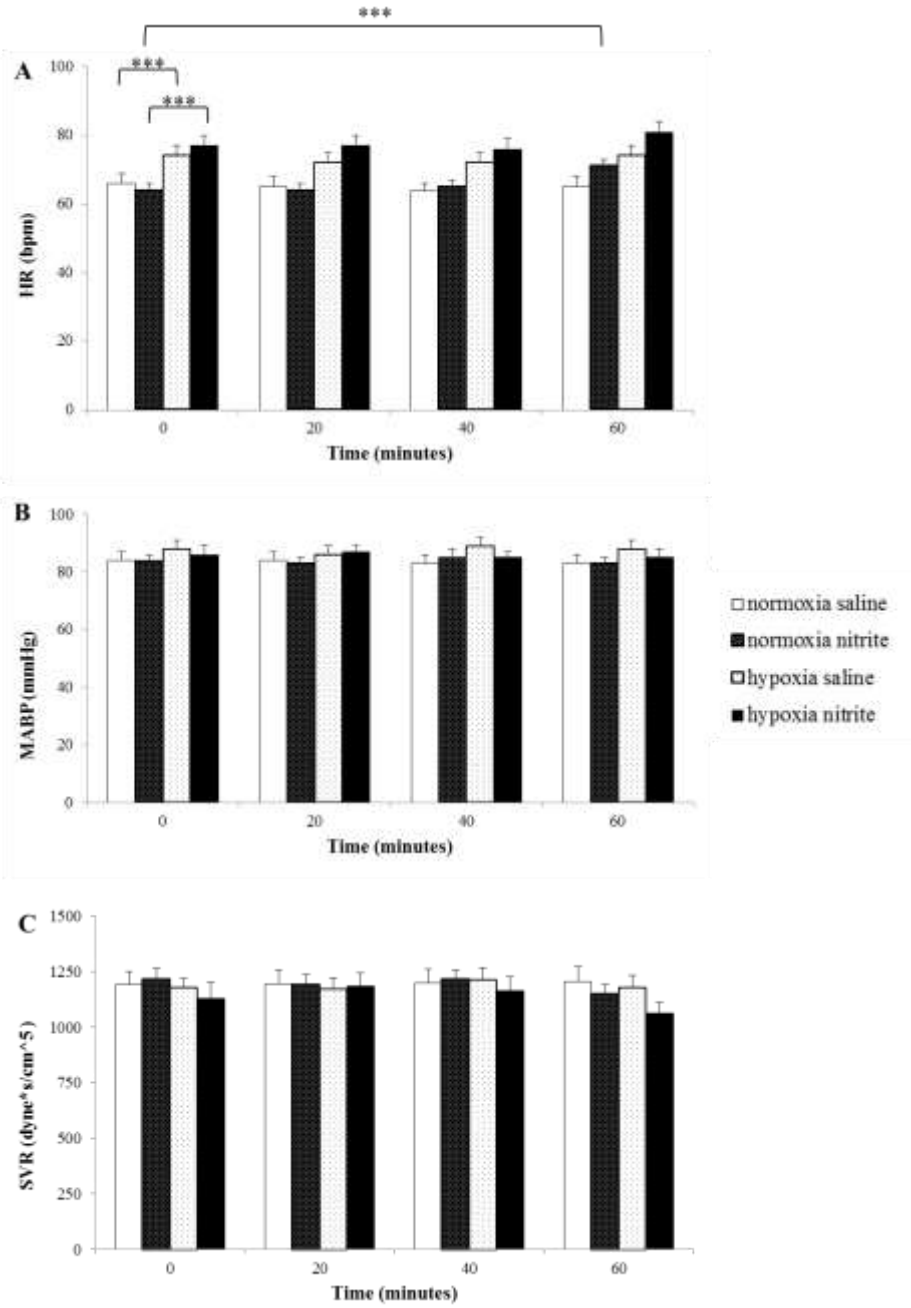


Figure 2.5: Peripheral haemodynamic parameters from subjects in study A during i.v. saline and 20minute escalating doses of i.v. sodium nitrite, during normoxia and hypoxia. (**A**) HR; (**B**) MABP and (**C**) SVR. ***p<0.001, n=19. 0, 20, 40 and 60minutes into nitrite infusion correspond to 0, 1, 10 and 50µg/kg/min of i.v. sodium nitrite, respectively.

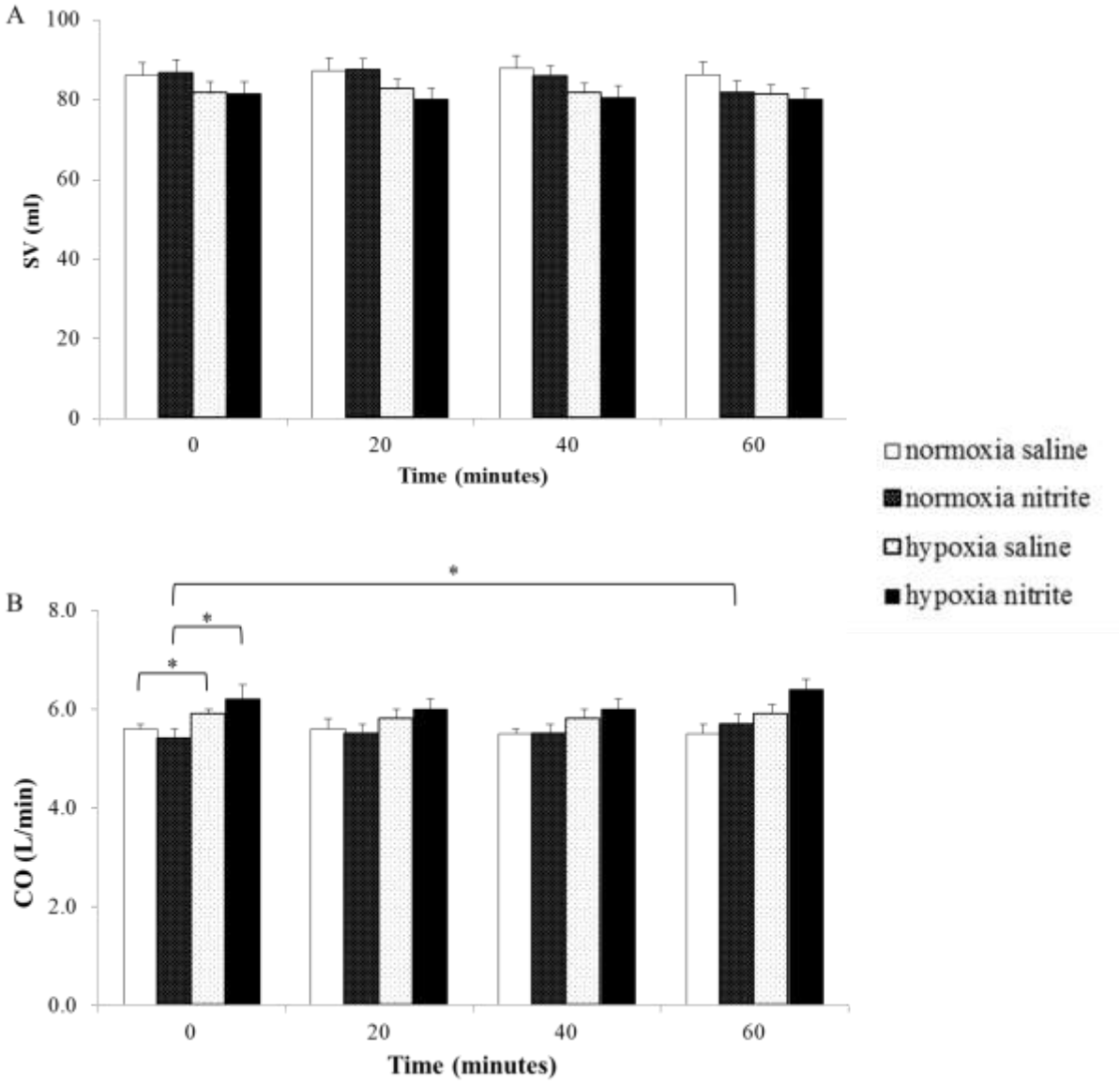


Figure 2.6: (A) SV and (B) CO from subjects in study A during i.v. saline and 20minute escalating doses of i.v. sodium nitrite, during normoxia and hypoxia. *$p<0.05$; n=19. 0, 20, 40 and 60minutes into nitrite infusion parallel 0, 1, 10 and 50µg/kg/min of i.v. sodium nitrite, respectively.

2.5.4 Effect of oxygen tension on venous acid-base balance

Table 2.4 shows venous pH and lactate data with placebo and nitrite infusion, during normoxia and hypoxia.

Venous pH remained steady during normoxia or hypoxia, without any significant change. Overall venous lactate was higher during hypoxia as compared to normoxia ($p<0.05$ normoxia *vs* hypoxia, n=19). Pre-infusion normoxia lactate concentration was 1.32±0.10mmol/L and 1.47±0.08mmol/L with saline and nitrite infusion, respectively. During hypoxia, baseline lactate was 1.57±0.08mmol/L and 1.89±0.22mmol/L* with saline and nitrite infusion, respectively (n=19, *$p<0.05$ compared to baseline normoxia levels).

Arterial blood gas analysis obtained at the end of the normoxia and hypoxia limbs of the study demonstrated no difference in arterial lactate between the two groups (1.03±0.06 mmol/L during normoxia and 0.98±0.05mmol/L during hypoxia, $p>0.05$, n=8)

	pH	Lactate (mmol/L)
Normoxia saline		
Baseline	7.366±0.004	1.32±0.10
20mins	7.364±0.004	1.28 ±0.09
40mins	7.362±0.004	1.25± 0.08
60mins	7.362±0.004	1.26± 0.07
Normoxia nitrite		
Baseline	7.359±0.005	1.47±0.08
1µg/kg/min	7.352±0.005	1.43±0.07
10µg/kg/min	7.356±0.005	1.19±0.28
50µg/kg/min	7.353±0.006	1.45±0.17
Hypoxia saline		
Baseline	7.379±0.006	1.57±0.08
20mins	7.370±0.006	1.59±0.15
40mins	7.378±0.006	1.57±0.11
60mins	7.377±0.008	1.60±0.19
Hypoxia nitrite		
Baseline	7.379±0.006	1.89±0.22*
1µg/kg/min	7.382±0.006	1.91±0.20
10µg/kg/min	7.378±0.006	1.64±0.15
50µg/kg/min	7.385±0.006	1.46±0.10

Table 2.4: Venous pH and lactate data from subjects in study 1 during normoxia and hypoxia. *p-value versus baseline normoxia is <0.05, n=19.

2.5.5 Haemodynamic effects of sustained infusion of sodium nitrite in healthy volunteers (study B)

Prolonged infusion (up to 6 hours) of 10µg/kg/min of sodium nitrite in healthy volunteers did not result in a significant change in either HR or MABP. HR was 64(58 - 86)bpm at baseline and remained at a similar level throughout the infusion (**figure 2.7A**; n=6, p>0.05). MABP (**figure 2.7B**) was 92(86 - 103)mmHg at baseline and 95(93 - 97)mmHg after 6 hours (n=6, p>0.05). Data is expressed as median (inter-quartile ranges).

2.5.6 Effect of nitrite on metHb accumulation in healthy volunteers

Venous metHb increased in a dose-dependent manner during the incremental short term nitrite infusion (study A) during both the normoxia and hypoxia interventions (**figure 2.8A**). Venous metHb increased from 0.3±0.03% at baseline to 0.7±0.08% at peak nitrite infusion rate during normoxia (n=19, p<0.001) and from 0.3±0.04% at baseline to 1.6±0.06% at peak nitrite infusion rate (n=19, p<0.001) during hypoxia.

During the 6-hour infusion of 10µg/kg/min of sodium nitrite in healthy volunteers, venous metHb increased in a dose-dependent manner from a median of 0.3(0.05 - 0.5)% at baseline to 2.1(1.3 - 2.5)% at 3 hours (p<0.05 compared to baseline, **figure 2.8B**). For the latter 3 hours, the metHb level appeared to plateau reaching a median of 1.9(1.5 - 2.4)% at 6 hours (p<0.05 compared to baseline). Data are expressed as median (inter-quartile ranges).

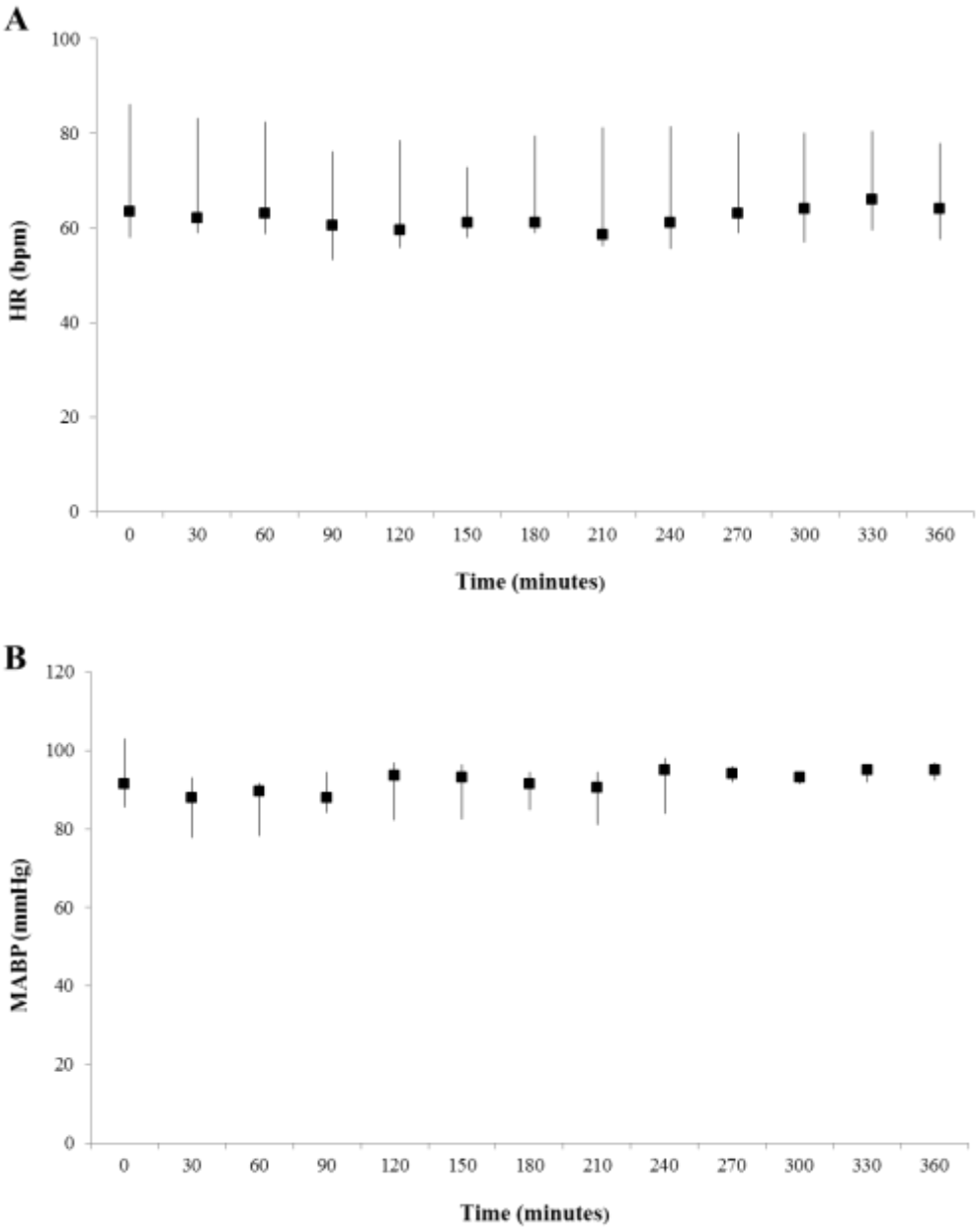


Figure 2.7: Median (inter-quartile) ranges showing (A) HR and (B) MABP during 6-hour infusion of i.v. sodium nitrite in healthy volunteers. No significant change was observed in either parameter. N=6. P>0.05 compared to baseline at all time-points for both HR and MABP.

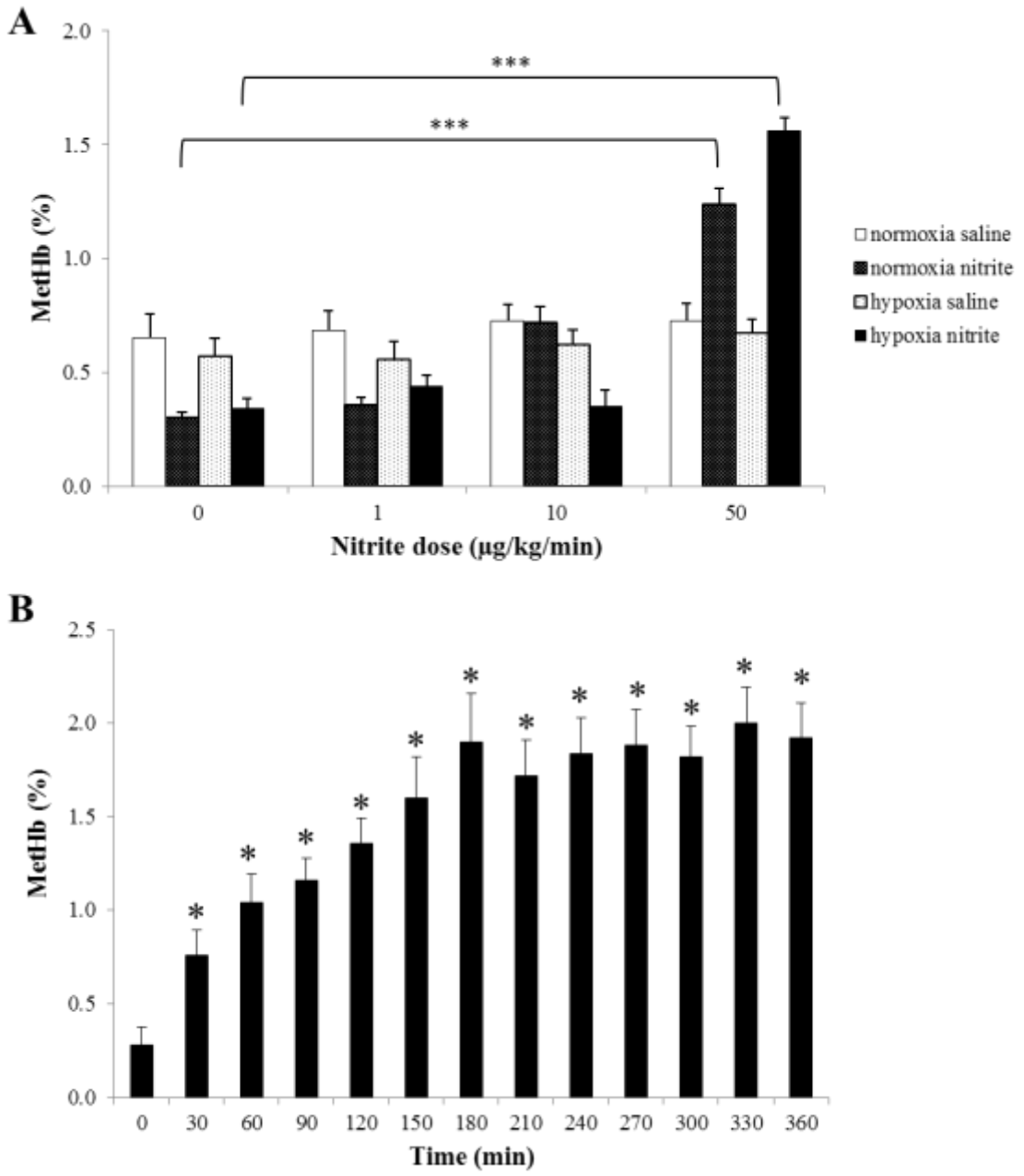


Figure 2.8: Venous metHb increase in healthy volunteers during (A) short incremental infusions of i.v. saline and nitrite during normoxia and hypoxia (n=19); data is expressed as mean±SEM; (B) prolonged (6-hour) infusion of i.v. sodium nitrite at 10µg/kg/min (n=6); data is expressed as median (inter-quartile ranges). *p<0.05 and ***p<0.001.

2.5.7 Effect of systemic nitrite on plasma nitrite, nitrate, RXNO and cGMP levels

In study A, plasma nitrite, nitrate and RXNO concentrations increased from baseline to peak (50µg/kg/min) nitrite infusion rate (**figure 2.9**). Plasma nitrite levels (**figure 2.9A**) also increased dose dependently from baseline values of 1.3±0.1µM and 1.3±0.1µM to 13.6±1.1µM and 12.9±1.4µM following 50µg/kg/min of i.v. sodium nitrite during normoxia and hypoxia, respectively ($p<0.001$, comparing baseline and post nitrite infusion). During normoxia, nitrate levels (**figure 2.9B**) increased from 19.8±3.4µM at baseline to 31.3±3.2µM following 50µg/kg/min of i.v. sodium nitrite ($p<0.01$). However, during hypoxia there was a smaller increase in nitrate levels from 18.3±1.9µM at baseline to 25.2±2.2µM following peak nitrite infusion rate which was not statistically significant ($p>0.05$). Plasma RXNO (**figure 2.9C**) increased from baseline values of 16.3±1.6nM and 14.1±0.9nM to 33.8±2.8nM and 27.9±1.7nM following 50µg/kg/min of i.v. sodium nitrite during normoxia and hypoxia, respectively ($p<0.001$, respectively).

During the sustained infusions of intravenous sodium nitrite (10µg/kg/min) in healthy volunteers, there was an increase in plasma nitrite (**figure 2.10A**) increased from 1.1(1.06 – 2.0)µM to a peak level at 3 hours of 9.8(7.2 – 10.1)µM ($p<0.05$, n=6). Plasma nitrate (**figure 2.10B**) continued to increases steadily throughout the duration of the infusion, from 20.6(17.2 – 29.0)µM at baseline to 78.4(72.6 – 92.0)µM after 6 hours ($p<0.05$, n=6). Plasma RXNO and nitrite which appeared to plateau after 120minutes. Plasma RXNO (**figure 2.10C**) increased from 12.8(10.8 – 19.3)nM at baseline to a peak of 32.5(21.3 – 33.0)nM at 6hours ($p<0.05$, n=6). Data are expressed as median (inter-quartile ranges).

Plasma cGMP levels were measured at baseline and following peak nitrite infusion rate (50µg/kg/min) in healthy volunteers during study A only (**figure 2.11**). There was no significant change from baseline in cGMP levels, during both normoxia and hypoxia. Baseline cGMP levels were 0.84±0.12fmol/well at baseline during normoxia and 0.78±0.06fmol/well at baseline during hypoxia. Following 50µ/kg/min of i.v. sodium nitrite, cGMP levels were 0.93±0.12fmol/well and 0.77±0.06fmol/well during normoxia and hypoxia, respectively ($p>0.05$ compared to baseline, $n=10$).

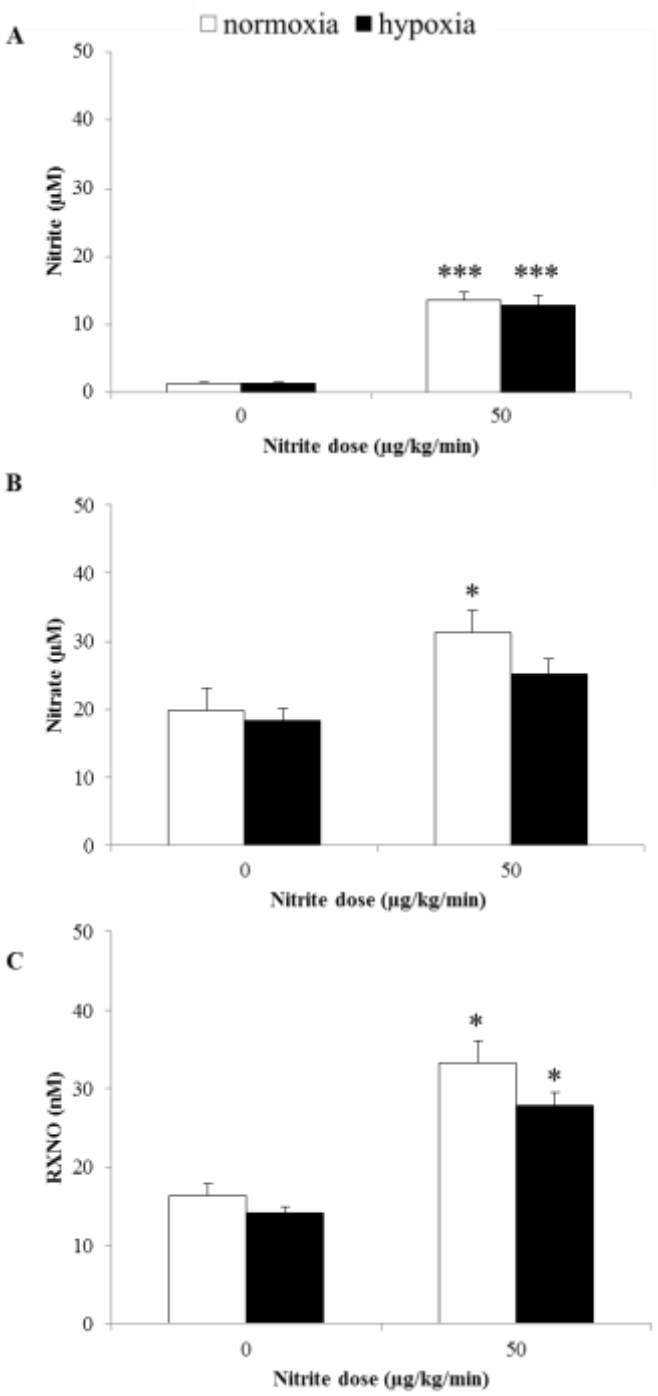


Figure 2.9: Plasma (A) Nitrite; (B) Nitrate and (C) RXNO concentrations from subjects in study A at baseline and following peak nitrite infusion rate of 50µg/kg/min. *p<0.05 and ***p<0.001 compared to baseline during the same oxygenation condition, n=10.

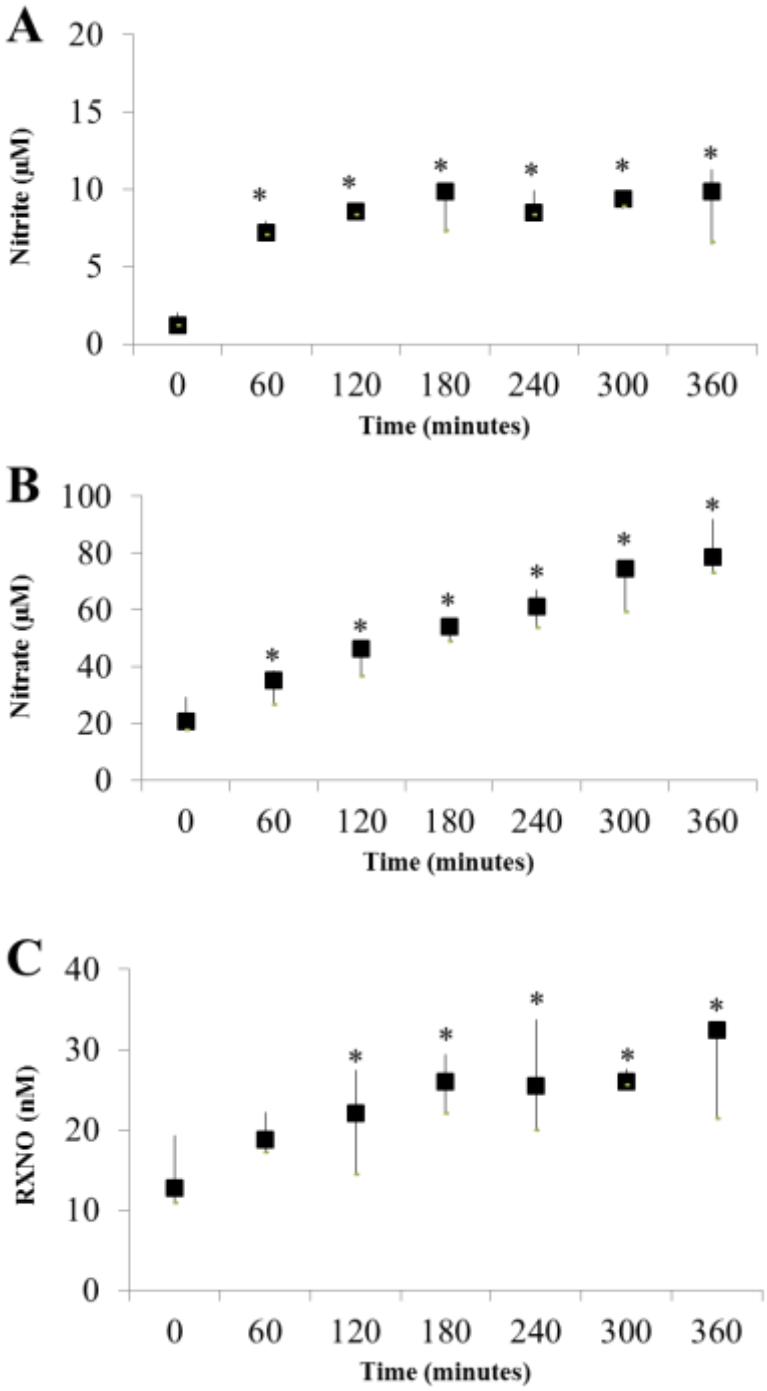


Figure 2.10: (A) Nitrite; (B) Nitrate and (C) RXNO concentrations from subjects in study B during a 6-hour infusion of 10µg/kg/min of i.v. sodium nitrite. *$p<0.05$, n=6. Data is expressed as median (inter-quartile ranges)

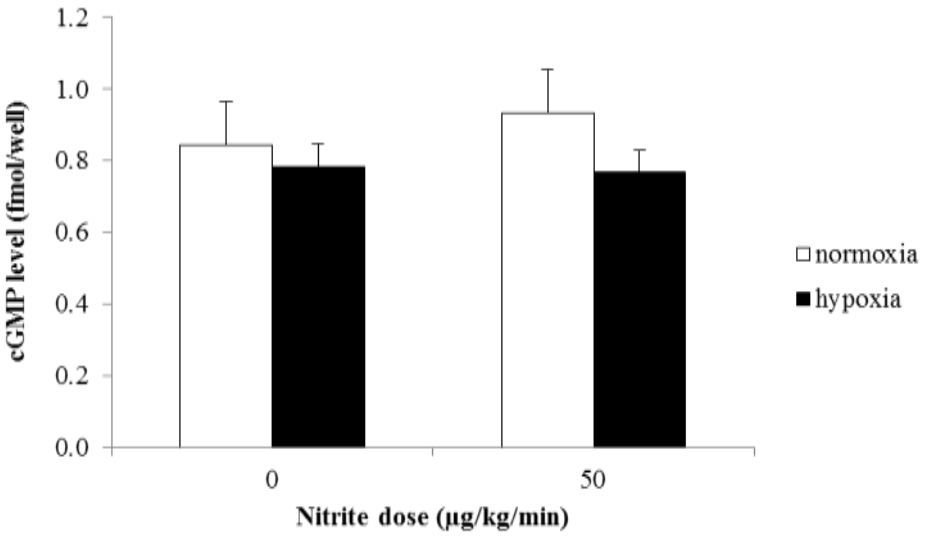


Figure 2.11: cGMP data from subjects in study A at baseline and following peak nitrite infusion rate of 50µg/kg/min, during normoxia and hypoxia. There was no significant difference in cGMP levels between baseline and peak nitrite infusion rate or oxygen states. P>0.05, n=10.

2.6 Discussion

This study followed-on from previous work undertaken by Maher et al. investigating the role of nitrite on forearm vasculature in healthy volunteers (46). Limited data is available on the systemic effects of nitrite. To date only two studies in the published literature (published after commencement of the study herein) have evaluated the role of systemic cardiovascular and peripheral haemodynamic effects of sodium nitrite in healthy volunteers (199, 381). Herein, the effects of nitrite were evaluated under both normoxic and hypoxic conditions, given the previously reported hypoxic augmentation of vascular effects during intra-arterial administration (46). Furthermore the current study utilised a 'placebo infusion' during both normoxia and hypoxia as a comparator against which to assess the haemodynamic effects of sodium nitrite infusion.

The preliminary study investigating the effects of up to 10, 30 or 60µg/kg/min in 7 healthy volunteers revealed no significant alteration in HR or MABP in any individual, although formal statistical analysis is unable to be performed due n≤ 3 at each infusion rate (**figure 2.3**). This pilot data allowed quantification of metHb accumulation and high infusion rates of 60µg/kg/min resulted in rapid accumulation of venous methHb up to 10% after 2.5hours. This informed the dosing for the main study discussed herein and a further study in heart failure patients (detailed in chapter 3). A lower starting infusion rate of 1µg/kg/min was utilised in case of significant hypoxic augmentation of nitrite.

In healthy subjects, there was no significant change in MABP or SVR under normoxic conditions at doses of up to 50µg/kg/min, but there was a modest increase in CO (**figure 2.6**), which was due to an increase in HR (**figure 2.5**). SV remained unchanged. At infusion rates of slightly less than just under 10µg/kg/min, Pluta et al. previously reported a transient,

modest fall in blood pressure in healthy subjects (381). Importantly unlike the current study, there was no placebo comparator against which to judge the effects of the nitrite infusion. Nossaman et al. recently showed that, in healthy open-chest rats, systemic sodium nitrite had an effect on blood pressure only at very high infusion rates, with a potency approximately 3 log orders lower than that of glyceryl trinitrate (GTN) (358). Contrary to our hypothesis that we would observe hypoxic augmentation of the vasodilator effects of sodium nitrite, which was based on previous evidence of hypoxic augmentation of the effects of intra-arterial nitrite infusion (46), there were no significant haemodynamic effects even at the highest infusion rate of sodium nitrite under hypoxic conditions. Furthermore, Pluta et al. reported modest increases in metHb (approx. 5%) during sustained infusion of the highest infusion rate of nitrite used in their study (>500µg/kg/hour which is approximately 9µg/kg/min) (381). In this study, short term infusion of even the highest infusion rate of sodium nitrite (50µg/kg/min) resulted in only modest increases in methHb. Consistent with the observations of Pluta et al. sustained infusion of 10µg/kg/min caused only modest increases in metHb (<2%) but we found that sustained infusion of 30µg/min and 60µg/min resulted in very marked increases in metHb (approximately 8% at 6 hours with 30µg/kg/min and abandoned at 2.5 hours with 60µg/kg/min because metHb increased above 10% in both subjects). This data suggests great caution should be applied (with regular metHb monitoring) if sustained infusion of more than 10µg/kg/min is to be administered.

The increase in HR observed with nitrite during normoxia (**figure 2.5**) was not observed during normoxia saline infusion or during either infusion in hypoxia. This effect cannot be attributed to arterial blood pressure or SVR as these parameters remained unchanged with nitrite infusion. Whether nitrite has a direct effect on baroreceptor responsiveness or directly on the myocardium itself was not investigated in this study. In a previous human study, NO

has been reported to modulate autonomic control, possibly through augmentation of cardiac vagal tone (388). A wealth of animal data exists suggessing a role for NO in autonomic control of heart rate and effect on cardiac autonomic efferents at the level of the heart (389). Alternatively NO may have a direct effect on cardiac autonomnic ganglia resulting causing a rise in heart rate. Systemic administration of NO donors has been shown to induce tachycardia, independent of baro-reflec activation. (390, 391). This may be a direct effect on the pacemaking current (I_f) found within myocytes, which are intrinsic for controlling activation of the sinoatrial node and therefore heart rate (392, 393). Potentially, either through release of NO or directly, nitrite may modulate autonomic control of heart rate or through direct effects on the heart. No study exists investigating this phenomenon. This would be an important question to address in future studies administering systemic infusions of nitrite to humans.

Plasma cGMP did not alter significantly in the healthy subjects and this would be in keeping with the lack of haemodynamic effects observed in this group, since cGMP is an index of soluble guanylate cyclase (sGC) activation. Plasma nitrite and nitrate increased in a dose dependent manner both healthy volunteers during the short-term infusions (**figure 2.9**). There was no significant difference in baseline values of plasma nitrite during normoxia and hypoxia in contrast to findings by others (199). This could be due to the shorter period of hypoxia in the current study, whereas Ingram and colleagues allowed a 3hour equilibration period during hypoxia, prior to commencement of nitrite infusion.

In addition to oxygen tension, acid-base status is well recognised to affect nitrite reduction to NO (117). In this study, pH was unaffected by hypoxia (**table 2.4**). Overall lactate levels

were higher during hypoxia compared to normoxia, as described previously (394). Despite these changes in lactate and a substantial increase in plasma nitrite concentration from baseline to peak nitrite dose of 50µg/kg/min, there was a lack of haemodynamic effect, in contrast to observations in the forearm vasculature with intra-arterial nitrite infusion, during normoxia and hypoxia (26, 46, 199). In these studies, increases in plasma nitrite concentration of between 2 to 10-fold resulted in localised vasodilatation especially during hypoxia. In the current study, plasma nitrite concentration increased 10-fold following both short, incremental infusions of nitrite and sustained infusion of nitrite at 10µg/kg/min, without any change in haemodynamic parameters. In contrast, studies exploring the role of dietary nitrate/nitrite intake observed a significant reduction in blood pressure following ingestion of 3.5mmol of oral nitrate resulting in a modest 1.5 fold increase in plasma nitrite (162). Despite attempts to standardise the study protocol, differences in dietary nitrate intake and nitrite metabolism between individuals may be a possible explanation for this lack of observed changes. Moreover, delivery of nitrite orally may result in a differing pharmacokinetic profile compared to intravenous nitrite, resulting in a sustained or larger, total nitrite exposure of the whole body over a given period of time. Furthermore, Ingram and colleagues reported that infusion of sodium nitrite prevented hypoxia-induced vasoconstriction in healthy subjects an hour after cessation of the infusion at a time when plasma nitrite had returned to baseline (199). This may provide a rationale to investigate pulsed high dose sodium nitrite therapy in heart failure that may potentially have sustained favourable haemodynamic effects with only modest associated methaemogobinaemia.

Study limitations

For greater accuracy and reliability, invasive methods of measuring cardiac haemodynamic parameters would ideally have been used in the healthy volunteers. Pulmonary vascular and

cardiac haemodynamic measurements can only be reliably obtained by invasive measurements. However, the use of such investigative tools in these healthy, subjects was deemed unjustified for the purpose of research alone. Hypoxia was administered via a face mask as previously employed by Maher et al (46). This did not allow a period of equilibrium as employed by others (199) and therefore may not have allowed for the physiological effects of hypoxia to stabilise.

Conclusions

In healthy subjects only minor haemodynamic effects were observed under both normoxic and hypoxic conditions, despite substantial increases in plasma nitrite concentrations. This is in contrast to what was expected from previous findings in the literature. However, this study provided important insight into the safety of tolerability of sodium nitrite infusion in human subjects to assess this further in patients with heart failure.

Chapter 3: Systemic Nitrite Infusion in Heart Failure

3.1 Introduction

Heart failure is a common cause of admission to hospital, intensive care and of death (356, 357). It is thought to affect approximately 900, 000 people in the UK (395). Most treatment of heart failure consists of chronic management of patients over the long-term to alleviate fluid overload, prevent acute decompensation and reduce mortality (396). In acute decompensation, patients with heart failure can become extremely unwell with acute pulmonary oedema requiring emergency treatment. In these patients, intensive therapy often includes diuretics and vasodilators. In some circumstances, inotropic agents may also be required to help improve cardiac function, albeit at the expense of increased myocardial lactic acidosis and with no improvement in mortality (397). For the majority of heart failure patients, whom are generally elderly with multiple co-morbidities, medical (drug) therapy remains the mainstay of treatment. In the Western world, this population is predicted to increase, predominantly due to an ageing population with coronary artery disease (398). With the degree of health burden that heart failure poses, novel therapeutic agents are continually being sought to treat this chronic condition.

In acutely decompensated or worsening chronic congestive cardiac failure, vasodilating drugs are commonly used to reduce both preload and afterload on the congested myocardium, decrease myocardial oxygen demand and increase coronary blood flow (399). One such vasodilator includes organic nitrate such as glyceryl trinitrate (GTN) which have formed the mainstay of treatment for many years, particularly in acutely decompensated heart failure. Trials investigating the role of intravenous nitrate in acute heart failure have demonstrated improvement in clinical condition and prevention of adverse consequences such as myocardial infarction and need for mechanical ventilation, when compared to other therapies for acute heart failure such as furosemide and non-invasive ventilation (400-402). However,

organic nitrates suffer from several shortcomings which limit their efficacy. Firstly, they markedly dilate resistance as well as capacitance vessels, resulting in hypotension which often constrains dose escalation. Secondly, patients can suffer from a significant headache which occasionally necessitates withdrawal of treatment with nitrates. Thirdly, resistance and the development of tolerance are significant clinical issues, which limit the efficacy and duration of treatment (403, 404). Furthermore, there is no evidence to suggest nitrates improve mortality in acute heart failure. Despite these drawbacks, GTN use is associated with long-standing clinical experience and remains an important drug of choice in acutely decompensated heart failure with no worthy alternative.

It is well recognised that venodilation decreases right-sided filling pressures and increases stroke volume (SV), probably via diminution of diastolic ventricular interaction (DVI). DVI is the term given to the situation in which the filling of the left ventricle is reduced by external constriction from the raised right ventricular diastolic pressure (RVEDP) which, confined within the relatively non-distensible pericardium, impedes left ventricular (LV) filling and reduces SV. In heart failure, DVI is often significant. Paradoxically, decrease of right ventricular (RV) volume and pressure (e.g. by venodilation) reduces this interaction and augments LV filling, resulting in an increased in SV (359, 405). Therefore, a profile of relatively selective dilatation of systemic capacitance and pulmonary resistance vessels would be potentially attractive in the treatment of decompensated heart failure (HF) since it would likely increase cardiac output (CO), and decrease ventricular diastolic pressures with less reduction in blood pressure than would be seen with an arterial vasodilator. In this regard, nitrite could potentially have a role in heart failure treatment.

A vasodilatory role for nitrite is well established. Nebulised nitrite resulted in a 65% reduction in hypoxia-induced pulmonary hypertension in newborn lambs (351). This effect was closely associated with the degree of pH reduction and deoxyhaemoglobin content. In an intact rat chest model, 100μmol/kg of i.v. nitrite resulted in a significant reduction in systemic arterial and pulmonary arterial pressures (358). These changes were attenuated by inhibition of soluble guanylate cyclase (sGC), suggesting that nitrite-mediated vaso-relaxation occurs via release of NO. In humans, following intra-brachial infusions of nitrite, Maher and colleagues, observed only modest effects on resistance vessel tone in normoxia. In contrast, there was potent dilatation of the forearm capacitance bed (presumably due to the lower ambient oxygen tension in venous blood). In contrast, under hypoxic conditions nitrite profoundly vasodilated the forearm resistance bed (46). In healthy subjects, Ingram and colleagues demonstrated significant reductions in systemic arterial and pulmonary artery pressure with low dose (1μmol/min over 30minutes) of i.v. nitrite, during hypoxia but not normoxia (199). These effects were associated with a no more than a 2-fold increase in plasma nitrite concentration. No changes in stroke volume (SV) or cardiac output (CO) were observed. In contrast, our work (presented in Chapter 2) did not demonstrate significant changes in haemodynamic parameters in healthy volunteers, during normoxia or hypoxia.

Since pulmonary arteriolar blood has a low oxygen tension, particularly in low output states it is also likely that nitrite reduction to NO will occur in the pulmonary vasculature causing pulmonary vasorelaxation. Through selective venodilation and pulmonary vasorelaxation, without systemic arteriolar vasodilation, sodium nitrite has the potential to relieve DVI in heart failure, increasing stroke volume without excessive hypotension. Furthermore, in primates nitrite did not induce tolerance unlike organic nitrates (406). However, a study in which nitrite was administered in the Langendorff rat heart demonstrated eNOS-independent

NO mediated negative inotropic and lusitropic effects that might be expected to reduce CO if these were replicated *in vivo* in man (407). A further study in the Langendorff rat heart demonstrated the converse (162). Angelone and co-workers reported that exogenous nitrite improved cardiac contractility through enhancement of the Frank-Starling mechanism. This improvement was accomplished primarily through intra-cardiac NO production via activation of the sGC/cGMP/PKG pathway, although S-nitrosylation may play a role in nitrite-mediated cardiac modulation also. These contrasting and variable findings necessitate further investigation of nitrite in humans and in disease states. This early study, presented herein investigates the acute haemodynamic effects of sodium nitrite in patients with severe, but stable heart failure.

3.2 Objectives

We sought to better understand the vascular and cardiac effects of systemic nitrite infusion in patients with severe heart failure (HF), evaluating the dose-response relationship for these effects during both brief and more prolonged infusions. We hypothesised that systemic nitrite infusion would increase CO and reduce right atrial pressure (RAP) and pulmonary vascular resistance (PVR) with relatively modest reductions in systemic vascular resistance (SVR) and mean arterial blood pressure (MABP) in patients with severe HF.

3.3 Power calculation

19 patients were recruited into this 'proof of principle' pilot study, with participants acting as their own control (baseline measures compared to three different doses of nitrite infusion), thus increasing the statistical power. This number was chosen to give a representative sample, based on our experience of physiological studies with nitrite and the typical patient groups

undergoing transplant assessment. The intended group size in the heart failure study was guided by the calculation that 19 participants would give a statistical power of 80% to detect a 0.9 L/min difference in cardiac output at a standard deviation of 0.9 L/min.

3.4 Methods

3.4.1 Participants

The study was granted full ethical approval (South Birmingham Local Research Ethics Committee 08/H1207/67). The investigation conforms to the principles outlined in the Declaration of Helsinki. HF patients, undergoing elective right heart cardiac catheterisation to assess suitability for cardiac transplantation were recruited from the Queen Elizabeth Hospital Birmingham NHS Trust. All of the subjects gave written, informed consent after satisfying the inclusion and exclusion criteria (**table 3.1**). The study was performed in a temperature-controlled, cardiac catheterisation laboratory at the Queen Elizabeth Hospital Birmingham NHS Trust. All subjects had a light breakfast and were requested to abstain from alcohol and caffeine-containing drinks and nitrite/nitrate rich food for 24 hours before the study (foods to avoid as part of nitrite/nitrate poor diet outlined in **table 2.1**).

Inclusion criteria

- Systolic heart failure of any cause
- Aged 18 years or over
- Admission to hospital for pulmonary artery catheterisation, under the transplant assessment protocol

Exclusion criteria

- Pregnant women or nursing mothers
- Pre-existing inotrope therapy
- Recent acute coronary syndrome
- G6PD Deficiency or high risk of G6PD deficiency

Table 3.1: Inclusion and exclusion criteria for study A and B.

3.4.2 Haemodynamic effects of short term incremental infusion of sodium nitrite in heart failure patients (study A)

19 patients with advanced HF (NYHA class III - IV), who were undergoing right heart catheterisation as part of planned haemodynamic assessment for cardiac transplantation, were recruited and underwent evaluation of the dose-response relation to short systemic infusions of sodium nitrite (as depicted in **figure 3.1A**). Subjects were placed in a supine position to allow insertion of a 7.5French, quadruple-lumen Swan-Ganz pulmonary artery catheter (Edwards Lifesciences, California, USA) by the Clinician (independent of the study) performing the cardiac catheterisation . This was introduced via an 8French introducer sheath into the right internal jugular vein (under local anaesthesia, 1% lidocaine). Positioning was confirmed by fluoroscopy and pressure waveform monitoring. The position of the patient remained unchanged for the duration of the study. Placement of the Swan-Ganz catheter allowed invasive cardiac measurements, as described in more detail in section 3.4.3.

Following placement of the Swan-Ganz catheter each subject was allowed to rest for 5 minutes following which baseline measurements were obtained. Heart rate (HR), Mean arterial blood pressure (MABP), right atrial pressure (RAP), right ventricular end-diastolic pressure (RVEDP), pulmonary artery pressure (PAP), pulmonary capillary wedge pressure (PCWP) and CO (thermodilution) were recorded. 10ml of venous blood was obtained from the pulmonary artery, via the distal port of the Swan Ganz catheter for immediate blood gas analysis and later analysis of plasma RXNO, nitrite, nitrate and cGMP, as described in section 3.4.5. Thereafter, subjects received three 5-minute infusions of sodium nitrite at incremental rates (1, 10 and 50µg/kg/min) into the right internal jugular vein sheath.

On completion of each infusion rate, HR, MABP, PAP, PCWP and CO (thermodilution) were recorded again. Estimation of CO by the Fick principle and changes in estimated trans–septal pressure gradient (TSG, left ventricular end-diastolic pressure – right ventricular end-diastolic pressure) were calculated at baseline and following peak nitrite infusion rate, as described in section 3.4.3. MABP was measured non-invasively using an automated sphygmomanometer and all subjects had continuous electrocardiogram and arterial oxygen saturation monitoring for the duration of the study (Phillips Intellivue MP70, Surrey, UK). As shown in **figure 3.1A**, venous blood samples were taken at baseline and following each infusion rate of sodium nitrite.

3.4.3 Cardiac and peripheral haemodynamic measurements

Placement of the Swanz-Ganz catheter allowed direct measurement of the following haemodynamic parameters:-

- Right atrial pressure (RAP)
- Right ventricular end-diastolic pressure (RVEDP)
- Pulmonary artery pressure (PAP)
- Pulmonary capillary wedge pressure (PCWP)
- CO (Thermodilution method)

Other haemodynamic parameters which were calculated include the following:-

- CO by the Fick principle
- End-diastolic trans-septal pressure gradient (TSG)
- Pulmonary vascular resistance (PVR)

- Systemic vascular resistance (SVR)

These are described in more detail below.

CO was calculated using both the Thermodilution Method and the Fick Principle. The Fick Principle of measuring CO was used because of the frequent presence of tricuspid regurgitation in these patients which leads to inaccuracy of thermodilution-measured CO and due to the tendency of thermodilution to overestimate CO in severe HF (163, 408). To avoid multiple insertions of the catheter into the pulmonary artery, RAP, RVEDP and other measures derived thereof (systemic vascular resistance and end-diastolic trans-septal gradient), were only available at baseline and at peak nitrite infusion rate.

The Fick principle states that the uptake or release of a substance (e.g. oxygen) by an organ (e.g. lungs) is the product of blood flow through that organ and the difference between the arterial and venous values of the same substance. This therefore allows calculation of CO by measuring oxygen uptake and the difference in oxygen consumption across the pulmonary bed using the equation below (409):-

$$CO = \frac{VO_2}{(CaO_2 - CvO_2)}$$

(VO_2= oxygen consumption/uptake; CaO_2 = oxygen content of arterial blood; CvO_2 = oxygen content of pulmonary venous blood).

For the purposes of this study due to the inherent difficulty in measuring oxygen consumption directly, CO was measured using the following equation:-

$$CO\ (L/min) = \frac{135\ (ml\ O_2/min/m^2) \times BSA\ (m^2)}{13 \times Hb \times (SaO2\text{-}CvO2)}$$

(BSA = body surface area)

Venous blood from the pulmonary artery was obtained for CvO_2 measurement. Peripheral arterial oxygen saturation (SaO_2) were used as a measure of CaO_2 (410).

TSG is the difference between left ventricular end-diastolic pressure (LVEDP) and RVEDP. TSG determines the end-diastolic position of the inter-ventricular septum (411). In the presence of pulmonary hypertension or congestive HF, volume loading of the right ventricle (RV) can result in flattening or leftward displacement of the inter-ventricular septum, with a decrease in or reversal of end-diastolic TSG (411). With the putative selective venodilatation and pulmonary vasorelaxation conferred by nitrite in patients with HF, end-diastolic TSG is expected to increase. End-diastolic TSG was derived at baseline and following peak nitrite infusion using the following equation:-

$$TSG = PCWP - RVEDP$$

PCWP was used as surrogate of LVEDP.

Pulmonary vascular resistance (PVR) and systemic vascular resistance (SVR) were calculated using the following equations:-

$$PVR = \frac{\text{mean PCWP} - \text{mean PAP}}{CO}$$

$$SVR = \frac{MABP - RAP}{CO}$$

CO = cardiac index

3.4.4 Haemodynamic effects of sustained infusion of sodium nitrite (study B)

8 additional heart failure patients received a 3 hour infusion of 10µg/kg/min of intravenous sodium nitrite to evaluate the sustained haemodynamic effects of sodium nitrite in this patient group (as depicted in **figure 3.1B**). This infusion rate was established to be safe in healthy volunteers, as outlined in Chapter 2.

Subjects were prepared and a Swanz-Ganz catheter inserted as described previously. Following baseline measurements, subjects received i.v. sodium nitrite with haemodynamic measurements at 5, 15, 30, 60 minutes and every 30 minutes thereafter. Each measurement was followed by venous blood sampling (as depicted in **figure 3.1B**) and analysed as described in section 3.4.5.

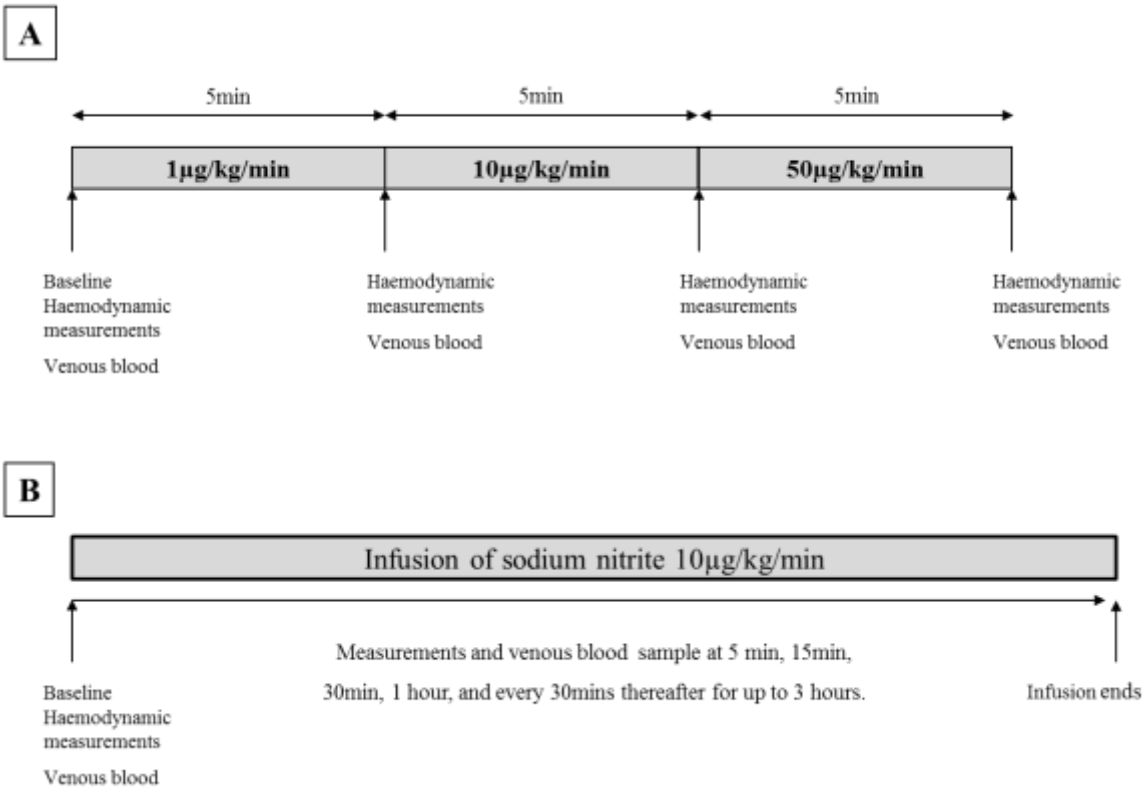


Figure 3.1: Study protocol. (**A**) HF subjects received 3 incremental doses of i.v. sodium nitrite at 1, 10 and 50µg/kg/min over 5 minutes each; (**B**) 8 HF subjects received a sustained infusion of 10µg/kg/min of i.v. sodium nitrite over 3 hours.

3.4.5 Blood sampling

Blood samples were obtained, from the distal port of the pulmonary artery catheter at the time points indicated in **figure 3.1**. 4mL of blood was transferred into an EDTA collection tubes (K3 EDTA, BD Vacutainers) containing 400µL of the thiol alkylator N-ethylmaleimide (Merck, Germany; 1mM final concentration) and centrifuged at 2000rpm at 4ºC, as described elsewhere (386). Plasma samples were immediately snap-frozen in liquid nitrogen and stored at -80ºC for subsequent analysis of plasma nitrite/nitrates and protein-bound NO (RXNO), as described in **Chapter 2, section 2.4.5**.

4mL of blood was transferred into an EDTA collection tube without any additive (K3 EDTA, BD Vacutainers) and centrifuged at 2000rpm at 4ºC. Plasma samples were immediately snap-frozen in liquid nitrogen and stored at -80ºC for subsequent analysis of cGMP, as described in **Chapter 2, section 2.4.5**. For study A, these analyses were performed at baseline and peak nitrite infusion rate of 50µg/kg/min only.

A further 2ml of blood was analysed immediately using a blood gas analyser (Bayer Rapidlab 865, Siemens, Tarrytown, NY) for metHb quantification.

3.4.6 Statistical analysis

The data were analysed using SPSS version 21.0 software (SPSS Inc., Chicago, USA). The data are expressed as mean ± standard error of mean (mean±SEM), unless otherwise stated. Probability values of <0.05 were considered statistically significant. One-way repeated measures ANOVA with consecutive Bonferroni post-hoc was used to test for changes from baseline in haemodynamic parameters during the short infusions of incremental sodium

nitrite in subjects who received all 3 doses of i.v. sodium nitrite (study A). A paired t-test was used for non-repeated data. Non-parametric data (during the sustained infusion of sodium nitrite, study B) was analysed using a Friedman's test with Wilcoxon signed-rank test for post-hoc analyses (study B). Changes in TSG were correlated with changes in stroke volume, CO and PCWP using Pearson's correlation.

3.5 Results

The patients had a diagnosis of HF of various aetiologies and all patients were on optimal standard heart failure therapy (**table 3.2**).

In study A, administration of all three nitrite infusion rates of 1, 10 and 50µg/kg/min was achieved in 9 out of 19 HF patients.

	Study A		Study B
	n=19	n=9	n=8
Age, years (mean±SD)	47±14	47±15	50±12
Gender, m	13	5	7
Body mass index (kg/m^2, mean±SD)	26±4	28±4	26±4
HR (bpm)	78±4	79±5	67±3
MABP (mmHg)	73±2	74±3	78±4
Baseline mean RAP (mmHg)	11.0±1.8	13.7±3.1	11.5(6.3 - 23)
Baseline mPAP (mmHg)	29± 2	31± 3	30(20 - 37)
Baseline mPCWP (mmHg)	20± 2	22±3	-
Baseline CO (L/min)	3.6±0.3	3.7±0.3	3.1(2.8 – 4.2)
Aetiology of heart failure			
Dilated cardiomyopathy	9	6	6
Ischaemic cardiomyopathy	5	1	2
Valvular heart disease	1	-	-
Other	4	1	-
Medication			
ACEI/AT2 receptor blocker	19	9	8
Beta blocker	13	7	6
Loop diuretic	19	9	6
Other diuretic	12	5	2
Aspirin/Warfarin	15	4	6

Table 3.2: Baseline characteristics of the HF patients.

3.5.1 Haemodynamic effects of short, incremental infusions of nitrite in heart failure patients (study A)

During short term incremental infusion rates of nitrite in HF patients, HR (**figure 3.2A**) decreased marginally from 79±5bpm at baseline to 76±5bpm following 50µg/kg/min of nitrite (n=9, $p<0.05$). HR remain unchanged following 1, 10 and 50µg/kg/min of nitrite (n=9, $p>0.05$). MABP remained unaltered throughout the duration of the study in 9 HF patients who received all 3 doses of i.v. sodium nitrite (**figure 3.2B**). However, in the 19 HF patients who received 50µg/kg/min of i.v. sodium nitrite only, there was a modest but statistically

significant reduction in MABP from 73±1.6mmHg at baseline to 70±1.3mmHg at peak nitrite infusion rate (**figure 3.2C**, n=19, $p<0.05$).

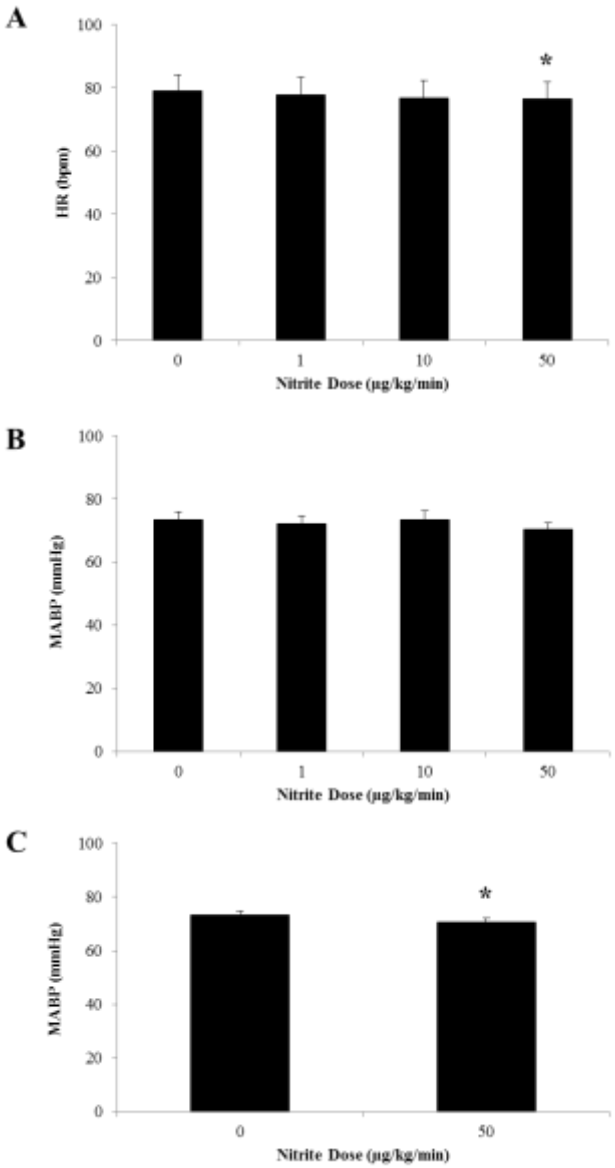


Figure 3.2: (**A**) HR decreased marginally from baseline following administration of 50µg/kg/min i.v. sodium nitrite to HF patients ($p<0.05$, n=9); (**B**) MABP remained unaltered at each dose in the 9 HF subjects who received 1, 10 and 50µg/kg/min i.v. sodium nitrite ($p>0.05$, n=9); (**C**) MABP decreased significantly at peak nitrite infusion rate, in HF patients following peak nitrite infusion rate of 50µg/kg/min (n=19, $p<0.05$). *$p<0.05$ compared to baseline.

At the highest infusion rate only, nitrite infusion significantly decreased PVR (**figure 3.3A**) by 30% from 184±23dyne*s/cm^5 to 129±15dyne*s/cm^5 (n=18, p<0.05) whereas SVR fell by 11% from 1356±82dyne*s/cm^5 to 1203±64dyne*s/cm^5 (**figure 3.3B**; n=18, p<0.05). These changes were associated with a significant reduction in mean PAP from 29± 2mmHg at baseline to 26± 2mmHg during 50µg/kg/min of i.v. sodium nitrite (n=19, p<0.05). Mean PCWP did not change (20± 2mmHg at baseline to 19± 2mmHg following peak nitrite infusion, n=19 and p>0.05). Escalating doses of i.v. sodium nitrite in 9 subjects demonstrated non-significant decreases in both PVR and SVR.

At peak infusion rate there was a significant (36%) reduction in mean RAP (**figure 3.4A**) from 11.0±1.8mmHg to 7.0±1.5mmHg following peak nitrite infusion rate (n=19, p<0.01). CO (Fick) increased by 10% from 3.8±0.2L/min to 4.3±0.2L/min from baseline to peak nitrite infusion rate (**figure 3.4B**, n=18; p<0.001). There was a trend towards an increase in CO (thermodilution) from baseline in 9 HF subjects at all doses with escalating i.v. nitrite infusion (**figure 3.4C**, n=9, p>0.05 compared to baseline).

Estimated TSG increased significantly from 9.1±1.9mmHg to 14.8±1.8mmHg at peak nitrite infusion rate (n=18, p<0.01). Change in TSG correlated positively with change in CO (**figure 3.5**, r=0.70, p=0.002) and change in stroke volume (r=0.73, p=0.001). Change in PCWP did not significantly correlate with change in CO (r=0.04, p>0.05).

All subjects were normoxic. Arterial oxygen saturations remained steady during nitrite infusion. Baseline saturations were 96.0±1.0% and did not vary with escalating doses of nitrite infusion (96.0±0.8%, p>0.05, n=9) or in patients only receiving the highest nitrite dose (97±0.2%, p>0.05, n=19).

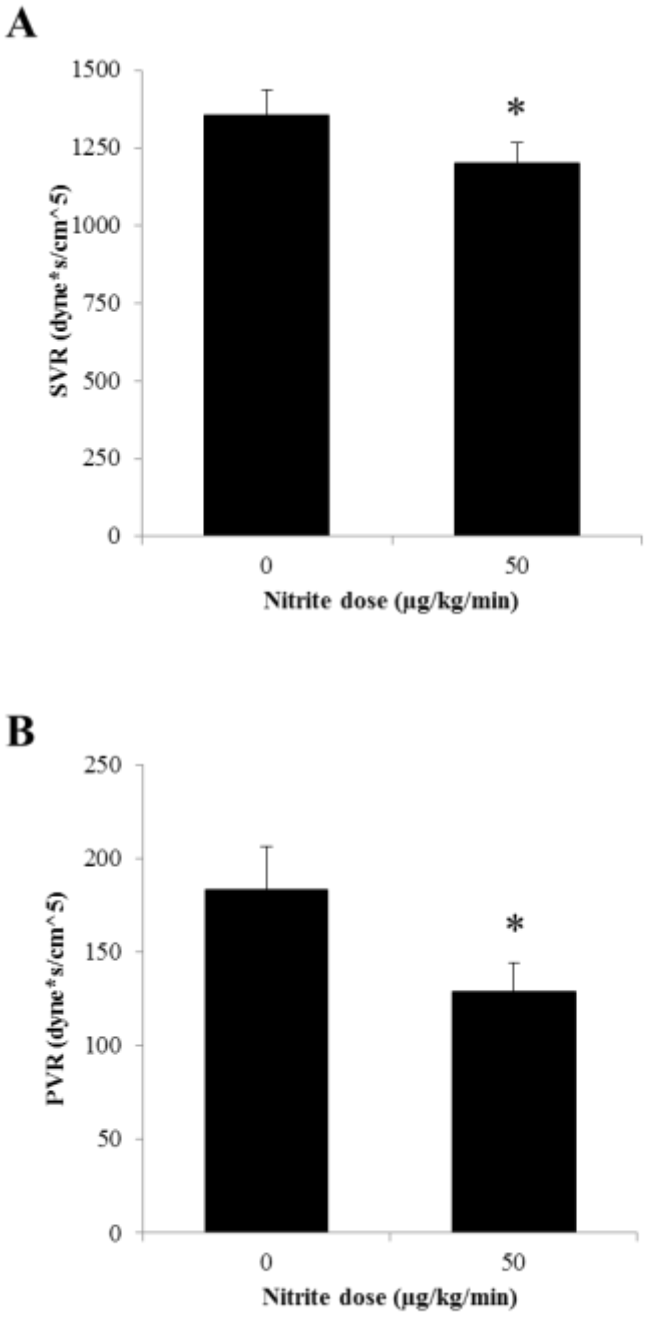


Figure 3.3: Both (**A**) PVR and (**B**) SVR decreased significantly from baseline at peak nitrite infusion rate in HF patients. PVR decreased to a greater degree than SVR. N=18, *$p<0.05$ compared to baseline.

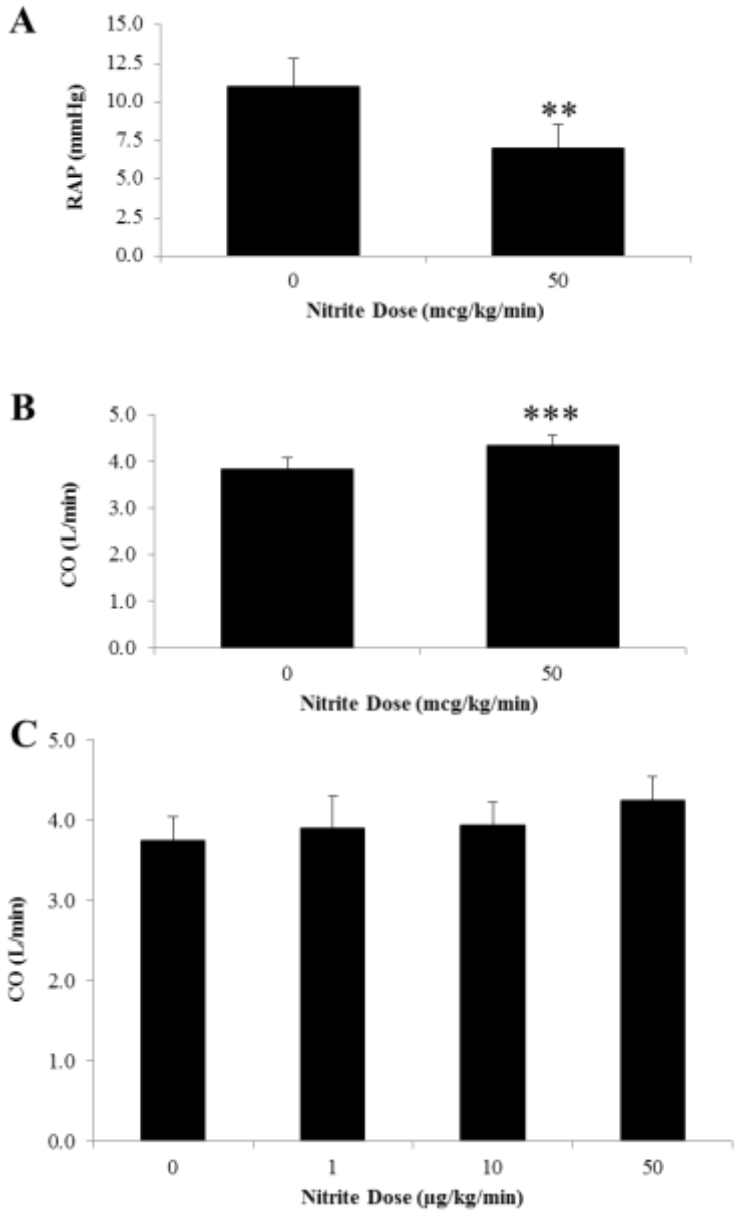


Figure 3.4: (**A**) RAP increased significantly from baseline at peak nitrite infusion rate (50µg/kg/min) in HF patients (n=19); (**B**) CO (Fick) increased significantly from baseline at peak nitrite infusion rate (50µg/kg/min) in HF patients (n=18); (**C**) CO (thermodilution) showed a trend towards increase from baseline with escalating nitrite (n=9, $p>0.05$). **$p<0.01$ and ***$p<0.001$ compared to baseline.

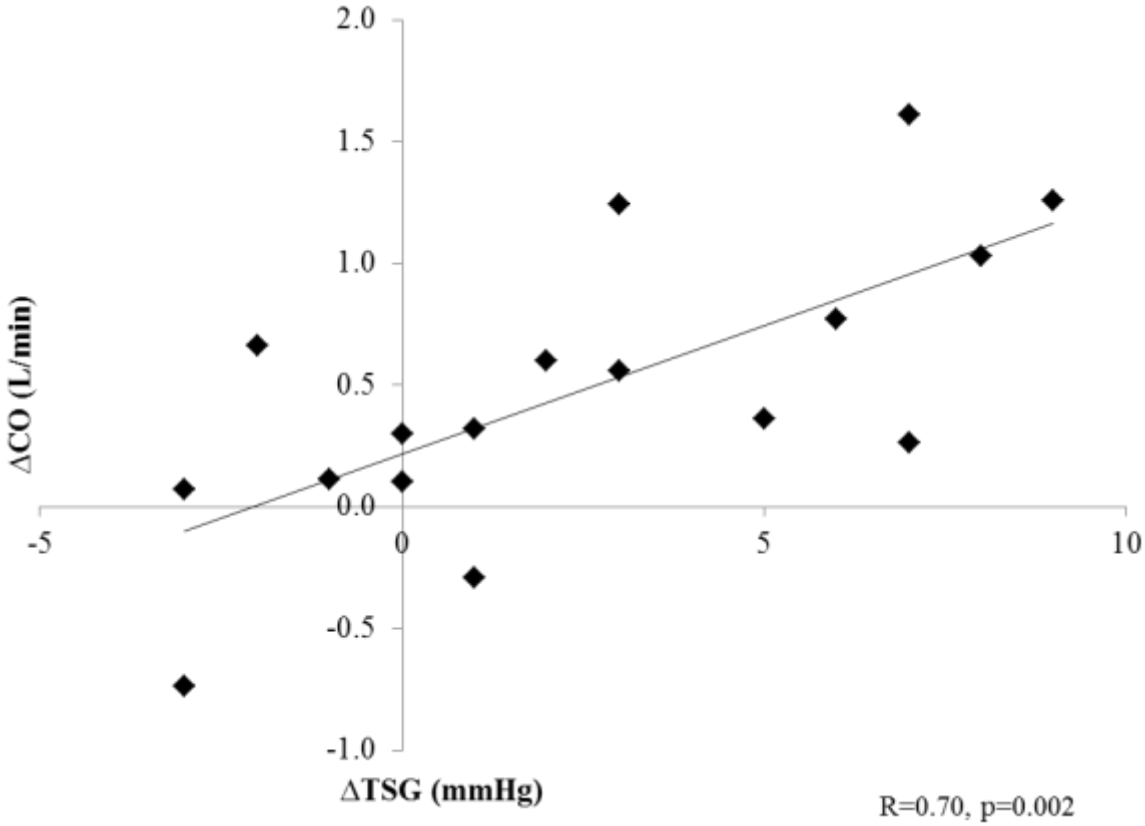


Figure 3.5: Change in TSG (baseline to peak nitrite infusion rate of 50µg/kg/min) correlated positively with change in CO (R=0.70, p=0.002, n=16).

3.5.2 Haemodynamic effects of sustained nitrite infusion of 10µg/kg/min in heart failure patients (study B)

Following prolonged infusion of 10µg/kg/min of i.v. sodium nitrite over 3 hours, HR and MABP did not alter. HR was 68(58 - 74)bpm at baseline and remain unchanged throughout the duration of the nitrite infusion ($p>0.05$, n=8, **figure 3.6A**). Similarly, MABP was 79(65 - 88)mmHg at baseline and remain unchanged throughout the duration of the nitrite infusion ($p>0.05$, n=8, **figure 3.6B**). Arterial oxygen saturations remained steady during nitrite infusion. Baseline saturations were 96.0±0.1% and did not vary throughout the infusion ($p>0.05$, n=8).

Mean PAP (mPAP) was 30(20 - 37)mmHg at baseline and decreased significantly from baseline after 30minutes to 23(15 - 34)mmHg ($p<0.05$, n=8). Thereafter it remain unchanged and was 22(16 - 42)mmHg after 180 minutes ($p>0.05$ from baseline, n=8). There was a trend towards a reduction in both SVR and PVR, but these changes were not significant. SVR (**figure 3.7A**) decreased non-significantly by 20% from 1711(1382 - 1778)dyne*s/cm^5 at baseline to 1369(1189 - 1682)dyne*s/cm^5 after 30minutes ($p>0.05$) and to 1375(1007 - 1902)dyne*s/cm^5 after 180 minutes ($p>0.05$, n=8). PVR decreased non-significantly from 144(68 - 261)dyne*s/cm^5 at baseline to 110(83 - 120)dyne*s/cm^5 after 30minutes ($p>0.05$) and thereafter increased to near baseline values of 16 (107 - 252)dyne*s/cm^5 after 180 minutes (**figure 3.7B**; n=8).

Figure 3.8A shows the change in RAP observed with sustained nitrite infusion. There was again a non-significant trend towards a reduction in RAP with nitrite infusion. Baseline RAP

decreased from 11.5(6.3 - 23)mmHg to 9(4.5 - 21.5)mmHg after 30minutes ($p>0.05$) and to 8.5(6.3 - 24)mmHg after 180 minutes ($p>0.05$, $n=8$). Congruently, there was a small, non-significant increase in CO (**figure 3.8B**). CO was 3.1(2.8 - 4.2)L/min at baseline and increased to 3.6(2.9 - 5.8)L/min/m2 after 180 minutes ($p>0.05$, $n=8$).

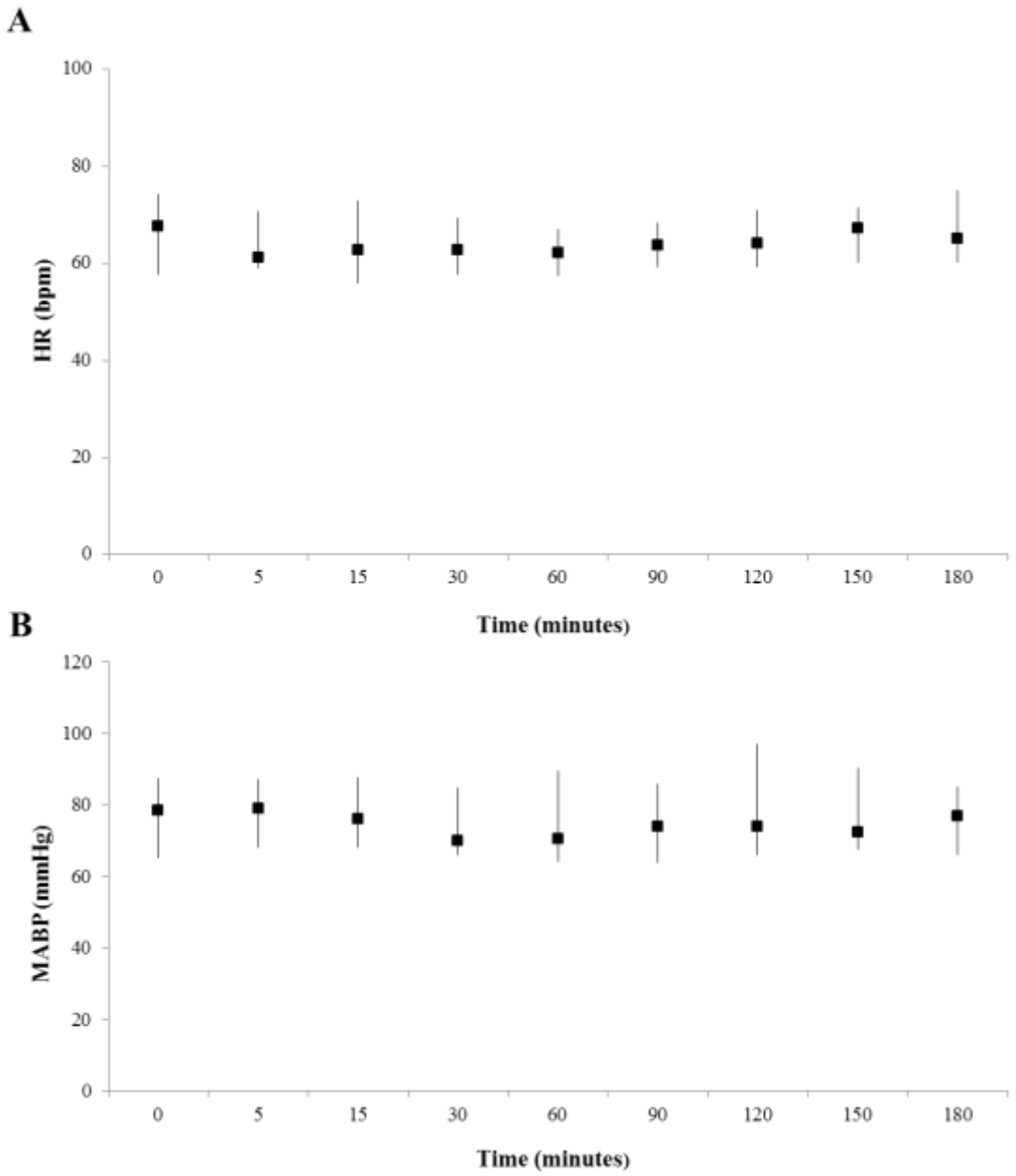


Figure 3.6: Both (**A**) HR and (**B**) MABP remained unchanged throughout the 3-hour i.v. nitrite infusion in HF patients (n=8, p>0.05, respectively). Data is expressed as median (interquartile ranges).

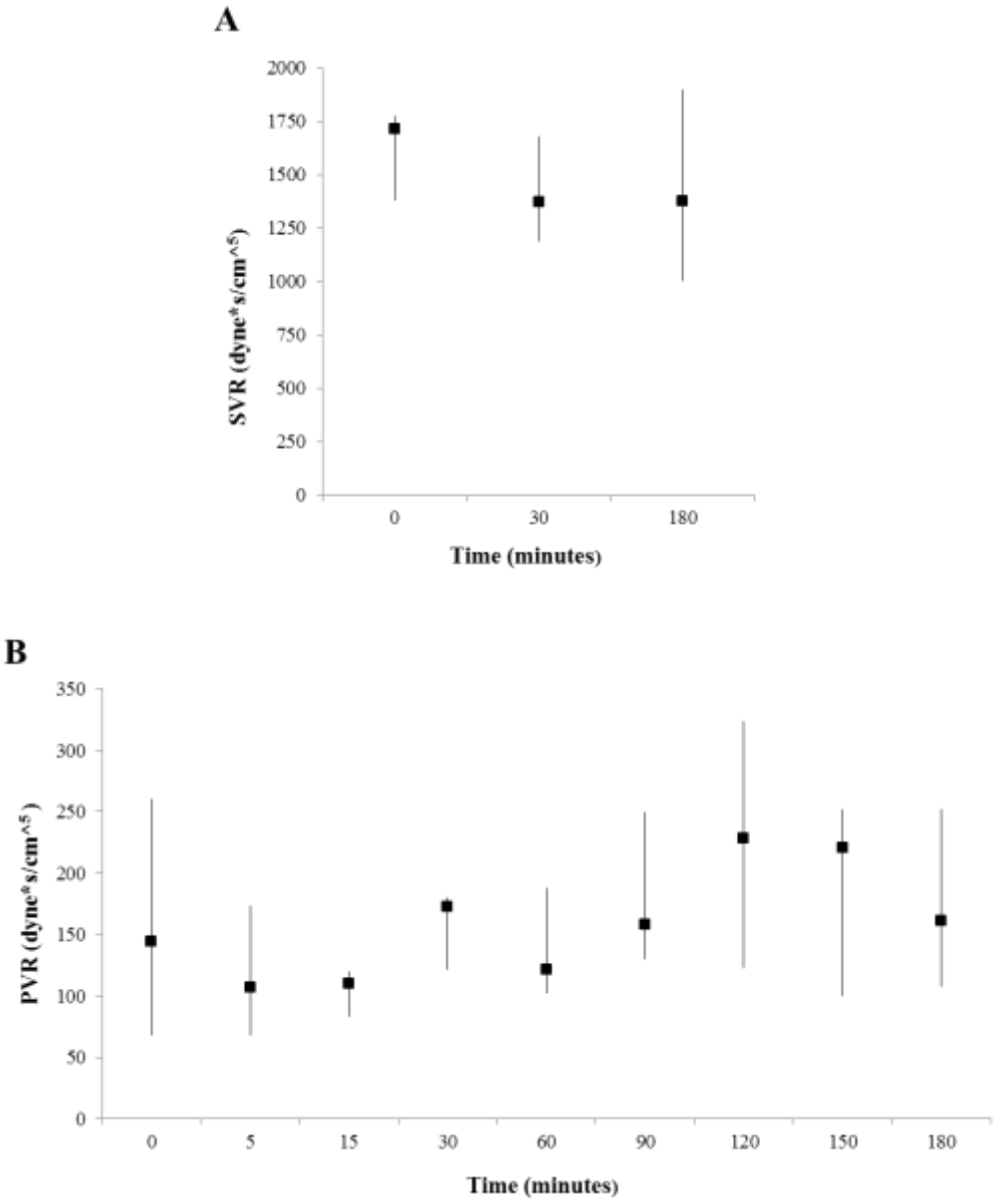


Figure 3.7: There were non-significant reductions in both (**A**) PVR and (**B**) SVR during 3-hour i.v. nitrite infusion in HF patients (n=8, p>0.05, respectively). Data is expressed as median (interquartile ranges). To avoid multiple insertions of the Swanz-Ganz catheter, SVR data is only available for the 3 time-points specified.

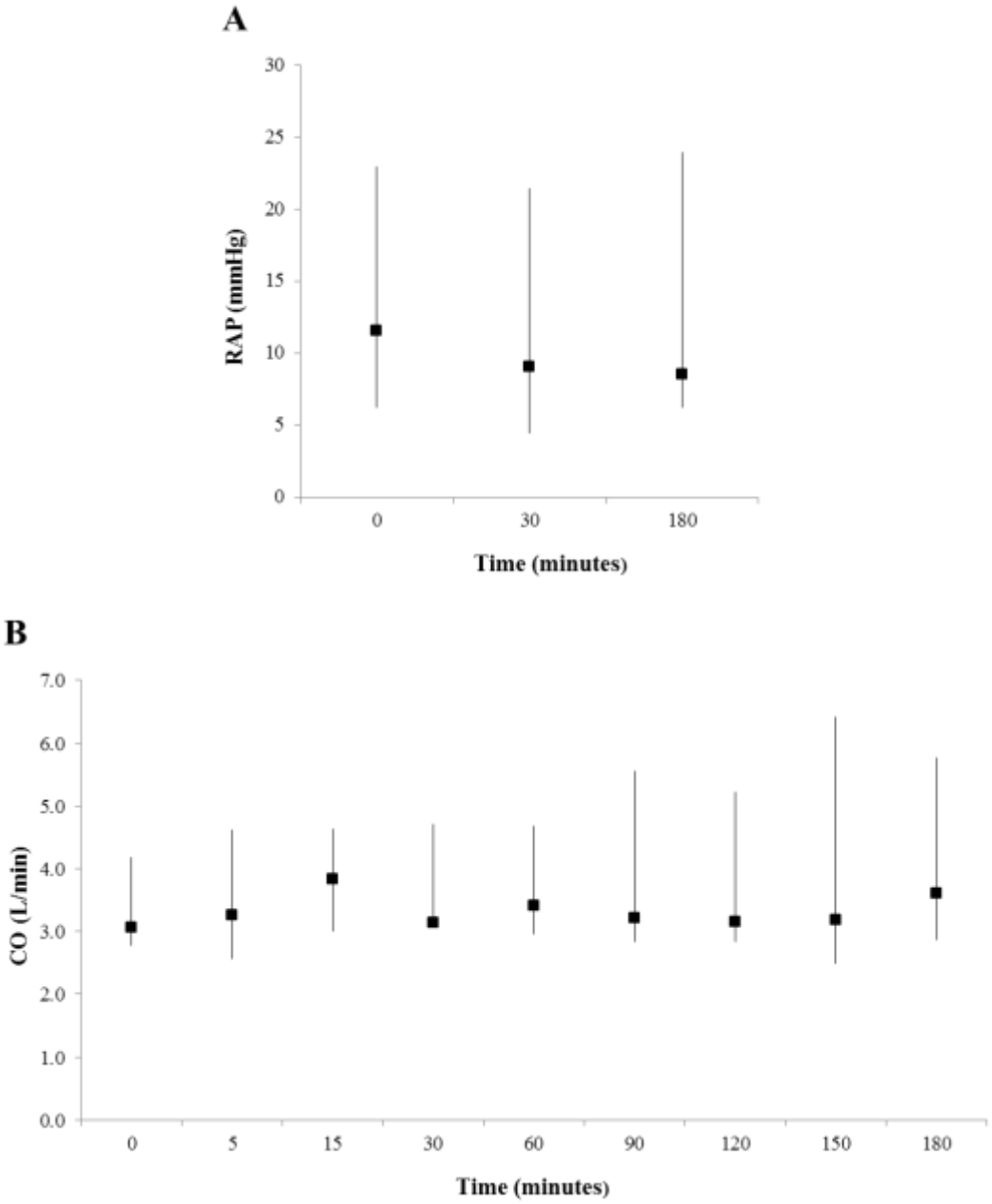


Figure 3.8: (**A**) There was a non-significant reduction in mean RAP following i.v. nitrite infusion in HF patients; (**B**) CO remained unaltered over the 3 hours (n=8, p>0.05). Data is expressed as median (interquartile ranges). To avoid multiple insertions of the Swanz-Ganz catheter, RAP data is only available for the 3 time-points specified.

3.5.3 Effects of nitrite infusion on venous metHb in heart failure patients

As expected, venous metHb levels rose in a dose-dependent and time-dependent manner with short, incremental infusions of nitrite (**figure 3.9A**) and following sustained nitrite infusion of 10µg/kg/min over 3 hours (**figure 3.9B**).

MetHb levels increased significantly following peak nitrite dose from 0.33±0.03% at baseline to 1.01±0.013% following 50µg/kg/min of i.v. sodium nitrite ($p<0.001$, $n=9$). Following sustained nitrite infusion, metHb increased significantly from baseline in a time-dependent manner (**figure 3.9B**). Baseline metHb increased from 0.5(0.3 - 0.5)% to a maximum of 1.5(0.8 - 2.0)% ($p<0.05$, $n=8$). After 60 minutes, the metHb level appeared to plateau.

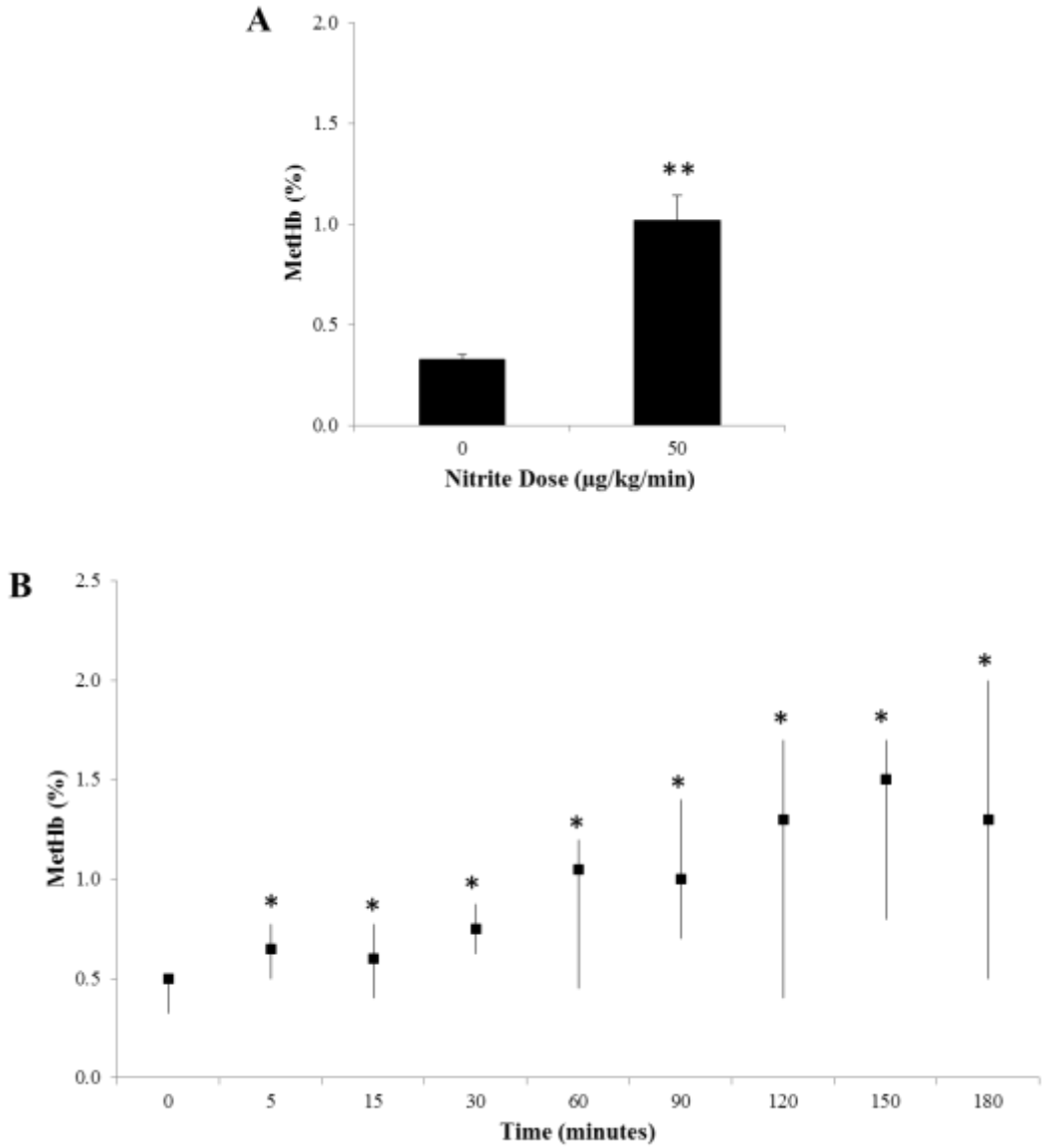


Figure 3.9: (**A**) Venous metHb increased in a dose-dependent manner with short, incremental infusions of nitrite in HF patients (n=9, **p<0.001, data is expressed as mean±SEM); (**B**) Similarly, there was a time-dependent increase in venous metHb with prolonged nitrite infusion, in HF patients (n=8, *p<0.05, data is expressed as median (interquartile ranges)).

3.5.3 Plasma nitrate, nitrite and RXNO levels in heart failure patients following nitrite infusion

Plasma RXNO, nitrite and nitrates all increased significantly from baseline to peak nitrite dose of 50µg/kg/min following a short (5-minute infusion) in HF patients (**figure 3.10**). Plasma RXNO levels were 12.2±1.3nM and increased to 37.7±5.9nM after administration of 50µg/kg/min i.v. sodium nitrite (**figure 3.10A**; n=18, p<0.0001). Baseline nitrite concentrations increased from 1.6±0.7µM to 16.0±2.8µM following the peak nitrite infusion rate (**figure 3.10B**; n=18, p<0.0001). Nitrate levels increased from 26.1±2.8µM to 37.1±3.6µM, respectively (**figure 3.10C;** n=18, p<0.0001).

Following prolonged (3-hour) infusion of 10µg/kg/min of i.v. sodium nitrite in HF patients, plasma RXNO concentration did not change significantly (**figure 3.11A**; n=7, p>0.05 compared to baseline). Plasma nitrites increased significantly from baseline values of 1.9(1.4 – 5.0)µM to a peak concentration of 9.6(6.3 – 14.8)µM after 120minutes (**figure 3.11B**; p<0.05 compared to baseline, n=7). After this time, plasma nitrite levels appeared to plateau and remained significantly above baseline levels. After 3 hours plasma nitrite concentration was 8.9(5.3 – 10.9)µM (n=7, p<0.05 compared to baseline). Plasma nitrate levels rose in a similar manner to nitrite (**figure 3.11C**). Baselines nitrate concentration increased from 24.7(18.93 – 52.0)µM to 54.3(31.7 – 59.7)µM after 120minutes and plateaued thereafter (n=7, p<0.05 compared to baseline).

In study A, baseline cGMP levels were 2.7±0.6fmol/well and after a 5-minute infusion of 50µg/kg/min of i.v. nitrite were 2.3±0.4fmol/well (n=11, p>0.05).

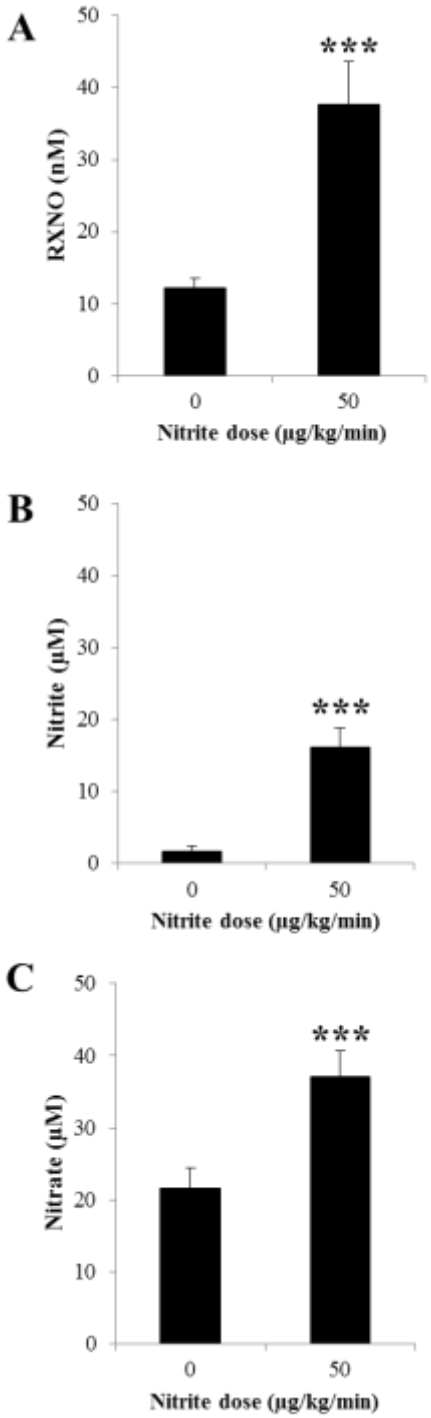


Figure 3.10: Plasma (**A**) RXNO, (**B**) Nitrite and (**C**) Nitrate increased in a dose-dependent manner from baseline (n=10). N=18, ***p<0.0001.

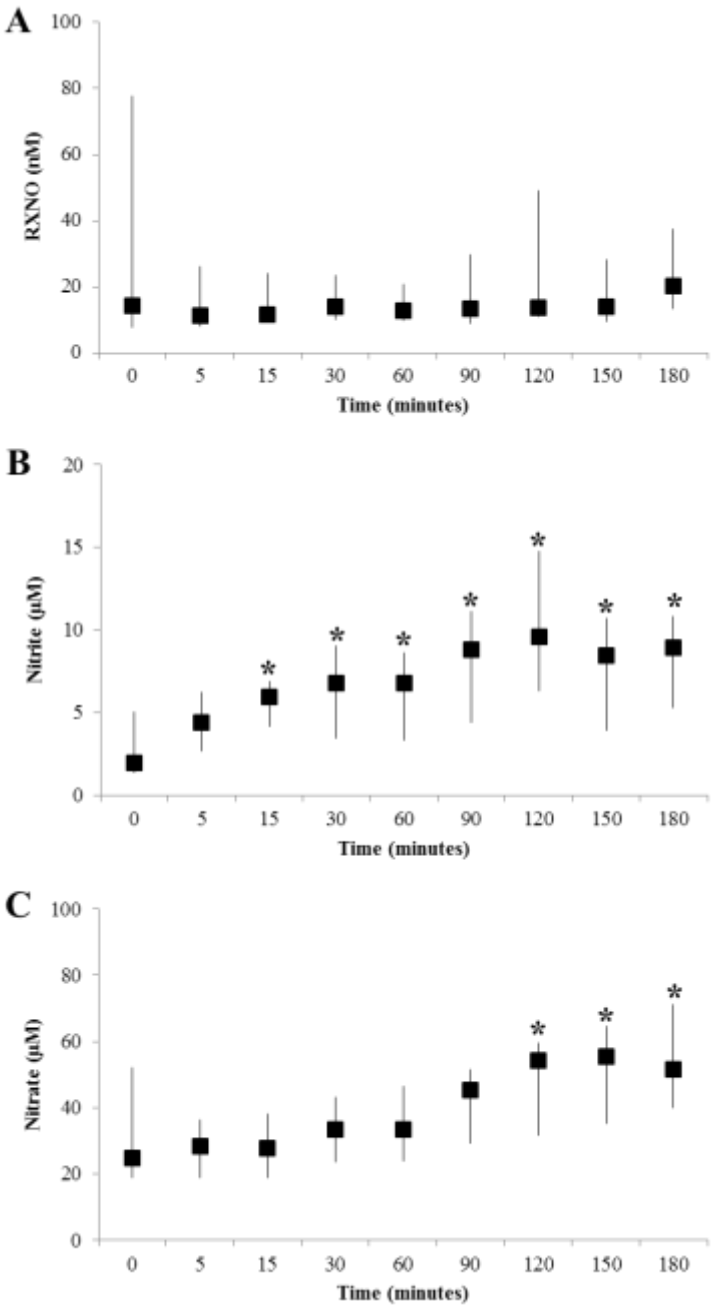


Figure 3.11: (A) Plasma RXNO concentrations remained stable throughout the 3hour (10µg/kg/min) nitrite infusion; (B) Plasma nitrite levels increased significantly from baseline after 30minutes and appeared to plateau after 120minutes; (C) Plasma nitrate levels increased from baseline after 120minutes and plateaued thereafter. N=7, *$p<0.05$. Data is expressed as median (inter-quartile range).

3.6 Discussion

To date this study is the first to assess the haemodynamic effects of systemic intravenous sodium nitrite infusion in patients with heart failure. In contrast to the findings in healthy subjects (as presented in Chapter 2), in patients with HF i.v.sodium nitrite demonstrated a highly favourable haemodynamic profile during short term administration, with an increase in CO in these patients though this was only significant at an infusion rate of 50µg/kg/min. There was a substantial reduction in PVR (30%) but only a modest reduction in SVR (11%) at this dose. Since CO increased and SVR fell only modestly, the fall in blood pressure was very small. This would be a very attractive haemodynamic effect for the treatment of decompensated heart failure. The use of current vasodilators such as GTN in these patients is often limited by the development of hypotension (403).

The observed substantial reduction in PVR in heart failure patients is consistent with a recent report of a substantial reduction in PVR with chronic administration of sodium nitrite in the rat monocrotaline model of pulmonary hypertension (412) and with the observation in man that a low infusion rate of sodium nitrite substantially reduced pulmonary artery pressure in acute hypoxia–induced pulmonary hypertension (199). Ventilation-perfusion mismatch is increased in patients with heart failure (413). Since nitrite is a hypoxia dependent vasodilator it might be expected to preferentially dilate arterioles supplying relatively under–ventilated alveoli and this could worsen ventilation–perfusion mismatching resulting in arterial desaturation. However, despite a substantial fall in PVR there was no observed reduction in systemic arterial oxygen saturation.

This study provides important mechanistic insights into how nitrite increased CO in patients with heart failure. RAP was markedly reduced during systemic nitrite therapy, presumably as a consequence of the fall in PVR, and potentially due to its venodilator action (46). In contrast, the reduction in PCWP was somewhat less. This resulted in an increase in the estimated TSG despite a reduction in PCWP. The increase in TSG observed during nitrite therapy implies amelioration of this DVI with an increase in effective left ventricular preload and hence an increase in stroke volume via the Frank-Starling mechanism, implying amelioration of diastolic ventricular interaction (DVI) (359, 414). Furthermore, the increase in CO was significantly correlated with the change in TSG ($R = 0.70$ $p=0.002$) but not with the change in PCWP. A recent study undertaken in the Langendorff rat heart reported that nitrite had negative inotropic and lusitropic effects via e-NOS–independent NO generation (407). Although we did not measure contractile state directly, our findings do not support a substantial negative inotropic effect in either healthy subjects or in patients with heart failure, in contrast to the study in the Langendorff model.

The substantial haemodynamic effects of sodium nitrite in patients with heart failure, albeit at a high dose were not replicated in healthy subjects as described in Chapter 2. This was the case even when subjects were administered i.v. nitrite under hypoxic conditions. NO resistance is well described in patients with heart failure in both platelets and blood vessels and in some patients may reduce the therapeutic effects of intravenous NO donors (414, 415). If nitrite bioactivity is predominantly mediated via reduction to NO as is commonly believed, then our study does not suggest marked NO resistance in these patients with heart failure (118). On the contrary, we have observed a much greater response in patients with heart failure compared to healthy controls.

Despite the favourable haemodynamic effects of short term administration of sodium nitrite at a dose of 50µg/kg/min in patients with heart failure, there would be substantial challenges to its use as a therapy for decompensated heart failure. Whilst we showed that sustained infusion of 10µg/kg/min sodium nitrite in both healthy volunteers and patients with heart failure only moderately increased methaemoglobin levels, this infusion rate did not cause a significant increase in cardiac output in heart failure patients during either short term or sustained infusion. Infusion of 60µg/kg/min in healthy subjects caused a substantial increase in methaemoglobin such that infusion was terminated in both subjects at 2 hours when methaemoglobin levels reached 10% (Chapter 2). Ingram and colleagues reported that infusion of sodium nitrite prevented hypoxia induced vasoconstriction in healthy subjects an hour after ceasing infusion at a time when plasma nitrite had returned to baseline (199). This may provide a rationale to investigate pulsed high dose sodium nitrite therapy in heart failure that may potentially have sustained favourable haemodynamic effects with only modest associated methaemogobinaemia.

Plasma cGMP did not alter significantly in heart failure patients at a haemodynamically effective infusion rate. This could be suggestive of cGMP-independent vascular relaxation to NO which is purported to be seen mostly at high concentrations of NO donors (416). Baseline plasma cGMP levels were elevated in the heart failure patients compared to healthy volunteers, as well recognised by others (417).

Plasma nitrite and nitrate increased in a dose dependent manner during the short-term infusions (**figure 3.10**). Despite similar increases within each group, there was a marked difference in haemodynamic effects between healthy volunteers (Chapter 2) and heart failure patients at peak nitrite infusion rate of 50µg/kg/min. Previously, Pinder et al. have

demonstrated, using rabbit aortic rings, that the effects of nitrite are not completely dependent upon nitrite reduction back to NO (418). Whether the effects observed in the heart failure patients could be explained by an NO-independent mechanism of nitrite –mediated vascular relaxation requires further exploration.

Study limitations

An important limitation of this study is the small number of patients studied. This may account for some of the perceived lack of benefit, particularly at the lower infusion rates of 1µg/kg/min and 10µg/kg/min. A full dose ranging study would have helped to establish if these gains (PVR reduction and improved CO with modest SVR reduction) would be replicated at doses of nitrite which do not induce significant methaemoglobinaemia. The subjects consisted of heart failure patients with varying aetiologies, which reflects the patient group being assessed for cardiac transplantation at University Hospitals Birmingham NHS Trust. Furthermore, there was no placebo group, therefore we were unable to correct for any non-nitrite related effects on cardiovascular or peripheral haemodynamic measurements in particular.

Conclusions

Sodium nitrite infusion has favourable haemodynamic effects in patients with heart failure during short term administration but this is only observed with high doses, potentially at higher, clinically significant maethaemoglobin levels. Although, sustained infusion at a lower rate did not induce clinically important methaemoglobinemia, significant haemodynamic effects were not observed. This study provides important initial insight into the role of nitrite in heart failure patients and further, placebo-controlled trials are required to ascertain the efficacy and mechanisms of putative nitrite therapy in heart failure. Alternate dosing

strategies including pulsed therapy should be explored. Furthermore, the issue of nitrite tolerance was not explored in this acute study and would be an important issue to address in future studies.

Chapter 4: Effects of Glyceryl Trinitrate on Human Resistance Vessels during Normoxia and Hypoxia

4.1 Introduction

Organic nitrates such as glyceryl trinitrate (nitroglycerin, GTN) have been in clinical use for approximately 130 years for the treatment of angina pectoris with the first literature report in the late 1800s (419). Notwithstanding modern pharmacological advances, they remain in widespread use for several cardiovascular conditions including acute coronary syndromes and heart failure. However, studies exploring the mechanisms of GTN bioactivity have only been conducted relatively recently. The discovery that endogenous NO production via GTN contributes to relaxation of vascular smooth muscle has only been made within the last 35 years (5, 129, 420, 421). Since then much interest has focussed on the exact mechanisms of action of GTN-mediated vascular dilatation, which to date still remain to be fully determined.

GTN is a pro-drug which is thought to exert its vascular effect through release of NO or a nitrosothiol intermediary. An earlier concept hypothesised that GTN is bioactivated non-enzymatically through its interaction with thiols or sulfhydryl (-SH)-containing cellular receptors (129, 422). Reaction of GTN with thiol groups could result in formation of an S-nitrosothiol intermediary and NO, with subsequent activation of soluble guanlyase cyclase (sGC) and vascular smooth muscle relaxation (129, 423). However, there is some controversy surrounding this notion as it has not been fully established whether the mechanistic processes seen *in vitro* result in GTN bioactivation *in vivo* (172).

An alternative and more contemporary theory suggests that GTN bioactivation is catalysed via an enzymatic mechanism. Evidence to date suggests that the most likely candidate for this process is mitochondrial aldehyde dehydrogenase (ALDH2) (168). Firstly, Chen and colleagues demonstrated the biotransformation of GTN to 1,2-GDN and nitrite *in vitro* in mouse macrophages. They proposed the following stoichiometric reaction for this:-

$$GTN + Ered \rightarrow 1,2\ GDN + NO2^- + Eox$$

(Ered = reductase enzyme; Eox = oxidising enzyme)

Secondly, there was an attenuation of GTN-mediated vascular relaxation in rat thoracic aorta following application of the ALDH inhibitors, cyanamide and chloral hydrate. Moreover, in anaesthetised rats receiving systemic infusions of i.v. GTN, administration of cyanamide and chloral hydrate attenuated the hypotensive effect of GTN, suggesting that nitrate tolerance may be caused by ALDH inhibition (largely the mitochondrial ALDH2 isoform) (168). Further work undertaken by the same group, confirmed these findings in ALDH2-knockout mice (170). Others have also confirmed the role of ALDH2 in the bio-activation of organic nitrates in subsequent *in vitro* and *in vivo* animal studies (169), (168, 170). Mackenzie and colleagues corroborated this by exploring forearm vascular responses to GTN (assessed via venous occlusion plethysmography) in healthy volunteers. They found that ALDH inhibition (either through oral disulfiram administration or through more specific intrinsic ALDH2 inactivity in subjects lacking the glu504lys ALDH2 mutation) resulted in an attenuation of GTN-mediated forearm vasodilatation (171).

Much of the clinical benefit conferred by GTN is attributed to its ability to dilate veins and large arteries at low doses, resulting in redistribution of blood flow to the venous capacitance bed (424-426). It is widely believed that in heart failure, this reduces preload and offloads a congested heart, allowing a modest improvement in cardiac output with a resulting improvement in clinical symptoms (427, 428). In support of this concept, administration of GTN has been shown to reduce right atrial pressure and left-ventricular end-diastolic pressure in studies (429-431). At high doses, GTN causes arteriolar dilatation resulting in reduced

afterload (432, 433). However, mechanistic studies directly comparing the arterial *v*s venous effects of GTN have reported variable results. Exposure of bovine intrapulmonary arteries and veins to GTN resulted in a greater vascular relaxation in veins compared to arteries (434). Similarly, in rabbit femoral and mesenteric vessels, veins were observed to be more sensitive to GTN than arteries at low concentrations of GTN (426). A related effect has been observed in human forearm resistance vessels compared to dorsal hand veins (435). In contrast, canine coronary arteries exhibited a much greater degree of GTN-mediated vasodilatation than mesenteric or saphenous veins (436). This greater arterial vasodilatation has been reported by others also (437, 438). A human study found GTN to be equipotent in the human forearm vasculature (439). These inconsistent reports may be attributable to differing protocols applied to a diverse range of blood vessels from a variety of animal species.

Despite incomplete knowledge regarding the mechanisms of action of organic nitrates and lack of randomised controlled clinical trials with a proven mortality benefit, the medical experience with nitrates is long-standing and hence their use in clinical practice is well established (440). Nevertheless, alternatives are constantly being sought. Nitrite as a NO donor with its relative venoselectivity and attribute of specific hypoxic vasodilatation, could potentially offer an alternative therapy to the well-established organic nitrates. The data regarding enhanced vascular effects of nitrates during hypoxia is especially scanty. In experiments utilising *ex vivo* porcine coronary arteries, Fukuda and colleagues reported hypoxia-potentiated vascular relaxation following exposure to GTN (441). Agvald et al investigated the effect of reducing oxygen tension on NO release following administration of GTN. Using anaesthetised rabbits, they demonstrated an incremental increase in NO liberation following i.v. GTN administration as graded hypoxia was commenced. They reported a >300% increase in NO production during hypoxia. To eliminate the scavenging

effects of haemoglobin, they performed the experiments using isolated buffer-perfused lungs and observed similar results. They observed nitrite reduction to NO during hypoxia (but not normoxia), in the absence of haem proteins (442).

In humans, greater peripheral haemodynamic effects have been observed following oral GTN administration in healthy subjects living at high altitude presumed to be due to moderate hypoxia, compared to those living at low altitude (443). With the paucity of data, it is unknown whether organic nitrates truly enhance vasodilatation during hypoxia or whether this phenomenon is unique to nitrite. Currently, the dosage and regimen of GTN treatment is similar for all patients regardless of underlying indication for vasodilator therapy and oxygen tension. This knowledge could yield important information for future studies investigating both nitrates and nitrite in cardiovascular disease as therapy could be tailored to patients with greater efficacy and improved clinical outcomes taking into consideration oxygen tension. Potentially, this may be important for subjects living at high altitude, critically ill patients with reduced oxygen saturations e.g. heart failure or those with myocardial infarction with associated cardiogenic shock.

4.2 Objectives

The objective of this study was to establish whether organic nitrates (GTN) exhibit hypoxia-specific vasodilatation in humans, similar to that seen with nitrite. This was determined in the intact forearm vasculature of healthy volunteers, using venous occlusion plethysmography.

4.3 Methods

4.3.1 Participants

10 healthy volunteers were recruited into this study. Subjects were recruited from the University of Birmingham. The study was granted full ethical approval (South Birmingham Local Research Ethics Committee 08/H1207/283). The investigation conforms to the principles outlined in the Declaration of Helsinki. All of the subjects gave written, informed consent after satisfying the inclusion and exclusion criteria (see below). The study was performed at the University of Birmingham Clinical Research Block in a quiet vascular laboratory that was temperature-controlled at 22 - 24ºC. All subjects had a light breakfast and were requested to abstain from alcohol and caffeine-containing drinks and nitrite/nitrate rich food for 24 hours before the study (foods to avoid as part of nitrite/nitrate poor diet outlined in **table 2.1**).

Inclusion criteria included age over 18 years, history of no-smoking and no regular medication (except the oral contraceptive pill). All subjects underwent a cardiovascular examination and had a normal electrocardiogram (ECG). Women of child-bearing potential or nursing mothers, people with BMI≥30kg/m^2 and migraine sufferers were excluded from the study.

4.3.2 Study protocol

The study protocol is depicted in **figure 4.1**. 10 healthy volunteers attended on one occasion. Each subject received three 5-minute intra-arterial infusions of glyceryl trinitrate (GTN, Lipha Pharmaceuticals Ltd, UK) at escalating doses of 4, 8 and 16nmol/min using non-

adsorptive tubing– once whilst breathing room air (normoxia) and once whilst breathing 12% oxygen (hypoxia), in random order. Forearm vasodilatation was assessed using venous occlusion plethysmography. Each series of infusions was separated by a 20-minute washout period to allow GTN-mediated vascular relaxation to return to baseline values.

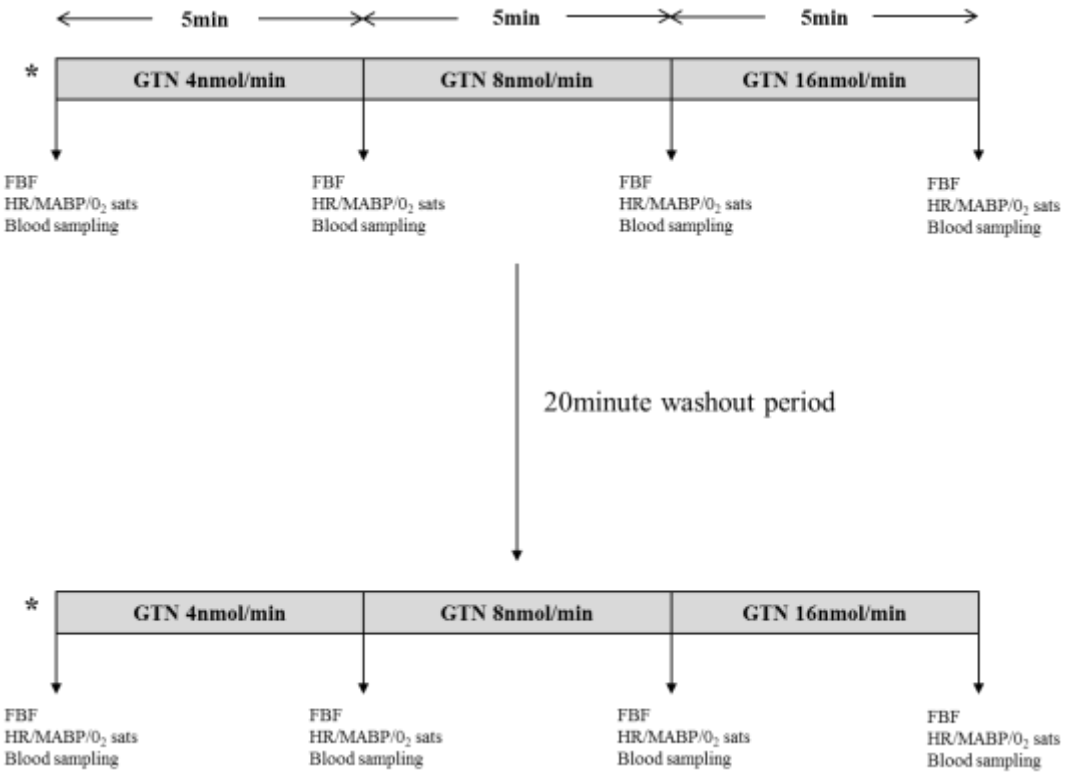
Subjects were placed in a semi-recumbent position enabling the administration of hypoxia to participants. An intravenous cannula (20-gauge) was inserted into a vein in both antecubital fossae. Changes in blood flow, using mercury in-silastic strain gauges were recorded in both arms (DE Hokanson, Bellevue, Wash), as detailed in *section 4.3.3*. GTN was administered intra-arterially *via* a 27-gauge arterial needle (Coopers Engineering, UK) mounted onto a 16-gauge epidural catheter and sealed with dental wax, as depicted in **figure 4.2A**. This was inserted aseptically into the brachial artery of the non- dominant arm under local anaesthesia (1% lidocaine) and was kept patent by intra-arterial (i.a) 0.9% saline infused at 1ml/min before initiation of GTN. Each subject underwent placement of a finger cuff for continuous blood pressure recording, an upper arm cuff for the oscillating blood pressure measurement, and chest electrodes for continuous electrocardiogram monitoring using the TaskForce Monitor® (CN Systems, Graz, Austria). Oxygen saturation levels were monitored continuously with pulse oximetry (Nellcor, Pleasanton, CA, USA). Measurement of forearm blood flow (FBF) is described in more detail in section 4.3.3. The experimental set-up is depicted in **figure 4.2B**.

As depicted in **figure 4.1**, following 10 minutes of stabilisation, subjects received three doses of intra-arterial GTN (4, 8 and 16nmol/min for 5 minutes each; infusion rate 1ml/min using non-adsorptive tubing) as employed by Kharbanda et al. (444). GTN was infused into

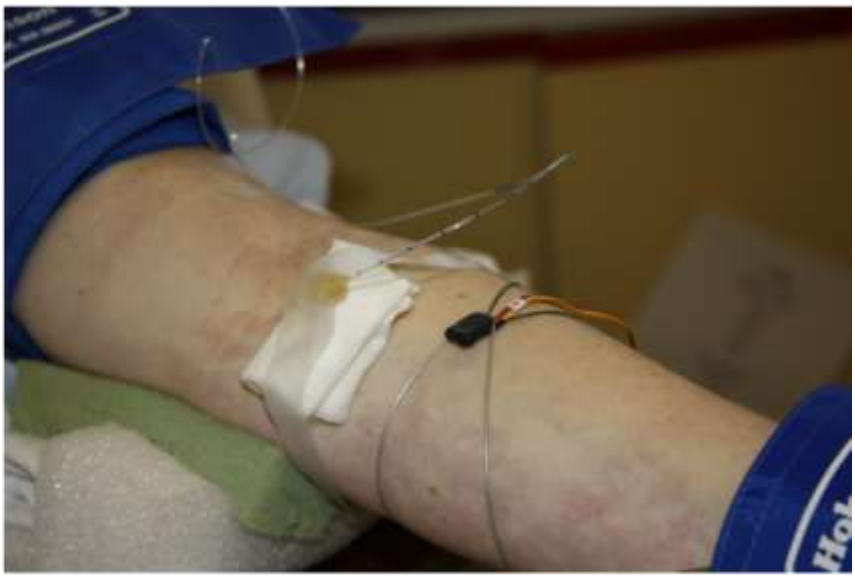
the brachial artery of the non-dominant arm and FBF was measured in both arms after each dose, as detailed in section 4.3.3.

6mL of venous blood was obtained from the infused arm cannula. 4mL was stored for later analysis of plasma nitrite/nitrates and 2mL was analysed immediately for plasma pH and lactate, as described in section 2.4.5.

Figure 4.1: Study protocol. *One series of infusions was administered during normoxia and the other during hypoxia, in random order.



A



B

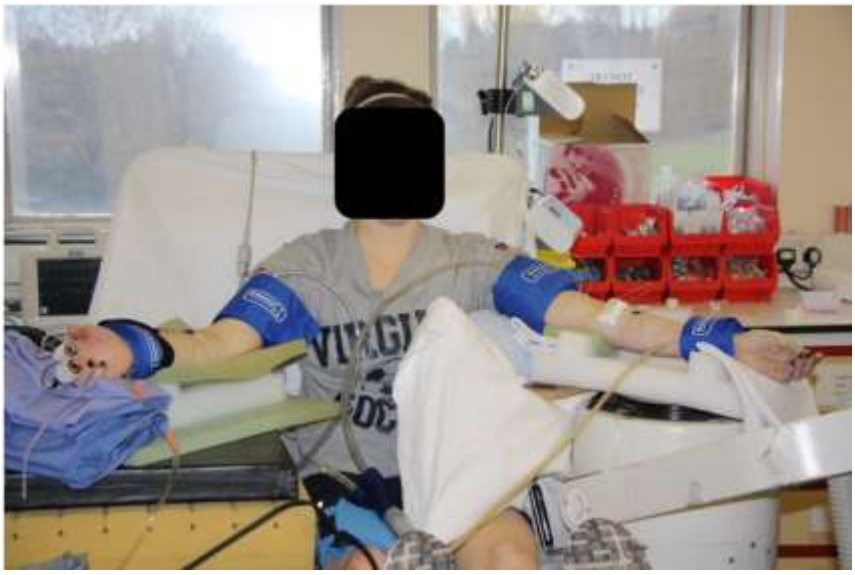


Figure 4.2: (A) Cooper's needle for intra-arterial GTN infusion into the brachial artery; (**B**) Typical experimental set-up of subjects in the study.

The third infusion of intra-arterial GTN (16nmol/min) was followed by a 20 minute washout period, during which time intra-arterial 0.9% saline was infused to ensure patency of the Cooper's needle. After 20minutes, the study then proceeded with three further 5minute infusions of intra-arterial GTN at 4, 8 and 16nmol/min, as described above.

During one series of infusions, the subjects were rendered hypoxic by inspiring 12% oxygen *via* a Hudson facemask, as employed by Maher et al. (46). Target oxygen saturation as measured by pulse oximetry was 83-88%, as described in Chapter 2, section 2.3.3. Upon achieving target oxygen saturation a further 10minute stabilisation period was observed. Following steady state hypoxia, three 5minute infusions of intra-arterial GTN at 4, 8 and 16nmol/min were administered, as described above. Oxygenation status was determined in random order.

Heart rate (HR), mean arterial blood pressure (MABP) and arterial oxygen saturations were recorded at baseline and following each dose of GTN during normoxia and hypoxia.

4.3.3 Measurement of forearm blood flow

Forearm blood flow (FBF) is a well-established measure of vascular relaxation/dilatation, as described in the literature (445). Mercury in-silastic strain gauges (DE Hokanson, Bellevue, Washington, USA) were placed on the widest part of the forearm and these allow detection of any changes to forearm circumference, as a result of variations in blood flow to the limb. A venous occlusion cuff was placed on the upper arm and one on the wrist, of each arm. During each recording, the wrist cuff was inflated to supra-systolic arterial blood pressure (200mmHg) to exclude the hand for the circulation. Blood flow in the hand is predominantly

through the skin and therefore fluctuates widely. This was followed by inflation of the upper arm cuff to 40mmHg to allow venous occlusion but without affecting arterial inflow. As blood flow into the limb increased, it caused the limb to swell at a rate proportional to the arterial inflow. Therefore the rate of swelling was used to measure the rate of arterial flow rate. The venous cuff was inflated for 10 seconds and then deflated for 5 seconds. This step was repeated three times for each recording in both arms simultaneously. FBF was calculated using LabChart 5 software (2006, AD Instruments, UK) in mL/100mL of tissue per minute and expressed as a ratio of change from baseline in FBF from infused:control arm (ΔFBF ratio). **Figure 4.3** depicts a typical recording of FBF obtained from one of the study subjects.

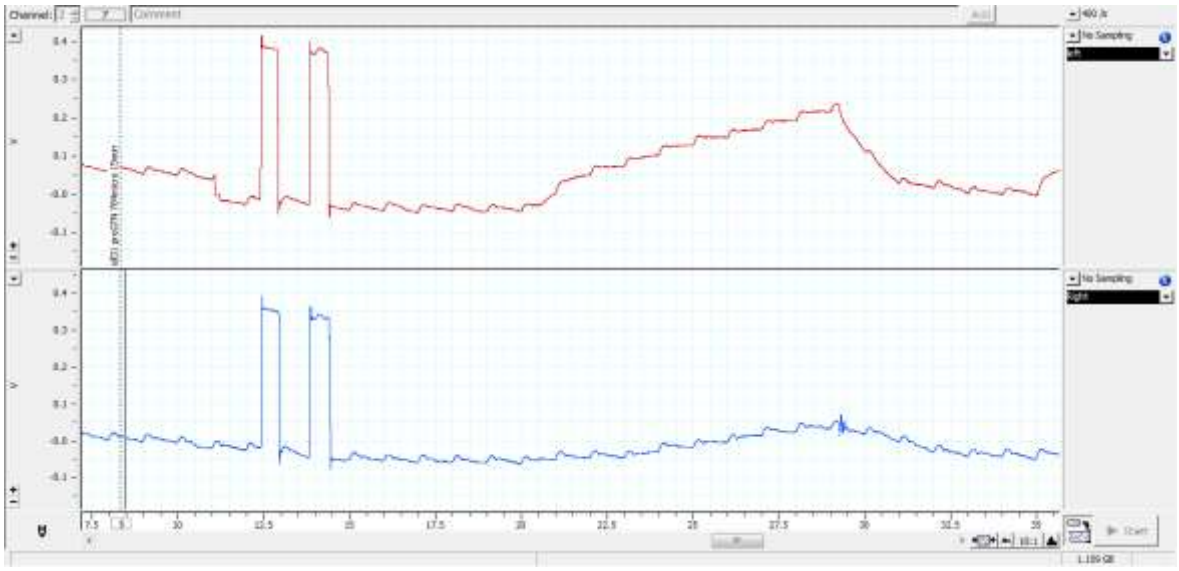


Figure 4.3: Typical FBF recording obtained during a recording.

4.3.4 Blood sampling

For analysis of plasma nitrite/nitrate, 4mL of blood was transferred into an EDTA collection tube (K3 EDTA, BD Vacutainers) pre-treated with N-ethylmaleimide (10mM; Merck, Germany). These samples were centrifuged at 2000rpm at 4ºC followed by immediate snap-

freezing in liquid nitrogen and storage at -80ºC for later analysis of plasma nitrite/nitrates, as described in section 2.4.5. A further 2ml of venous blood was analysed immediately using a blood gas analyser (Bayer Rapidlab 865, Siemens, Tarrytown, NY) for measurement of pH and lactate.

4.3.5 Statistical analysis

Repeated measures ANOVA with consecutive Bonferroni post hoc was used to test for changes from baseline in all parameters during normoxia and hypoxia, with each subject acting as their own control. The data are expressed as mean ± standard error of mean (mean±SEM), unless otherwise stated. Probability values of <0.05 were considered statistically significant. Statistical analysis was undertaken using SPSS (version 21.0 SPSS Inc., Chicago, USA).

4.4 Results

4.4.1 Baseline characteristics

Table 4.1 outlines the subject characteristics.

	N=10
Age, years (mean±SD)	22±2
Gender, m	5
Body mass index (kg/m^2, mean±SD)	21±2
Forearm circumference (cm)	24±2
HR (bpm)	64±3
MABP (mmHg)	79±4

HR, heart rate; MABP, mean arterial blood pressure. Data is expressed as mean±SEM unless stated otherwise.

Table 4.1: Baseline characteristics of study participants.

4.4.2 Effect of oxygenation on GTN-mediated vasodilatation

There was a dose-dependent increase in FBF ratio with incremental infusions of intra-arterial GTN during normoxia and hypoxia (**figure 4.4**). During normoxia, there was a 3.5 fold increase in FBF ratio from baseline following peak GTN infusion rate ($p<0.05$, n=10). Absolute FBF ratio was not significantly different during normoxia or hypoxia, although there was a trend towards a lower FBF ratio in hypoxia. At baseline normoxia, FBF ratio was 1.8±1.1 and at baseline in the hypoxia arm was 1.6±0.8 ($p>0.05$, n=10). During hypoxia, a smaller increase in FBF ratio was observed. FBF ratio increased by 1.8 times from baseline following maximal GTN infusion rate ($p<0.05$, n=10). However, there was no statistically

significant difference between ΔFBF ratio between normoxia and hypoxia at any infusion rate of GTN ($p>0.05$, n=10).

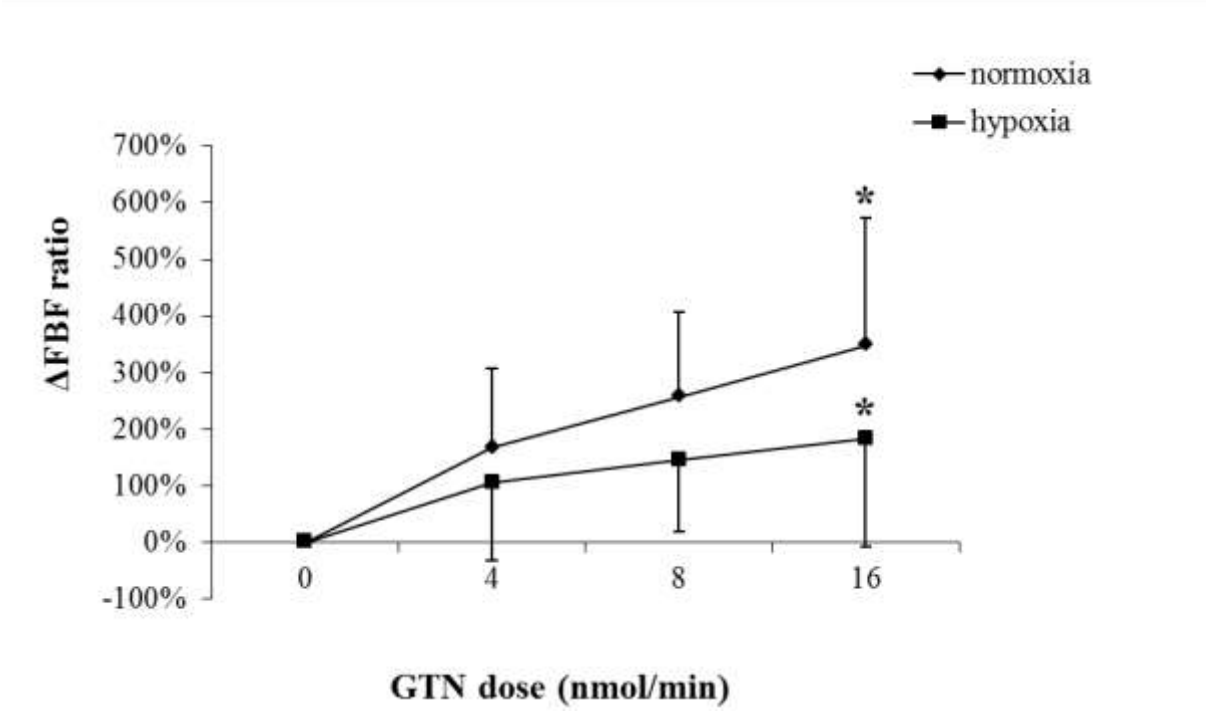


Figure 4.4: There was a dose-dependent increase in ΔFBF ratio during both normoxia and hypoxia. *$p<0.05$ compared to baseline, n=10. Data is expressed as mean±SD.

4.4.3 Haemodynamic data

During normoxia, arterial oxygen saturations were 99.0±0.3% (at baseline and remained unchanged throughout the duration of the infusions (**figure 4.5A**, $p>0.05$, $n=10$). During hypoxia, baseline oxygen saturations of 86.0±0.5% were achieved and remain steady for the duration of the infusions ($p>0.05$, $n=10$). As expected, there was a significant difference between arterial oxygen saturations during normoxia and hypoxia at all infusion rates ($p<0.05$, $n=10$ for all infusion rates of GTN).

During both normoxia and hypoxia, there was no significant change in HR from baseline (**figure 4.5B**). However, baseline HR differed significantly between normoxia and hypoxia and this difference remained constant throughout the duration of the GTN infusion. Baseline HR was 64±3bpm during normoxia and 71±3bpm during hypoxia ($p<0.05$, $n=10$).

MABP remained constant (**figure 4.5C**) for the duration of the study, during both normoxia and hypoxia, with no difference being observed between the two oxygenation states.

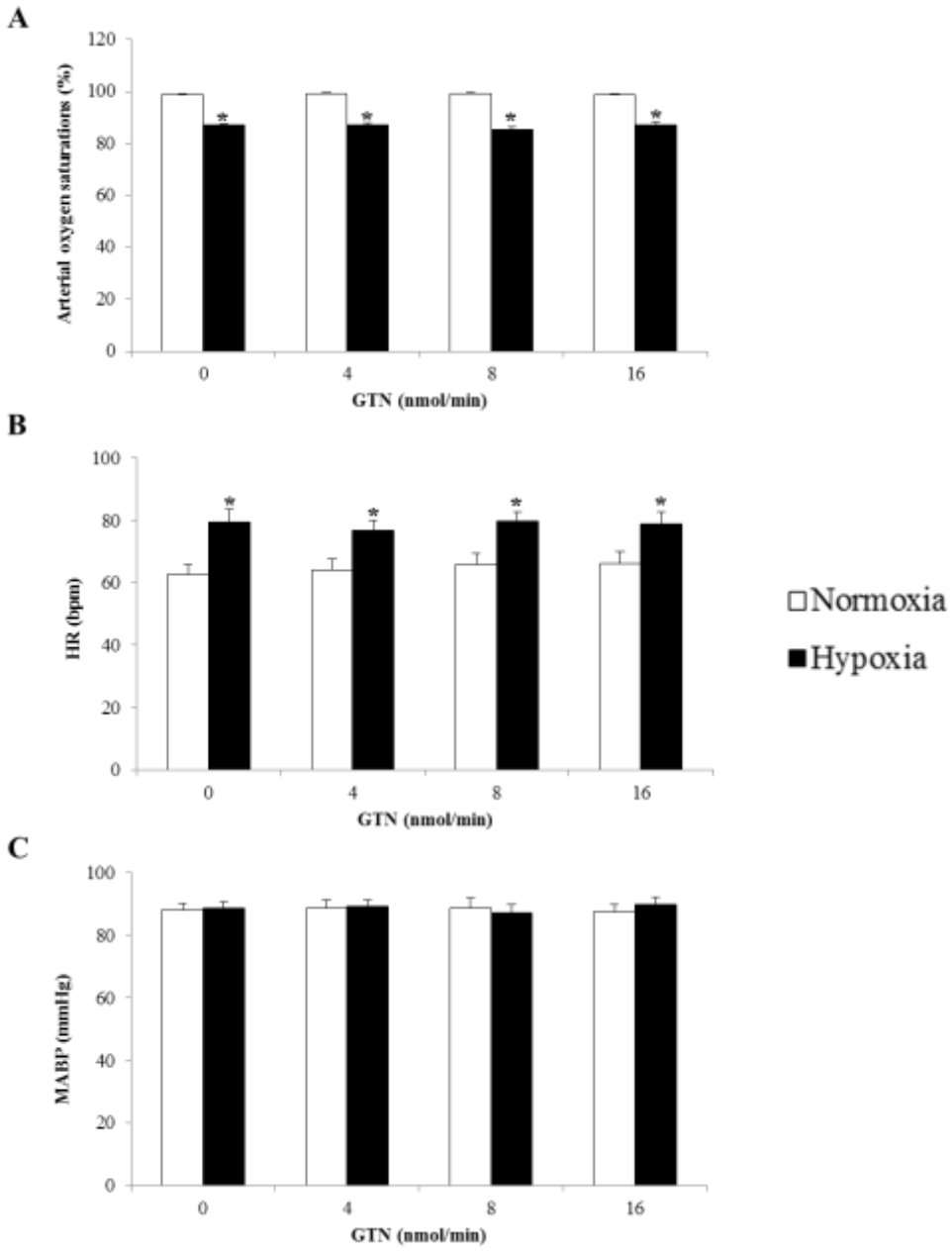


Figure 4.5: (**A**): Arterial oxygen saturations were significantly different all time-points between normoxia and hypoxia ($p<0.05$, n=10); (**B**) HR was significantly different all time-points between normoxia and hypoxia ($p<0.05$, n=10); (**C**) There was no significant change in MABP for the duration of the GTN infusions at any stage or with differing oxygenation conditions ($p>0.05$, n=10). *$p<0.05$ normoxia *vs* hypoxia for equivalent GTN dose.

4.4.4 Acid-base balance

There was no evidence of significant acidosis with hypoxia. Whole blood venous pH or lactate did not alter at baseline (prior to onset of GTN infusion) or following completion of the GTN infusion (**Table 4.2**).

	normoxia		hypoxia	
	Pre-infusion	Post-infusion	Pre-infusion	Post-infusion
Arterial oxygen saturations (%)	99.0±0.3	99.0±0.2	87±0.5*	87±0.9*
pH	7.36±0.03	7.37±0.04	7.37±0.04	7.36±0.03
Lactate (mmol/L)	1.4±0.5	1.4±0.5	1.5±0.6	1.4±0.3

Table 4.2: pH and lactate values at baseline and following completion of intra-arterial GTN infusion. *p<0.05 normoxia *vs* hypoxia for equivalent GTN dose.

4.4.5 Plasma nitrite and nitrate

Plasma nitrite and nitrate levels were analysed. During normoxia, nitrite levels in the infused arm did not alter significantly for the duration of the GTN infusion (p>0.05 compared to baseline, n=7; **figure 4.6A**). Baseline nitrite concentration was 1.3±0.1μM and increased to 1.5±0.2μM following 16nmol/min of intra-arterial GTN. During hypoxia, baseline nitrite concentration was significantly lower compared to hypoxia (1.3±0.1μM *vs* 0.5±0.1μM during normoxia *vs* hypoxia; p<0.05, n=7). There was a dose-dependent increase from baseline in nitrite levels following intra-arterial GTN during hypoxia (p<0.05 following 8 and 16nmol/min of intra-arterial GTN, n=7).

During normoxia, nitrate levels did not alter for the duration of the GTN infusion (**figure 4.6B**). During hypoxia there was a trend towards an increase in plasma nitrate levels with escalating doses of intra-arterial GTN however, this was not statistically significant. Baseline nitrate levels differed between normoxia and hypoxia (34.8±11.0μM *vs* 25.7±7.6μM, respectively) but this did not reach statistical significance (p>0.05, n=8).

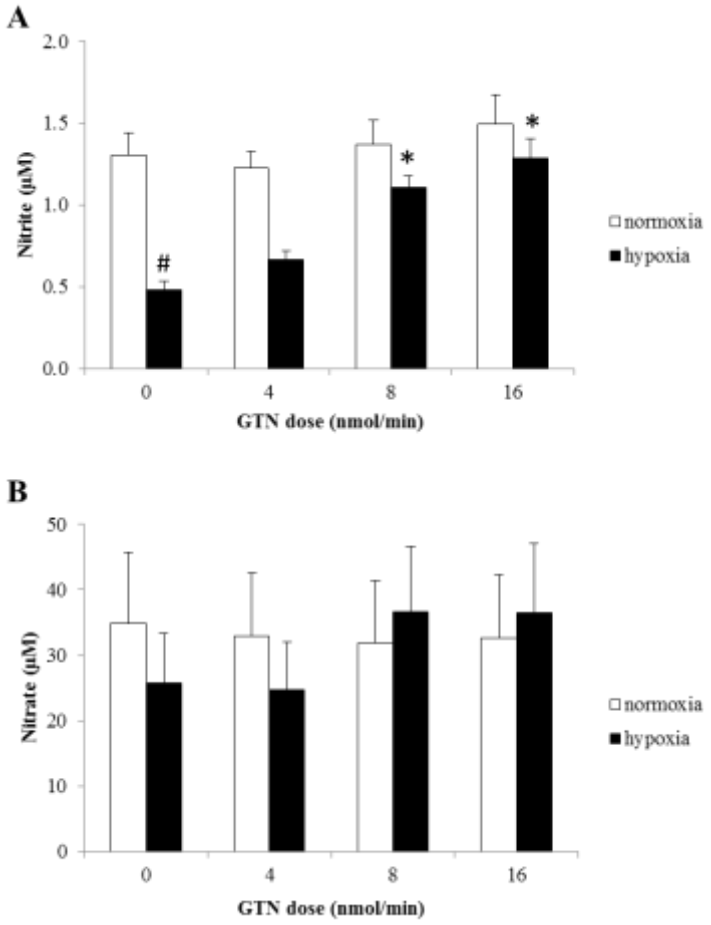


Figure 4.6: (**A)** Plasma nitrite levels following intra-arterial GTN infusion during normoxia and hypoxia (n=7); (**B**) Plasma nitrate levels following intra-arterial GTN infusion during normoxia and hypoxia (n=8). *p<0.05 compared to baseline; #p<0.05 normoxia *vs* hypoxia.

4.5 Discussion

Acute hypoxia alone is well recognised to be associated with relaxation of forearm vasculature. Surprisingly, in our study there was a tendency to a lower FBF ratio (suggestive of attenuated vasodilatation) even at baseline, following stabilisation of target oxygen saturations (**figure 4.4**). One study reported that in healthy subjects, hypoxia (target oxygen saturations of 85%) was associated with a 24% increase in FBF (446). Markwald's findings corroborate those of several other groups previously (447-450). Therefore, the current study did not explore the effects of hypoxia alone on resistance vessels as it was decided that this has been well established. In view of the unanticipated findings above, it is recognised that a hypoxic control limb to the study would have provided useful insight to the forearm vascular response of the study subjects in our cohort. The aim of this study was to determine if GTN-mediated vasorelaxation differed during normoxia and hypoxia. The data presented herein does not demonstrate an accentuated GTN-mediated vasodilatory effect during hypoxic conditions. The forearm vasculature tended to dilate to a lesser degree in response to intra-arterial GTN during hypoxia as compared to normoxia. During hypoxia there was a smaller increase in FBF ratio (~1.8 fold during hypoxia compared to ~2.5 fold during normoxia) suggestive of attenuated forearm resistance vessel vasodilatation as compared to normoxia (**figure 4.4**). Augmentation of sympathetic vasoconstrictor activity is thought to diminish the degree of hypoxic vasodilatation seen, which in turn is believed to be due to a balance between sympathetic activity and local vasodilatory factors (such as NO) (180, 450, 451). In the current study, activation of sympathetic vasoconstrictor activity during hypoxia may explain the trend towards a lower baseline FBF ratio in the hypoxia limb of the study (1.5±0.2 and 1.8±0.4 during hypoxia and normoxia, respectively, $p>0.05$) and the diminished GTN-mediated vasodilatation seen, during hypoxia. Conversely, this inability of intra-arterial GTN to overwhelm possible sympathetic activity was not observed with nitrite. Intra-arterial

infusion of nitrite has been previously shown to cause an enhanced vasodilatory effect in the forearm vasculature (without blockade of sympathetic drive), presumably overcoming any sympathetic vasoconstrictor responses seen during hypoxia. Cosby et al demonstrated an increase in forearm blood flow with intra-arterial nitrite infusion, which was further enhanced during exercise and associated with nitrite reduction to NO via deoxyhaemoglobin (26). In a study by our group, intra-arterial nitrite was associated with greater venodilatation (presumably secondary to the lower oxygen tension in veins) during normoxia with no discernible effect on resistance vessels. During hypoxia, resistance vessels dilatation was observed also (46).

A Russian study found accentuated peripheral vasodilatory effects following sublingual GTN administration in humans living at high altitude (compared to those living at low altitude), presumably secondary to moderate hypoxia (443). Our contradictory findings may be due to differences in GTN vasodilatory effects during chronic hypobaric hypoxia (where adaptations have occurred) compared to acute normobaric hypoxia (as delivered in the current study). Erzurum and colleagues found accentuated forearm blood flow in Tibetan residents living at altitude, compared to subjects living at sea-level. This was associated with 10-fold greater concentrations of bioactive NO products (nitrate and nitrite) within the plasma of Tibetans (452). In our study, despite greater forearm vasodilatory responses during normoxia compared to hypoxia following administration of intra-arterial GTN, plasma nitrite and nitrate levels did not change significantly from baseline during normoxia (**figure 4.6**). Nitrite levels in the infused arm were higher during normoxia (with a greater FBF ratio response) and remained elevated above hypoxic levels for all corresponding GTN infusion rates (**figure 4.6A**). This may support Chen and colleagues' report that GTN-mediated vasodilatation occurs via conversion to 1,2GDN and nitrite (168) and is consistent with findings by others

(453). Moreover, baseline nitrite concentration was significantly lower during hypoxia compared to normoxia, which may reflect nitrite consumption during hypoxia. Despite the lower baseline nitrite concentration during hypoxia, the increase from baseline was greater during hypoxia compared to normoxia (15% *vs* 160%, respectively), again suggestive of enhanced nitrite bioavailability following GTN administration. Physiological plasma nitrite concentrations are between 150-1000nM (26). We observed supra-physiological levels (up to ~1500nM during normoxia at maximal GTN infusion rate). Lack of correlation between vasodilatory response and plasma nitrite concentration has been reported previously by Ingram and colleagues, following administration of nitrite in healthy volunteers and may be suggestive of the existence of further, unknown mechanisms contributing to nitrite-mediated hypoxic vasodilatation (199).

MABP remain constant during both normoxia and hypoxia with no significant change between oxygenation states or with intra-arterial GTN infusion (**figure 4.5**). However, HR was significantly higher in the hypoxia group and remained so for the duration of hypoxia. This finding is in keeping with the effect of acute hypoxia and has been observed by others (449, 450).

Study limitations

The aim of this study was to directly compare GTN-mediated vasodilatation during normoxia and hypoxia, therefore a hypoxia-only group was not studied. Although, the effects of acute hypoxia on forearm vasculature have been described by others, it would have been useful to investigate the effect on our cohort due to the surprising findings of attenuated dilatation during hypoxia (446, 449, 450). Furthermore, we did not utilise sympathetic blockade to

diminish the sympathetic vasoconstrictor effect of hypoxia, which may account for the differing changes in GTN-mediated vasodilatation during normoxia and hypoxia.

Conclusions

GTN does not appear to display an accentuated vasodilatory effect during hypoxia, as reported with nitrite. Indeed, differing vasodilatory effects of NO-donors may be detected between species, degrees of hypoxia and different vascular beds within species. These findings provide important insight into the pharmacodynamic effect of GTN *in vivo* in man, and supports further research into nitrite as a putative NO donor for use in cardiovascular conditions.

Chapter 5: Nitrite-mediated Cardioprotection in Patients undergoing Coronary Artery Bypass Grafting Surgery

5.1 Introduction

Ischaemia-reperfusion injury (IRI) is a well-recognised paradox of reperfusion following a period of ischaemia, undoing some of the benefit gained by restoration of blood flow (94). Several examples of IRI exist in cardiovascular medicine, for example acute myocardial infarction (AMI) with thrombolysis/percutaneous coronary intervention (PCI), during cardiac surgery with cardio-pulmonary bypass, following organ transplantation and following cardiac arrest. These clinical conditions are commonly encountered and remain a significant health problem. Despite reductions in cardiovascular mortality, cardiovascular disease is still the largest killer in the UK (454). Approximately 100,000 people suffer an acute myocardial infarction in the UK every year. A significant proportion of patients with cardiovascular disease undergo cardiac surgery. Coronary artery bypass grafting (CABG) surgery usually with cardiopulmonary bypass and cardioplegic arrest is a common surgical procedure. Greater than 16,000 isolated, first-time elective CABG operations were performed in the UK in 2010-11 (455). The risk profile of patients undergoing cardiac surgery has increased over time due to more elderly patients having surgery along with a rise in the complexity of operations being undertaken (455). Approximately 60,000 people in the UK suffer an out-of-hospital cardiac arrest with the majority presumed to be cardiac in origin (456, 457). Irrespective of advances in modern medicine, survival rates, particularly from out-of-hospitals cardiac arrest remain poor, variable and largely unchanged over the years (458). Consequently, due to the potential for methods to protect against IRI to improve clinical outcomes in these commonly encountered circumstances, the impetus to find ways to reduce the lethal effects of IRI has been the ‘holy grail’ of countless research studies and has formed the basis of numerous literature reviews on the subject (209-211, 459, 460).

The role of nitrite in cardioprotection is being increasingly recognised with several experimental studies proposing that nitrite may protect the myocardium against IRI. A

number of *in vitro* studies have demonstrated that nitrite is cardio-protective and some of these have been reviewed in Chapter 1 (27, 97, 329, 331-333, 461). I*n vivo* animal studies have corroborated these findings by demonstrating a reduction in myocardial injury in several animal models (334, 335). In particular, Gonzalez and colleagues explored the role of low-dose nitrite infusion administered to dogs during myocardial ischaemia and reported an up to 50% reduction in myocardial infarct size following intravenous nitrite administered during the ischaemic period (336). Additionally, Doganci and colleagues recently administered intra-coronary nitrite (0.5µg/kg) immediately prior to ischaemia in a pig model of AMI (462). The authors reported favourable haemodynamic recovery following restoration of blood flow with an associated reduction in biochemical markers of oxidative stress and myocardial injury. In a further murine cardiac arrest model, a single bolus dose of i.v. nitrite (bolus dose of 0.13mg/kg) during ischaemia improved cardiac function, survival and neurological outcomes (339).

These promising findings led to some recent translational studies in man to investigate the role of nitrite-mediated protection. Administration of dietary nitrate in the form of beetroot juice (with a resultant increase in plasma nitrite) to 10 healthy subjects was associated with diminution of ischaemia-induced endothelial dysfunction in a forearm resistance vessel model of IRI (338). This finding was later corroborated by Ingram and colleagues with the similar reduction in ischaemia-induced endothelial dysfunction in the brachial artery of healthy volunteers, when nitrite is administered pre-ischaemia. Preliminary data from an on-going Phase I human trial of intravenous nitrite therapy in human cardiac arrest survivors exploring the safety of tolerability of intravenous nitrite administration, has suggested that nitrite is safe to administer to humans in doses that confer cardio-protection in animals (340). The majority of studies have investigated nitrite peri- or post-conditioning, whereas data

exists that nitrite may confer myocardial protection when delivered up to hours before the ischaemic insult also (332, 333, 335, 463).

Despite an increasing number of studies detailing nitrite-mediated protection against IRI, there is a relative paucity of data outlining the exact mechanisms of this protection, particularly in humans. There is some evidence to suggest that NO-independent nitrite-mediated protective activity may occur via modulation of mitochondrial function (333). The importance of mitochondria as the final target for protective signaling cascades such as the 'Reperfusion Injury Salvage Kinase' (RISK) pathway, (as described in section 1.7.2.4) is well established (295). The RISK pathway (**figure 1.4**) comprises a series of pro-survival kinases which include phosphatidylinositol-3-OH kinase (P13K–Akt), extra-cellular signal-regulated kinases (Erk 1/2) and endothelial nitric oxide synthase (eNOS) (102, 296). Several experimental studies report up-regulation of eNOS phosphorylation associated with myocardial conditioning, downstream of Akt activation (464-467). A recent study demonstrated that nitrite may confer protection against vascular injury through mitochondrial biogenesis, independent of an NO-mediated pathway (341). Furthermore, Perlman and colleagues provided some important insights into the role of nitrite in cardioprotection. The authors demonstrated that nitrite exhibits a dose-response relationship in affording cardio-protection in the Langendorff rat heart. Low (0.1mg/kg) and high (10mg/kg) doses of nitrite were cardio-protective whereas intermediate (1.0mg/kg) doses had no effect. Additionally, directly and through NO-release, nitrite alters tissue redox status, alters transcription factor signaling, protein expression and cardiac metabolism (176). Dezfulian's study, although investigating mechanisms of neuro-protection, have again demonstrated nitrite-mediated protection via modulation of mitochondrial function, and S-nitrosation independent of sGC-signaling pathways (340). To date, there is extremely limited data on mechanisms of

protection against IRI in the human myocardium, with no studies investigating the mechanisms of nitrite-mediated cardio-protection specifically. Furthermore, the optimal dose and optimal timing (i.e early *vs* late window of pre-conditioning) of nitrite-mediated cardioprotection in humans remains unknown. This requires further investigation and forms the basis of the work presented in this chapter.

An important setting of IRI in the clinical environment is that of 'on-pump' cardiac surgery with cardiopulmonary bypass (CPB) and cardioplegic arrest. Overall 30-day cardiac mortality in patients undergoing CABG surgery is low at 1-3% (455, 468, 469). Nonetheless, depression of myocardial function with post-operative myocardial stunning and peri-operative myocardial necrosis remains commonplace with up to 50% of peri-operative deaths attributed to a cardiac cause (470, 471). In one large study involving approximately 2500 patients undergoing CABG surgery, myocardial stunning with low cardiac output syndrome requiring inotropes or mechanical circulatory support, was observed in 9.2% of patients, and in this group mortality was 16% (472). Peri-operative myocardial necrosis, as evidence by elevated troponins or creatinine kinase is associated with a poor prognosis over the months to years after the operation (473-477). Therefore, the impetus to discover methods of protection during CABG surgery remains strong.

CPB requires cannulation of the right atrium and the ascending aorta, with re-direction of blood from the right atrium, via a cardio-pulmonary bypass machine into the ascending aorta, thus bypassing the myocardium. The aorta is cross-clamped with subsequent disruption of blood flow and oxygen transport to the myocardium leading to myocardial ischaemia, resulting in a degree of myocardial damage further compounded by IRI (478, 479). Studies investigating mechanisms of IRI have consistently demonstrated that oxidative stress plays an

important role in myocardial IRI in patients undergoing cardiac surgery with CPB and cardioplegic arrest, as evidenced in some studies by elevated levels of plasma 8-isoprostane (a well recognised marker of oxidative stress) (479-482). However, limited data exists on the exact mechanisms of myocardial protection in patients undergoing cardiac surgery. One human study proposed that post conditioning in human atria (in patients undergoing coronary artery bypass grafting surgery) occurs via the RISK pathway (483). In a recent study, Cappellano et al observed up-regulation of RISK-pathway associated proteins (extracellular signal-regulated kinases 1 and 2, Erk 1/2) in patients undergoing valvular cardiac surgery (484). During cardiac surgery, the timing of ischaemia is known in advance, allowing investigation of pre-conditioning agents to be undertaken. Hence, the setting of CABG surgery lends itself well to investigate the possible mechanisms by which nitrite exerts its cytoprotective effects. Additionally, there is no data to date outlining the role of the RISK pathway in nitrite-mediated cardioprotection specifically and thus the current study attempts to elucidate this further.

5.2 Objectives

The aim of this double-blind, 'proof of principle' study was to determine the molecular mechanisms that might lead to cardioprotection by nitrite in humans, in the setting of CABG surgery. The objectives of this study are as follows:-

1. To determine if nitrite confers cardioprotection through reduction of intra-operative myocardial injury (troponin) and reduction of intra-operative oxidative stress.
2. To determine the time-scale of nitrite-mediated cardioprotection against IRI e.g. early window and late window.

3. To determine the optimal dose of nitrite required for cardioprotection.
4. To determine whether the RISK pathway is involved in nitrie-mediated cardioprotection. Tissue and blood samples obtained at the time of surgery will help elucidate the molecular and metabolic mechanisms which play a role in the conditioning pathways involved in ischaemia-reperfusion injury.

5.3 Methods

5.3.1 Participants

The study was granted full ethical approval (South Birmingham Local Research Ethics Committee 09/H1207/7) and was registered with the UK Clinical Research Network (No. 9329; www.ukcrn.org.uk). The investigation conforms to the principles outlined in the Declaration of Helsinki. Patients, undergoing isolated coronary artery bypass grafting surgery were recruited from pre-admission clinics or cardiology/cardiac surgery wards at the Queen Elizabeth Hospital Birmingham NHS Trust. All of the subjects gave written, informed consent after satisfying the inclusion and exclusion criteria (**table 5.1**). All subjects were admitted onto the cardiac surgery/cardiology ward the day before surgery and were fasted from midnight prior to their surgery, as part of their surgical management. The study was conducted on the ward (pre-surgery nitrite infusion) and in dedicated cardiac theatres at the University Hospital Birmingham NHS Trust. All subjects were requested to adhere to the following advice prior to their surgery:-

1. Omit nitrate medication 72 hours prior to surgery.
2. To follow a nitrate/nitrite-poor diet for 2 days prior to surgery (**table 5.2**).
3. To abstain from alcohol and caffeine-containing drinks for 24 hours prior to surgery.

Table 5.1: Inclusion/exclusion criteria for recruitment into study.

Inclusion criteria

- Patients undergoing isolated first time multi-vessel CABG
- Aged 18 years or over

Exclusion criteria

- Significant psychiatric /neurological impairment that might prevent adherence to the requirements of the protocol or the ability to give informed consent
- Redo operation
- Age >80 years
- Pregnancy
- Renal Impairment requiring pre-operative renal support
- Diabetes Mellitus
- Intended heart valve or additional surgery
- Episodes of angina or ischaemia within 48hours prior to the procedure

Table 5.2: List of food items subjects were asked to abstain from, as part of a nitrate/nitrite poor diet.

Leafy vegetables e.g.	Cured meat	Pickled vegetables
Lettuce	Cured fish	Pickled meat
Beetroot		Pickled fish
Celery		
Radish		
Rhubarb		
Turnip		
Spinach		

5.3.2 Study protocol

44 patients, who were undergoing isolated CABG surgery for multi-vessel coronary artery disease (elective and urgent), were recruited and underwent evaluation of myocardial protection afforded by systemic nitrite infusions, administered 24 hours prior to onset of ischaemia (late window) or immediately before onset of ischaemia (early window). **Figure 5.1** outlines the flow of patients through the study. **Figure 5.2** outlines the study protocol. The study drug was administered in a double-blind manner and assigned randomly on a 1:4 basis. Randomisation was undertaken via a computer-generated randomisation schedule, undertaken by a person independent of the study. The schedule generated was placed in sequentially numbered sealed envelopes.

(1) Day before Surgery

Following admission to the hospital ward (the day prior to surgery), all subjects received a 30 minute infusion of i.v. sodium nitrite or placebo (0.9% saline, Infusion 1). Subjects rested in a semi-recumbent position on a hospital bed and an intravenous cannula (Venflon, 20 Gauge) was placed in each antecubital fossa. One intravenous cannula was used to administer the infusion, with blood obtained from the non-infused arm cannula. A blood pressure cuff and an oxygen saturation probe were placed on the infusion arm. After 5 minutes of rest, heart rate (HR) and blood pressure (BP) was recorded. 10ml of venous blood was extracted. 2mL was analysed immediately for metHb and 8mL stored for later analysis of plasma nitrite/nitrates and plasma 8-isoprostane, as described in section 5.3.3. Thereafter, a 30minute infusion of the study drug (nitrite/placebo) was initiated. After 30 minutes, peripheral

haemodynamic parameters were re-measured and a further 10ml of venous blood was obtained and analysed as described in section 5.3.3.

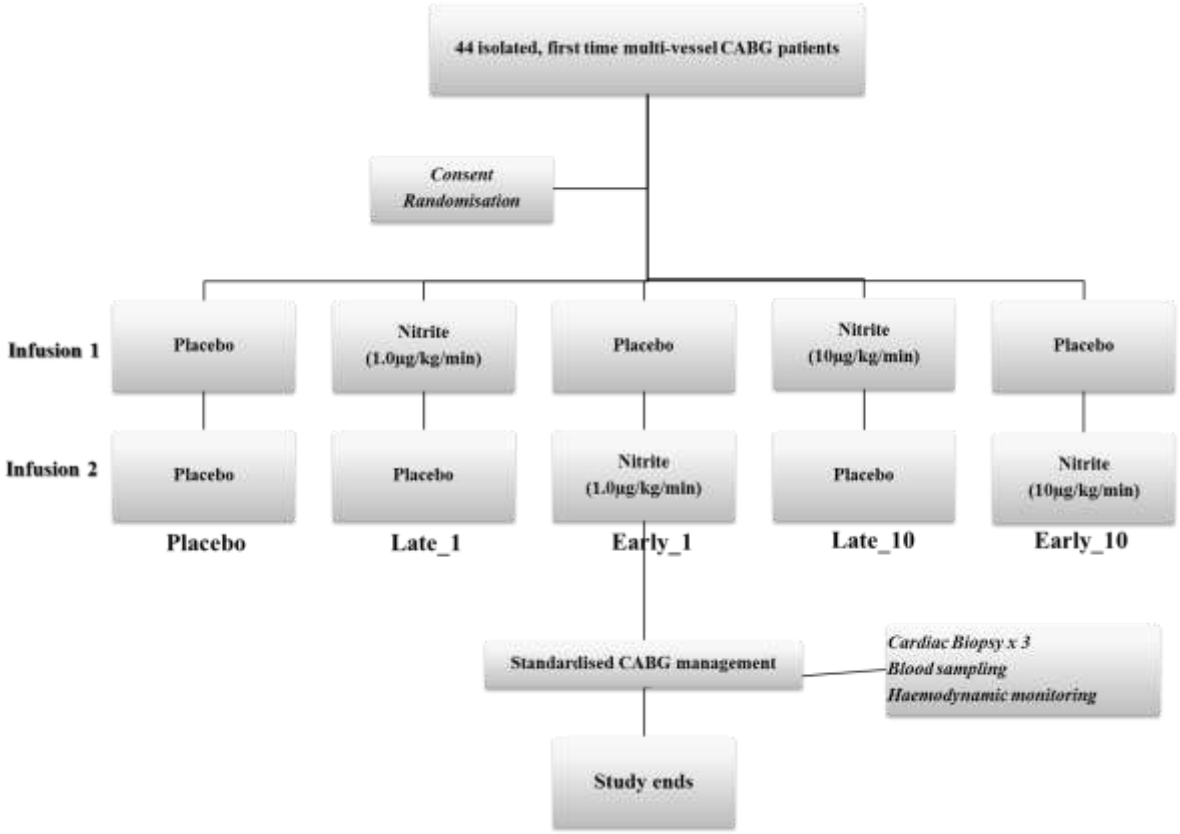


Figure 5.1: Flowchart depicting study design. Infusion 1: 30minute i.v. infusion of sodium nitrite (1µg/kg/min or 10µg/kg/min) or placebo (0.9% saline) at infusion rate of 1mL/min the day before surgery. Infusion 2: 30minute i.v. infusion of sodium nitrite (1µg/kg/min or 10µg/kg/min) or placebo (0.9% saline) at infusion rate of 1mL/min intra-operatively.

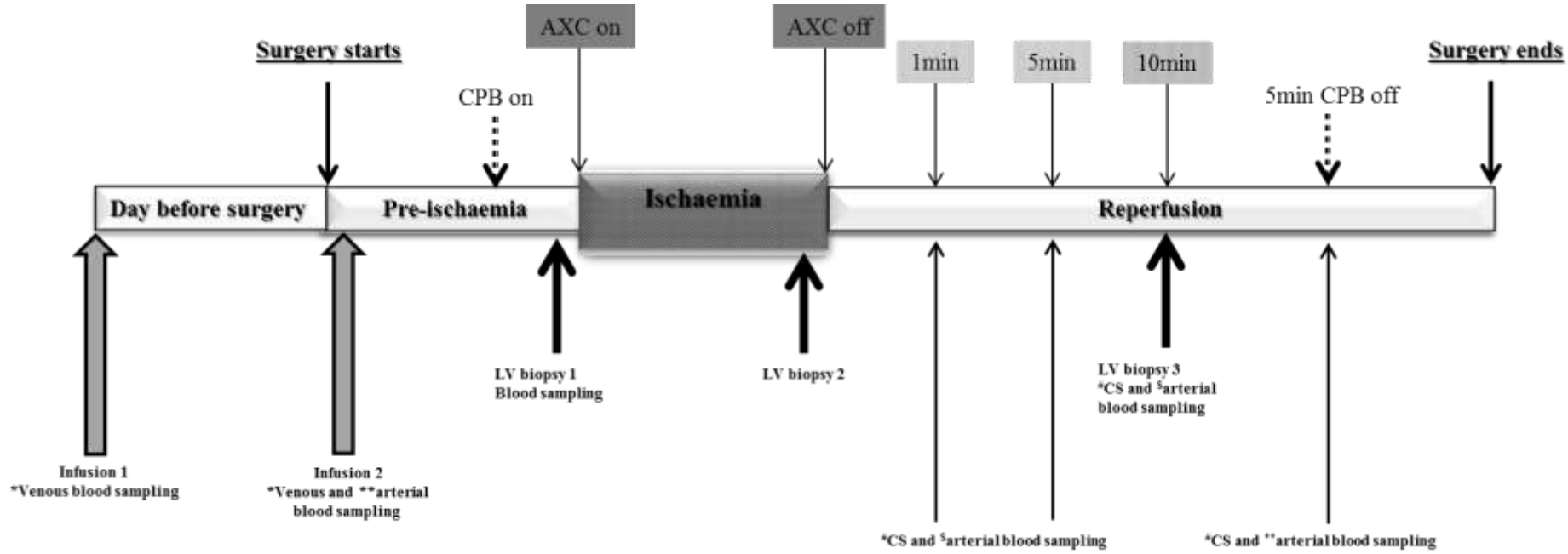


Figure 5.2: Study protocol. AXC, aortic cross-clamp; CPB, cardiopulmonary bypass; LV, left ventricle.

Infusion 1 and Infusion 2 = i.v. sodium nitrite (1 or 10µg/kg/min) or placebo (0.9% saline) for 30minutes at infusion rate of 1mL/min.

*Peripheral venous blood sampling and **peripheral arterial blood sampling. #Coronary sinus blood sampling and $arterial blood sampling (via cardiopulmonary circuit).

LV biopsy 1, pre-AXC (pre-ischaemia); LV biopsy 2, pre-reperfusion; LV biopsy 3, 10minutes post-reperfusion.

(2) Day of Surgery

On the day of surgery, subjects underwent general anaesthesia as per a standardised clinical protocol (**appendix 1**). Briefly, anaesthesia was induced with etomidate, fentanyl, and rocuronium with the use of propofol and alfentanil for maintenance, supplemented on cardio-pulmonary bypass by isoflurane. Following induction of anaesthesia, all subjects underwent placement of a 4French right internal jugular central venous line, a 7.5French, quadruple-lumen Swanz-Ganz catheter via the right internal jugular vein (Edwards Lifesciences, California, USA , a 20Gauge cannula (Becton Dickinson UK Ltd) into the right radial artery and a trans-oesophageal echocardiogram (TOE) probe (Philips CX50, USA). These procedures were performed by a Clinician (independent of the study) and positioning of the central venous and radial arterial catheters was confirmed by pressure waveform monitoring. The position of venous lines remained unchanged for the duration of the study and placement of the Swan-Ganz catheter allowed invasive cardiac measurements, as described in section 5.3.5.

Following transfer to the operating table, CABG surgery was commenced as per a standardised protocol (**appendix 1**). Briefly, a membrane oxygenator at 34°C was utilised to undertake CPB. Standard, clinical myocardial protection was undertaken using intermittent antegrade cold blood cardioplegia (St Thomas cardioplegia, Martindale Pharmaceuticals, Essex, United Kingdom) with an initial dose of 12 mL/kg followed by maintenance administration every 20 minutes. All anastomoses were made using a single cross-clamp technique. Discontinuation of CPB was undertaken when the patient's temperature reached 36°C (as measured by a nasopharyngeal temperature probe). All subjects underwent placement of epicardial wires for atrial or dual-chamber pacing, with a target heart rate of

90bpm. During surgery, if inotropic support was required, dopamine (3 to 10μg/kg/min), was commenced if the cardiac index (CI) was ≤2.2 L/min/m^2 in the presence of a central venous pressure (CVP) of 12 mmHg, pulmonary capillary wedge pressure (PCWP) of 14 mmHg, and heart rate (HR) of 90bpm. Escalation of inotrope therapy with epinephrine or norepinephrine or intra-aortic balloon pump (IABP) insertion was allowed at the clinician's discretion. Introduction of support was also permitted if the operating surgeon identified poor contractility at separation of CPB. Intraoperative boluses of phenylephrine were used to maintain a mean arterial blood pressure (MABP) of ≥55 mmHg during CPB and ≥65 mmHg after discontinuation of CPB and with associated low systemic vascular resistance (SVR). As part of the study protocol, during surgery a retrograde cardioplegia cannula was inserted into the coronary sinus (CS) for blood sampling only and placement confirmed by trans-oesophageal echocardiography.

Prior to sternotomy, cardiac and peripheral haemodynamic measurements were undertaken as detailed in section 5.3.5. A 12mL sample of venous blood (from the distal port of the Swanz-Ganz catheter) and a further 10mL sample of arterial blood (from the radial arterial cannula) were obtained. These samples were analysed immediately for methaemoglobin, pH and lactate using a blood gas analyser, and some stored for later analysis (section 5.3.3). Thereafter, a 30minute infusion of the study drug (nitrite/placebo, Infusion 2) was commenced via the right internal jugular central venous catheter, with the aim of completing this infusion within 30minutes of onset of ischaemia. Following completion of the study drug, repeat cardiac and peripheral haemodynamic measurements were recorded and venous and arterial blood samples obtained, as described above.

As the operation proceeded, biopsy and further blood samples were obtained at the time-points indicated in **figure 5.2** and outlined below:-

i. **Pre-ischaemia -** prior to placement of aortic cross-clamp (pre AXC), a left ventricular (LV) biopsy was obtained, as described below (LV 1). 12mL of coronary sinus venous blood and 10mL of arterial blood was obtained.

ii. **Pre-reperfusion**– prior to reperfusion (removal of AXC) – a further LV biopsy was obtained (LV 2).

iii. **Reperfusion 10minutes** - blood (coronary sinus and arterial via CPB pump) was obtained at 10minutes post-removal of aortic cross-clamp. A further LV biopsy (LV 3) was obtained at 10minutes post-reperfusion.

iv. **5 minutes post CPB** – 5minutes after discontinuation of CPB, further blood samples (coronary sinus and peripheral arterial) were taken.

During the surgery, cardiac and peripheral haemodynamic measurements (section 5.3.5) were undertaken at the following time-points:-

i. Pre-administration of intra-operative study drug.

ii. Post-administration of intra-operative study drug.

iii. Pre-CPB.

iv. 5minutes post discontinuation of CPB.

5.3.3 Blood sample analysis

Blood samples obtained intra-operatively were analysed for the following:-

1. Plasma nitrite/nitrate in venous and arterial (pre and post infusion 2) and CS and arterial blood, pre AXC, at 10minutes post-reperfusion (Rep 10) and 5minutes after discontinuation of CPB (Off CPB).
2. Plasma 8-isoprostane in CS and arterial blood obtained pre AXC, at 10minutes post-reperfusion and 5minutes after discontinuation of CPB.
3. Plasma troponin in coronary sinus blood, obtained pre AXC and 5minutes after discontinuation of CPB.
4. Immediate blood gas analysis for pH, lactate and metHb in venous/CS and arterial blood before and after infusion 2 and pre AXC, at 10minutes post-reperfusion and 5minutes after discontinuation of CPB.

(1) Plasma nitrite/nitrate analysis

For analysis of plasma nitrite/nitrate, 4mL of blood was transferred into an EDTA collection tube (K3 EDTA, BD Vacutainers) pre-treated with N-ethylmaleimide (10mM; Merck, Germany). These samples were centrifuged at 2000rpm at 4ºC followed by immediate snap-freezing in liquid nitrogen and storage at -80ºC for later analysis of plasma nitrite/nitrates, as described in Chapter 2, section 2.4.5.

(2) Plasma analysis of 8-isoprostane

To investigate the effect of nitrite on oxidative stress, plasma 8-epi-prostaglandin F2α (8-epi-PGF 2α) was measured. 8-epi-PGF 2α is a well recognised marker of oxidative stress (485). 4mL of blood was added to a second EDTA tube. These samples were centrifuged at 2000rpm at 4ºC. 2mL of plasma for measurement of 8-epi-prostaglandin F2α (8-epi-PGF 2α) was aliquoted to tubes, each containing 20µL of 0.005% butylated hydroxytoluene (BHT; a

free radical scavenger), snap-frozen in liquid nitrogen and stored at -80ºC for later analysis. The samples were purified and analysed for 8-epi-PGF 2α using a commercially available affinity sorbent and enzyme immunoassay kit as per the manufacturer's instruction (Cayman Chemical, Ann Arbor, MI, USA). Briefly, the plasma samples were thawed and centrifuged at 2000rpm at 4ºC for 5minutes to remove particles and diluted 1:5 with 0.1M phosphate buffered solution, (PBS/column buffer; pH 7.4). The affinity sorbent was washed twice with PBS. 1mL of plasma was added to 100µL of affinity sorbent and gently agitated for 60minutes, followed by centrifugation at 2000rpm for 5minutes. The sorbent was separated from the supernatant be pipetting/decanting, and washed with 1mL of PBS (0.1M, pH 7.4), centrifuged for a further 5minutes at 2000rpm followed by removal of excess supernatant. Following a further washing step with 1mL of ultra pure water, the 8-isoprostane was eluted from the affinity sorbent using 0.5mL of eluting solution (95% ethanol/5% ultra pure water). This solution was centrifuged at 2000rpm for 5minutes. The eluting solution containing the 8-isoprostane was transferred to a clean tube, with a further 0.5mL of eluting solution added and centrifuged again. The samples were evaporated to dryness under vacuum centrifugation and reconstituted in methanol. 50µL aliquots of sample per well were added to a 96-well plate in triplicate, containing 50µL 8-isoprostane AChE tracer and 50µL of 8-isoprostane antiserum. The plates were covered with plastic film and incubated at 4ºC for 18 hours. After rinsing the wells five times with wash buffer, 200µL of Ellman's reagent was added to each well and the plate contents were gently mixed in the dark (at room temperature) for 2 hours. A spectrophotometric microplate reader (410nm) was used to measure the absorbance of each well.

(3) Blood gas analysis

Lactate, pH and metHb quantification was performed immediately with 2ml of blood using a blood gas analyser (Bayer Rapidlab 865, Siemens, Tarrytown, NY).

(4) Troponin analysis

2mL of blood for troponin T (TnT) was transferred into a Lithium heparin bottle and sent to the Biochemistry laboratory (at University Hospitals Birmingham) for immediate analysis. This was undertaken using a commercially available assay (Elecsys 170, Roche Diagnostics, United Kingdom).

5.3.4 Western blot analysis for phosphorylation of eNOS and AkT in LV cardiac biopsies

LV biopsies were obtained at three time points – pre-ischaemia (LV 1), pre-reperfusion (LV 2) and 10minutes post-reperfusion (LV 3). These were immediately snap-frozen in liquid nitrogen and stored at -80ºC until used Western blot analysis of endothelial nitric oxide synthase (eNOS) and protein kinase B (Akt).

At the time of the Western blot analysis, the samples were homogenized under liquid nitrogen conditions with lysis buffer (pH 7.4) containing 2mM sodium orthovanadate, 5mM sodium fluoride, 1 x complete protease inhibitor tablet (Roche Applied Sciences, Upper Bavaria Germany). The samples were placed immediately back into liquid nitrogen, followed by two times freeze-thawing procedure. Once samples were thawed the cardiac lysate was

centrifuged at 13,000 rpm for 15 minutes at 4°C. The supernatant was transferred into a new eppendorff tube and placed on ice for protein content analysis.

Protein content was quantified using the BCA protein assay kit (Thermo Scientific, Waltham MA, USA). Briefly, assay reagents A and B supplied were mixed in a ratio of 20:1. Bovine serum albumin at concentrations of 0 -1.0µg/µL was used to create a standard curve. 5uL of each sample of homogenate was diluted in 10uL of lysis buffer. 5uL of cardiac lysate was added to the 96-well plate for triplicate analysis. 25ul of reagent A was added to each of the wells, followed by 200uL of reagent B. The plate was incubated for 15 min with foil. After 15mins, the absorbance was read at 750nm. The sample was compared against the standard curve and the samples were normalised to 25ug protein with 1 x Laemmli Buffer (Bio-rad, Hercules, CA, USA) with 5% mercaptoethanol and stored at -80ºC for Western blot analysis.

Western blotting protocol

Samples were allowed to thaw in ice, followed by boiling at 95°C for 5 min. The samples were centrifuged at room temperature at 1200 rpm for 10 min). 40uL of sample was loaded onto a SDS polyacrylamide gel electrophoresis (SDS-page) to separate the proteins based on molecular weight. The gels were run on at 180V for approximately 60 minutes or until the protein marker was seen to have run off the gel. Once completed, gels were removed from the tank and placed into cooled wet transfer tank (4ºC; Wet transfer buffer: 3g Tris Base, 14.4g Glycine, 200ml methanol, made up to 1L with ddH20).The transfer stacks were formed with a layer of fibre pad and pre-cut blotting paper (both soaked in transfer buffer) as the base for the gel. A polyvinylidene difluoride (PVDF) membrane (activated by soaking in 100% methanol for 10 minutes prior to rinsing in transfer buffer) was placed on top of the gel and the stack was completed with further layers of blotting paper and fibre pad, prior to rolling to

remove air bubbles. Transfers were conducted for 1.5 hrs at 30V with constant stirring using a magnetic flea.

After transfer, PVDF membrane were placed in blocking buffer containing 5% non-fat milk/0.05% PBS Tween*20 detergent for 2 hours at room temperature on the shaker. After 2 hours blocking, the PVDF membranes was incubated with the primary antibody (phospho-eNOSser1177 (1:500/1% non-fat milk and PBS/Tween; Cell Signalling Tech, Danvers, MA, USA); total eNOS (1:200; BD Bioscience, Franklin Lakes, New Jersey, USA); phospho-AKT ser473(1:1000; Cell Signalling Tech, Danvers, MA, USA) or total AKT (1:2000; Santa Cruz Biotech, Dallas, TX, USA). Primary antibody was incubated overnight on shaker at 4ºC.

The next day, primary antibody was removed and washed four times for 15 minutess each with PBS/Tween. After washing, the membrane was incubated on the shaker for 1 hour (room temperature) with the secondary antibody (horseradish peroxidase–conjugated donkey anti-rabbit immunoglobulin G (1:1000 in 5% milk/PBS Tween; GE healthcare, Amersham, UK). After 1 hour, the membrane was then washed with PBS/Tween 4 times for 15 minutes each.

ECL detection reagent (GE Healthcare, Amersham, UK) was mixed in a 1:1 ratio and pipette onto the membrane for 5 minutes at room temperature. The resulting chemiluminescence was detected using ECL film (GE Healthcare, Amersham, UK). Western blots were scanned and quantified using Gel-Pro analyser software. To determine equal loading of samples, glyceraldehyde-3-phosphate dehydrogenase (GADPH; 1:1000; Abcam, Cambridge, UK) was also conducted.

5.3.5 Cardiac and Peripheral Haemodynamic measurements

The following peripheral and cardiac haemodynamic parameters were recorded at the time points specified previously:-

i. Heart rate (HR)
ii. Mean arterial blood pressure (MABP)
iii. Pulmonary artery pressure (PAP)
iv. Pulmonary capillary wedge pressure (PCWP)
v. Systemic vascular resistance (SVR)
vi. Central venous pressure (CVP)
vii. CO (Thermodilution method)

5.3.6 Study drug

1µg/kg/min or 10 µg/kg/min sodium nitrite or 0.9% saline was infused at a rate of 1mL/min over 30minutes. Sodium nitrite was purchased from Martindale Pharmaceuticals (Brentwood, UK). 0.9% sodium chloride (Baxter Healthcare, USA) was used for the placebo infusion. The study drug was prepared by a member of clinical staff (nurse or theatre practitioner independent of the study) with the researcher blinded to this process.

5.3.7 Statistical analysis

The data were analysed using SPSS version 21.0 software (SPSS Inc., Chicago, USA). The data are expressed as mean ± standard error of mean (mean±SEM) or median (upper quartile ranges), unless otherwise stated. Probability values of <0.05 were considered statistically significant. Continuous data were analysed using repeated measures ANOVA with

consecutive Bonferroni post-hoc tests for serial measurements between subjects or paired t-test. Categorical data were compared suing Chi-Square test and ordinal data with Kendall-tau b. Due to a change in assay part way through the study (without informing the research team), later samples (from 20 subjects) were analysed for high sensitivity troponin (hsTnT) instead of troponin T. To allow for this, analysis of samples undertaken from a separate group of patients undergoing CABG for both troponin and hsTnT were used to construct a regression model (using Pearson's correlation) and this was used to predict TnT levels from the hsTnT values obtained.

5.4 Results

5.4.1 Patient characteristics

The characteristics of the patient's taking part in the study are outlined in **table 5.3**. All subjects underwent a standardised anaesthetic and surgical operation, as outlined previously The subjects were split into 5 groups: '**Placebo**' (0.9% saline), '**Late_1'** (sodium nitrite 1µg/kg/min the day before surgery, '**Early_1**' (sodium nitrite 1µg/kg/min during surgery), '**Late_10**' (sodium nitrite 10µg/kg/min the day before surgery) and '**Early_10**' (sodium nitrite 10µg/kg/min during surgery). Nitrite infusion was well tolerated in all patients with no adverse effects reported.

Table 5.3	**Placebo**	**Late_1**	**Early_1**	**Late_10**	**Early_10**	P value
Age, yrs (mean±SD)	68±9	61±2	66±3	61±3	63±3	ns
Male, n (%)	7(88)	7(78)	7(78)	8(89)	9(100)	ns
BMI (kg/m^2)	26±1	30±2	29±3	26±1	28±1	ns
Elective, n (%)	7(88)	6(67)	7(78)	5(56)	6(67)	ns
Angina CCS class	2(1 – 2)	2(2 – 2)	2(2 – 2)	2(2 – 2)	2(1 – 2)	ns
NYHA class	1(1 – 1)	1(1 – 1)	1(1 – 1)	1(1 – 2)	2(1 – 2)	ns
Preserved LV function, n (%)	7(88)	7(78)	9(100)	7(78)	6(67)	ns
Logisitic Euroscore	0.8 (0.7 – 1.0)	1.2 (1.0 – 1.6)	1.9 (1.2 – 2.4)	1.5 (1.2 – 2.8)	1.5 (1.0 – 3.1)	ns
Standard Euroscore	4(2 – 4)	1(1 - 2)	3(1 – 3)	2 (1 – 4)	2(1 – 4)	ns
Comorbidities						
Recent AMI, n(%)	3(38)	4(44)	4(44)	5(56)	5(56)	ns
Hypertension, n(%)	5(63)	5(56)	7(78)	4(44)	3(33)	ns
Hypercholesterolaemia, n(%)	5(63)	3(33)	5(56)	2(22)	3(33)	ns
Previous TIA/CVA, n(%)	1(13)	0(0)	0(0)	1(11)	0(0)	ns
Smokers, n(%)	5(63)	7(78)	4(44)	4(44)	4(44)	ns
Pre-operative bloods						
Hb (g/dL)	13.7±0.4	14.0±1.0	14.0±1.0	14.0±0.5	14.0±0.4	ns
Fasting glucose (mmol/L)	5.2±0.2	5.9±0.3	5.9±0.2	5.9±0.3	6.0±0.2	ns
Creatinine (µmol/L)	87±9	88±8	89±3	95±10	88±12	ns
Pre-operative medication						
Aspirin, n(%)	8(100)	9(100)	9(100)	7(78)	8(89)	ns
Statin, n(%)	8(100)	9(100)	8(89)	9(100)	9(100)	ns
B-blockers, n(%)	8(100)	8(89)	6(67)	7(78)	7(78)	ns
ACEI/ATII blockers, n(%)	7(88)	5(56)	8(89)	7(78)	7(78)	ns
Ca-channel blockers, n(%)	2(25)	1(11)	2(22)	2(22)	3(33)	ns
Diuretics, n(%)	3(38)	0(0)	1(11)	1(11)	2(22)	ns

Table 5.3 (previous page): Patient characteristics. There was no significant difference in baseline characteristics between patients in different groups. N=8-9, ns=$p>0.05$. Data is expressed as mean±SEM or median (inter-quartile ranges).

Pre-operative oral nitrate use was present in a minority of patients in each group. In the placebo group, 3(38%) of subjects were prescribed oral nitrate and this was discontinued 7(5 – 10) days prior to surgery. In the Late_1 group, 1(11%) subject received oral nitrates and this was discontinued 3(3 - 3) days before the day of surgery. Similarly oral nitrates were prescribed to 1(11%), 3(33%) and 1(11%) of subjects and these were discontinued 4(4 – 4) days, 4(1 – 3) days and 4(4 – 4) days in the three further groups Early_1, Late_10 and Early_10, respectively.

Time between study drug infusion and pre-ischaemia are outlined in **table 5.4**.

	Placebo N=8	**Late_1 N=9**	**Early_1 N=9**	**Late_10 N=9**	**Early_10 N=9**	P value
No. of grafts	4(3 – 4)	4(4 – 5)	4(3 – 4)	3(3 – 4)	3(3 – 4)	ns
CPB time (min)	121±9	138±8	125±12	107±7	103±8	ns
AXC time (min)	73±3	80±6	77±9	70±5	71±6	ns
Time from Inf 1 to AXC (hrs)	19.7±0.8	17.9±1.0	18.2±0.7	17.4±1.1	17.4±0.5	ns
Time from Inf 2 to AXC (min)	54±6	61±8	52±5	59±7	43±6	ns

Table 5.4 (above): Intra-operative details. There was no difference in ischaemia time or 'time of study infusions to ischaemia' between groups. Data is expressed as mean±SEM or median (inter-quartile ranges), ns=p>0.05.

5.4.2 Effect of nitrite infusion on heart rate and blood pressure

Figure 5.3 depicts the HR and MABP before and after infusion 1 and infusion 2. Administration of 1µg/kg/min and 10µg/kg/min of i.v. sodium nitrite did not result in alteration of either HR or MABP after 30minutes.

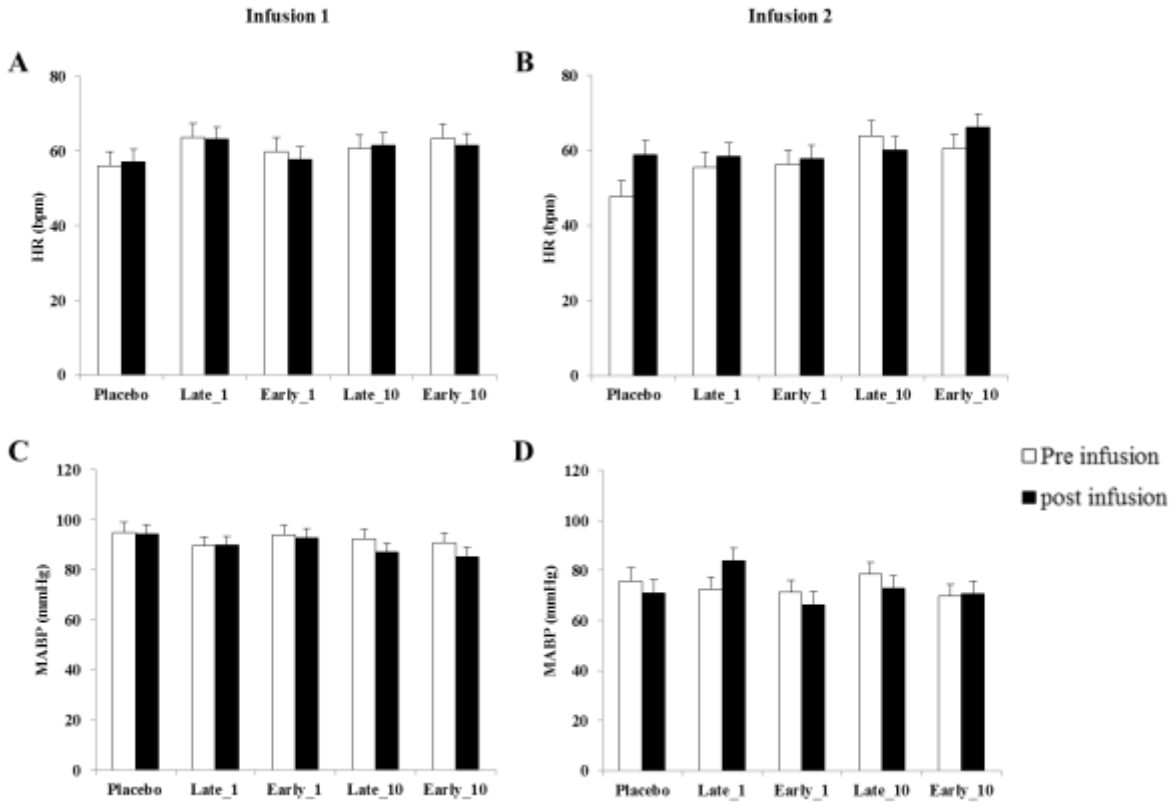


Figure 5.3: **A**) HR during infusion 1; **B**) HR during infusion 2; **C**) MABP during infusion 2; **D**) MABP during infusion 2. N=8-9, p>0.05. Data is expressed as mean±SEM.

5.4.3 Effect of nitrite infusion on intra-operative haemodynamic parameters

Intra-operative cardiac and peripheral haemodynamic variables, measured before commencement of and 5 minutes after discontinuation of cardio-pulmonary bypass (CPB) are shown in **figure 5.4**.

Intra-operative CO increased significantly from pre-CPB measurements to following discontinuation of cardiopulmonary bypass (post-CPB) , in the placebo group and the two groups that received nitrite the day before surgery (**figure 5.4 A**). Pre-CPB cardiac output increased from 4.2±0.4L/min to 5.3±2.3L/min, 4.3±0.4L/min to 5.2±2.1L/min and 4.2±0.4L/min 5.4±2.1L/min in the Placebo, Late_1 and Late_10 groups, respectively ($p<0.01$ for all, n=8 each group). There appeared to be an attenuation of CO increase in subjects receiving nitrite intra-operatively. CI exhibited a similar pattern to CO (**figure 5.4B**).

Although, there was a general reduction in mPAP pre and post CPB, this only reached significance in patients receiving 10µg/kg/min of nitrite intra-operatively (**figure 5.4C**). mPAP in the Early_10 group decreased from 22±2mmHg pre-CPB to 17±2mmHg post CPB ($p<0.05$, n=8 each group). There was a reduction in mPCWP within all groups (**figure 5.4D**). Pre-CPB, mPCWP was 14±1mmHg, 14±1mmHg, 16±1mmHg, 11±1mmHg and 14±1mmHg and fell to 9±1mmHg, 8±1mmHg, 8±1mmHg, 8±1mmHg and 9±1mmHg in the Placebo, Late_1, Early_1, Late_10 and early_10 groups, respectively ($p<0.05$ for all, n=8 each group).

SVR fell significantly pre and post CPB in all groups except those receiving intra-operative 10µg/kg/min of nitrite (**figure 5.4E**). Pre-CPB SVR measurements were 1268±240, 1279±132, 1248±137 and 1413±131 dyne*s/cm$^{\wedge 5}$ and deceased to 1082±122, 958±114,

989±115and 971±115 dyne*s/cm$^{\wedge 5}$ in the Placebo, Late 1, Early_1 and Late_10 groups, respectively ($p<0.05$, n=8 each group). There was no significant change in MABP pre and post CPB in any group (**figure 5.4F**).

Despite changes within groups between pre and post CPB measurements, as outlined above, there was no significant between-groups difference in any of the parameters, as measured by 2-way ANOVA ($p>0.05$, n=8 in each group).

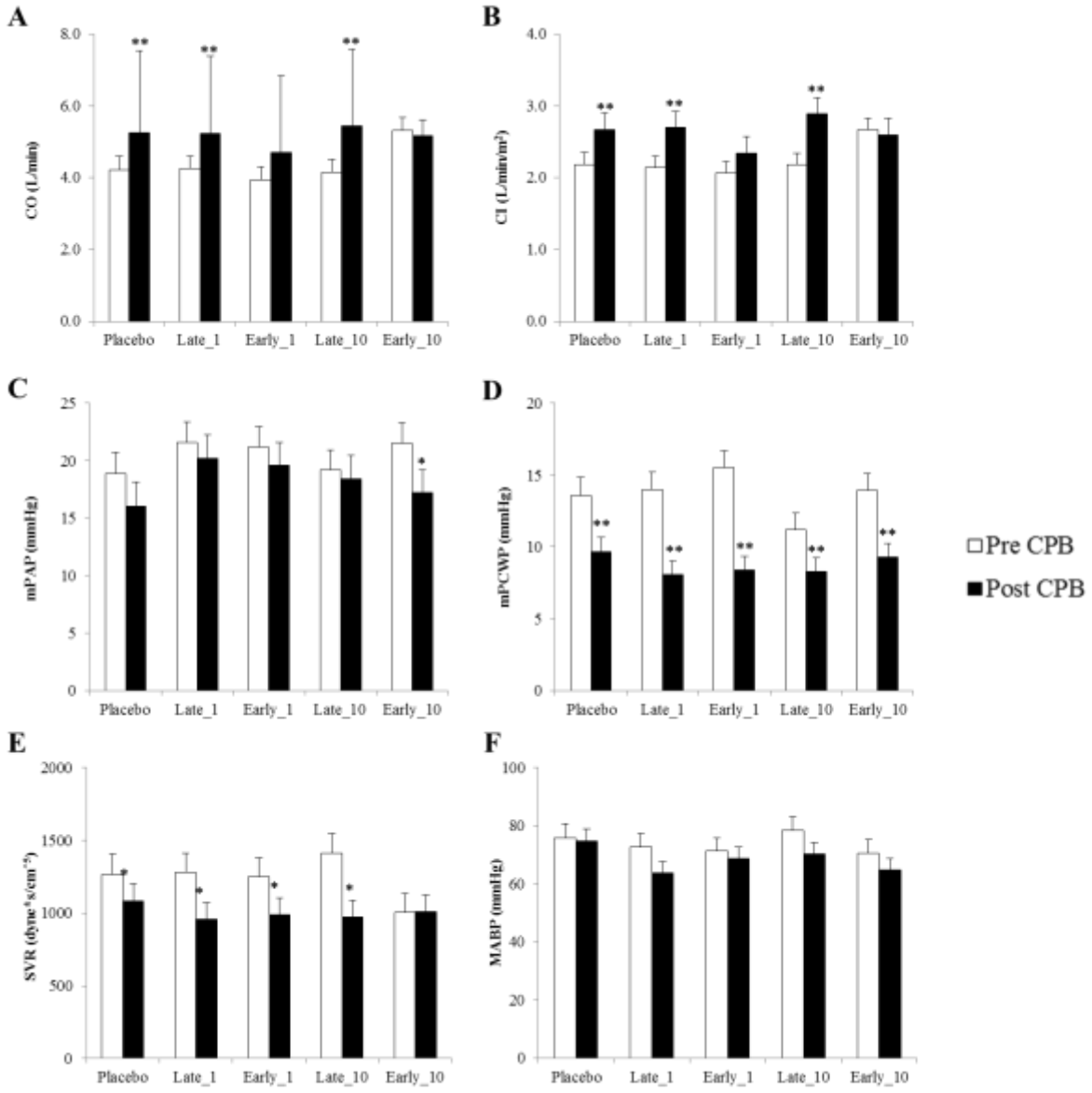


Figure 5.4: **A**) CO, **B**) CI, **C**) mean PAP, **D**) mean PCWP, **E**) SVR and **F**)MABP. *p<0.05, **p<0.01, ***p<0.001 comparing pre and post CPB measurements. N=8, Data is expressed as Data is expressed as mean±SEM.

5.4.4 Venous methaemoglobinaemia associated with nitrite infusion

Venous methHb increased significantly from baseline with i.v. infusion of 10μg/kg/min of sodium nitrite during both 'late' and 'early' infusions (**figure 5.5, A and B**). Following administration of 10μg/kg/min of sodium nitrite the day before surgery, venous metHb rose from 0.64±0.06% at baseline to 0.88±0.08 ($p<0.01$, n=8-9). Similarly, following intra-operative administration of 10μg/kg/min of sodium nitrite, baseline venous metHb was 0.63±0.06% and increased to 0.98±0.07% after 30minutes ($p<0.05$, n=8-9).

There was a small, statistically significant decrease in venous lactate levels during the placebo infusion administered the day before surgery. Initial lactate levels were 1.4±0.14mmol/L and decreased to 1.17±0.12mmol/L after 30minutes of i.v. 0.9% saline ($p<0.05$, n=8, **figure 5.5 C and D**). There was no significant change in acid-base balance (**figure 5.5 E and F**) or lactate levels associated with nitrite infusion, either the day before surgery or intra-operatively.

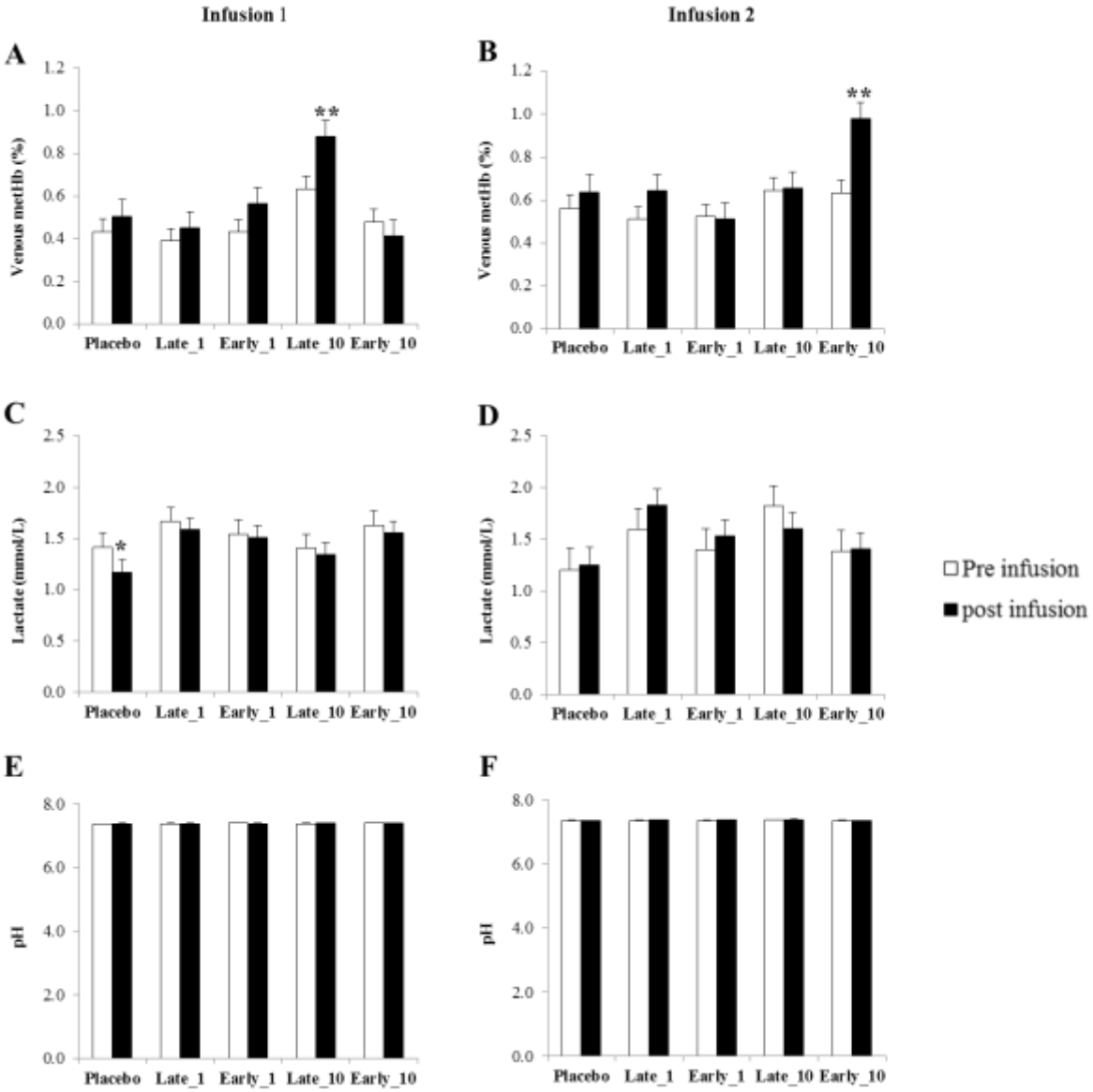


Figure 5.5: A) Venous metHb infusion 1; **B**) Venous metHb infusion 2; **C**) Lactate infusion 1; **D**) Lactate infusion 2; **E**) pH infusion 1; **F**) pH infusion 2. *p<0.05 and **p<0.01 comparing pre and post infusion values. N=8-9, data is expressed as mean±SEM.

5.4.5 Changes in intra-operative acid-base balance

During surgery, there was no change in pH between groups or within each group at any time-point (**figure 5.6 A and B**). Overall lactate levels were higher prior to onset of ischaemia (pre AXC) and fell following onset of reperfusion (**figure 5.6 C and D**, $p<0.001$ compared to placebo group for both arterial and coronary sinus samples). Pooled mean lactate levels prior to onset of ischaemia were 4.34±0.17mmol/L and 4.15±0.12mmol/L in arterial and coronary sinus blood, respectively. Following onset of reperfusion, lactate levels fell to 2.66±0.12mmol/L and 3.05±0.13mmol/L in arterial and coronary sinus blood, respectively ($p<0.001$, $n=44$). Thereafter, lactate levels did not alter significantly. Between groups analysis did not demonstrate any difference in change in lactate levels between the five groups at any timepoint.

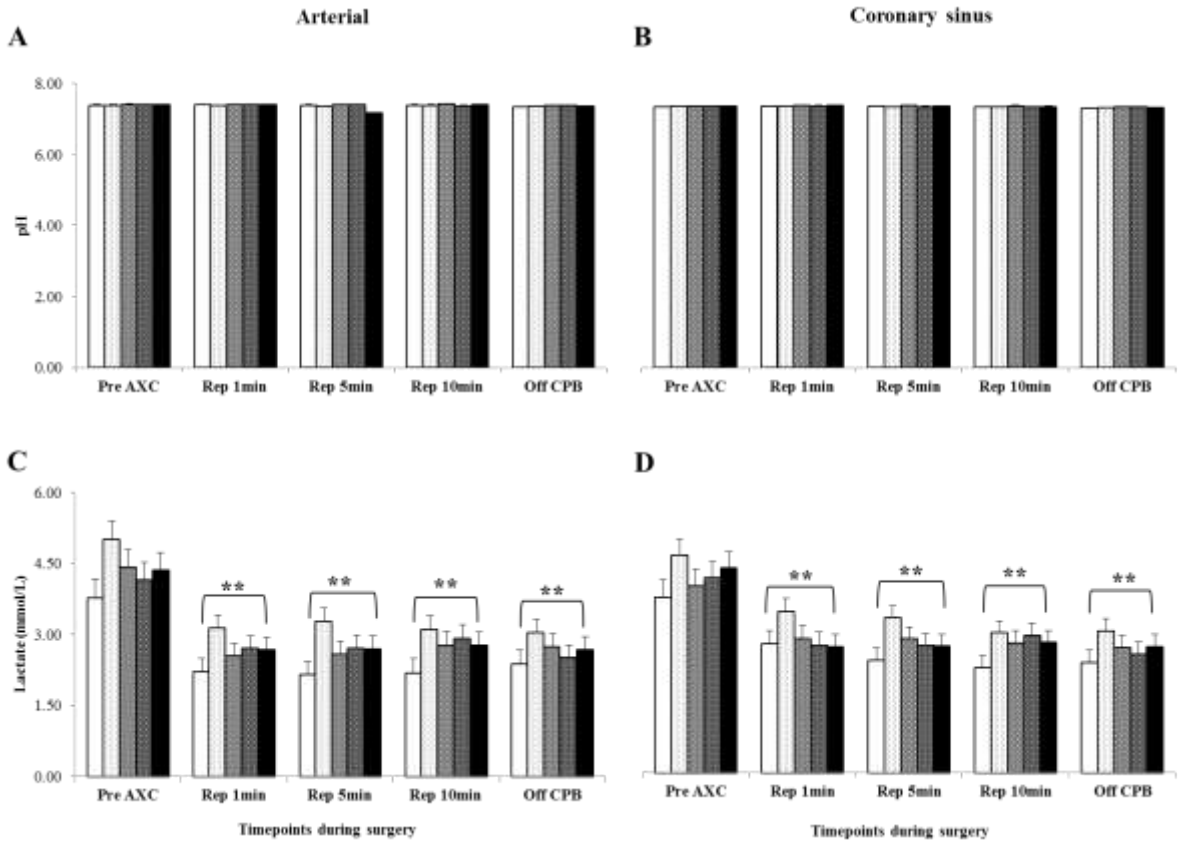


Figure 5.6: **A**) Intra-operative pH in arterial blood; **B**) Intra-operative pH in CS blood; **C**) Intra-operative lactate in arterial blood; **D**) Intra-operative lactate in CS blood. N=8-9, *p<0.01 compared to pre-ischaemia (preAXC levels). Rep 1min, 5min and 10min = 1minute, 5minutes and 10minutes post onset of reperfusion, respectively. Off CPB = 5 minutes after discontinuation of cardiopulmonary bypass. Data is expressed as mean±SEM.

5.4.6 Plasma nitrite and nitrate levels

Following administration of i.v. sodium nitrite, plasma nitrite and nitrate levels increased in a dose-dependent manner (**figure 5.7 A and C**). Administration of 1µg/kg/min of nitrite resulted in an increase in venous plasma nitrite from 1.1±0.4µM to 1.8±0.6µM the day before surgery (**figure 5.7A**) and 1.2±0.1µM to 2.0±0.4µM intra-operatively (*p<0.05 comparing pre and post infusion values, **figure 5.7C**). Administration of 10µg/kg/min of nitrite was associated with a greater increase in venous plasma nitrite levels from 1.3±0.2µM to 5.2±2.0µM (**p<0.01 comparing pre and post infusion values, **figure 5.7A**) the day before surgery and, 1.2±0.1µM to 6.1±0.3µM during surgery (***p<0.001 comparing pre and post infusion values, **figure 5.7C**). Peripheral arterial blood was also sampled intra-operatively. Following administration of 1µg/kg/min, arterial plasma nitrite increased from 1.7±0.4µM to 3.0±2.0µM (**p<0.01 comparing pre and post infusion values) with a greater increase seen following 10µg/kg/min of nitrite from 2.4±0.4µM to 10.5±0.2µM (***p<0.001 comparing pre and post infusion values). Baseline nitrite levels were higher in arterial plasma compared to venous plasma ($^{\#}$p<0.05).

Following nitrite infusion administered the day before surgery, there was a small dose-dependent increase in venous plasma nitrate levels following 1µg/kg/min of i.v. sodium nitrite from 31.3±4.2µM to 34.5±4.9µM (*p<0.05 comparing pre and post, **figure 5.7B**). 10µg/kg/min i.v. sodium nitrite was associated with an increase in venous plasma nitrate from 27.3±3.5µM to 37.2±3.6µM (**p<0.01 comparing pre and post). In contrast, intra-operative nitrite infusion did not yield any significant change in plasma nitrate levels (**figure 5.7D**). In subjects receiving 1µg/kg/min of i.v. sodium nitrite, pre-infusion nitrate concentration was 19.2±3.4µM and post-infusion nitrate concentration was 18.2±2.4µM

($p>0.05$). Intra-operative 10µg/kg/min i.v. sodium nitrite was associated with a non-significant increase in nitrate concentration from 20.3±3.2µM to 25.8±2.3µM ($p>0.05$). In arterial blood, nitrate concentration exhibited a similar pattern of change to that in venous plasma. 1µg/kg/min of i.v. sodium nitrite was not associated with a change in arterial plasma nitrate concentration and 10µg/kg/min of i.v. sodium nitrite was accompanied by a small, non-significant increase in arterial plasma nitrate concentration. Baseline plasma nitrate concentration was higher in arterial blood compared to venous blood ($^{\#}p<0.05$).

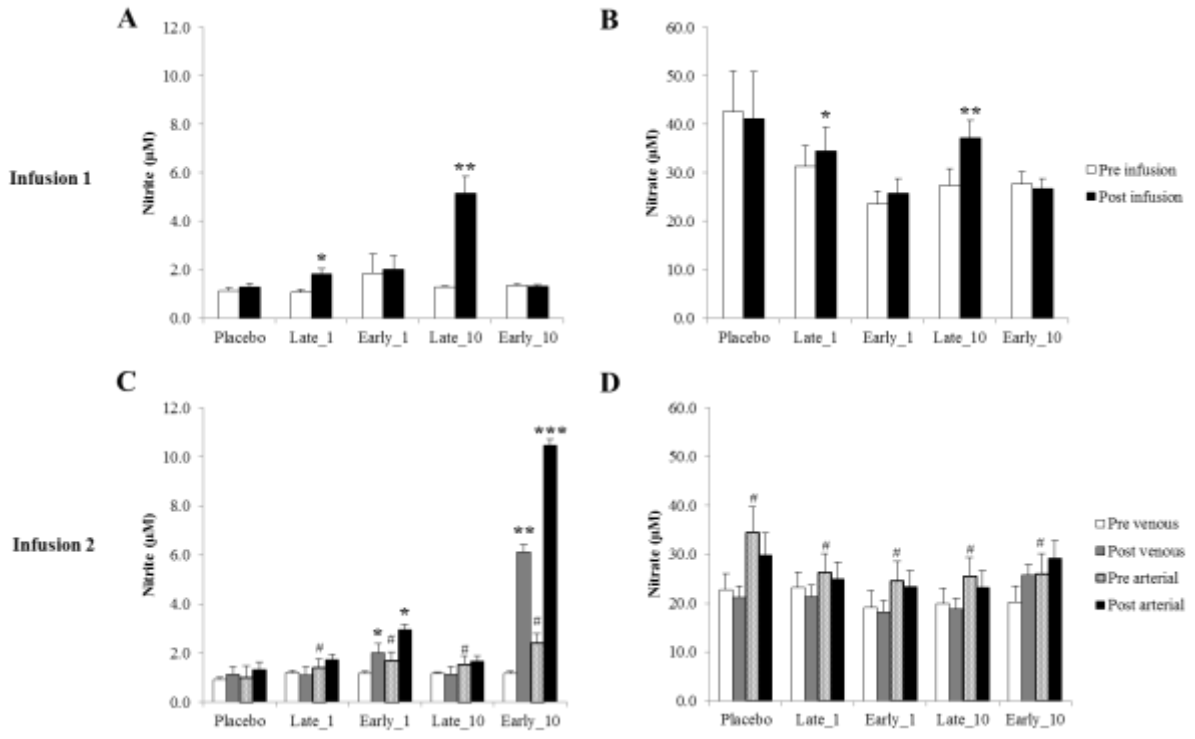


Figure 5.7: **A** and **B**) Venous plasma nitrate and nitrate levels Infusion 1; **C** and **D**) Venous and arterial plasma nitrate and nitrate levels Infusion 2. *p<0.05, **p<0.01 and **p<0.001 comparing pre and post infusion values. $^{\#}$p<0.05 comparing pre-infusion venous *vs* pre-infusion arterial levels. N=8-9, Data is expressed as mean±SEM.

Intra-operative plasma nitrite and nitrate concentration were also measured in coronary sinus and arterial blood, prior to onset of ischaemia (Pre AXC), 10 minutes after start of reperfusion (Rep 10) and 5 minutes after discontinuation of cardio-pulmonary bypass (Off CPB), as depicted in **figure 5.8**. Administration of 10µg/kg/min of i.v. sodium nitrite intra-operatively (Early_10 group) was associated with an higher pre-ischaemia nitrite concentration in both coronary sinus (CS) and arterial compared to the placebo group (***$p<0.001$, **figure 5.8 A and B**). Following onset of reperfusion, nitrite concentration fell to placebo levels ($^{\$}p<0.001$ compared to pre-ischaemia, **figure 5.8 A and B**) and remained at that level after discontinuation of CPB. 1µg/kg/min of i.v. sodium nitrite was not associated with a noticeably different pre-ischaemia nitrite concentration in CS or arterial plasma compared to the placebo group. Overall, pre-ischaemia nitrite concentration (both CS and arterial) was higher compared to 10 minutes post-reperfusion and after CPB discontinuation ($^{\#}p<0.01$, **figure 5.8 A and B**). Both CS and arterial plasma nitrate levels exhibited a similar pattern with non-significantly higher nitrate concentration in both the Placebo and the Early_10 groups at all time-points ($p>0.05$, **figure 5.8 C and D**). Overall, nitrate concentration were higher at all time-points in arterial plasma compared to CS plasma ($^{\S}p<0.001$ CS *vs* arterial, **figure 5.8 C and D**).

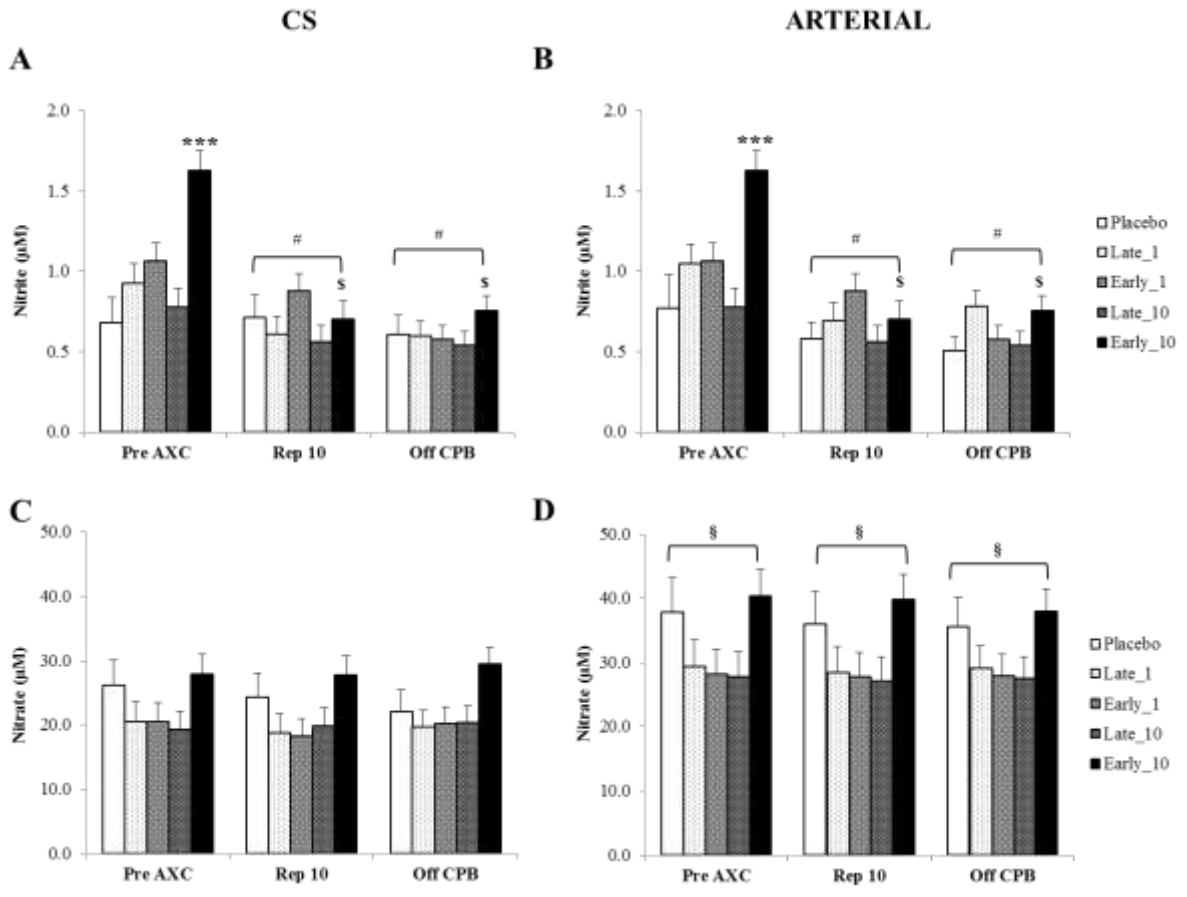


Figure 5.8: Intra-operative plasma nitrite and nitrate concentrations. A) CS nitrite, B) Arterial nitrite, C) CS nitrate and D) Arterial nitrate. Pre AXC, pre-ischaemia, Rep 10, 10minutes after onset of reperfusion and off CPB, 5 minutes after discontinuation of CPB. ***$p<0.001$ compared to Placebo group, #$p<0.02$ compared to pre-ischaemia, $^{\$}p<0.001$ compared to pre-ischaemia and $^{\S}p<0.001$ CS *vs* arterial. N=8-9, data is expressed as mean±SEM.

5.4.7 Intra-operative myocardial injury

Plasma troponin levels were measured intra-operatively prior to ischaemia (pre AXC) and 5 minutes after discontinuation of CPB (off CPB). As described previously, due to a change in troponin assay to hsTnT partway through the study, a regression model (**figure 5.9A**) was constructed and used to calculate troponin from hsTnT values obtained for the later 20 subjects.

TnT results are depicted in **figure 5.9B**. Following reperfusion, TnT concentration had increased significantly from pre-ischaemia levels (**p<0.01 and ***p<0.0001, compared to pre-ischaemia levels. Pre-ischaemia troponin levels were 0.06±0.04ng/mL (Placebo), 0.10±0.02ng/mL (Late_1), 0.05±0.02ng/mL (Early_1), 0.04±0.03ng/mL (Late_10) and 0.06±0.02ng/mL (Early_10). After discontinuation of CPB, troponin levels were 1.10±0.34ng/mL (Placebo), 0.93±0.18ng/mL (Late_1), 0.77±0.18ng/mL (Early_1), 0.49±0.24ng/mL (Late_10) and 0.48±0.19ng/mL (Early_10). Although, the degree of increase appeared to be attenuated with increasing dose of i.v. sodium nitrite, this difference did not reach statistical significance.

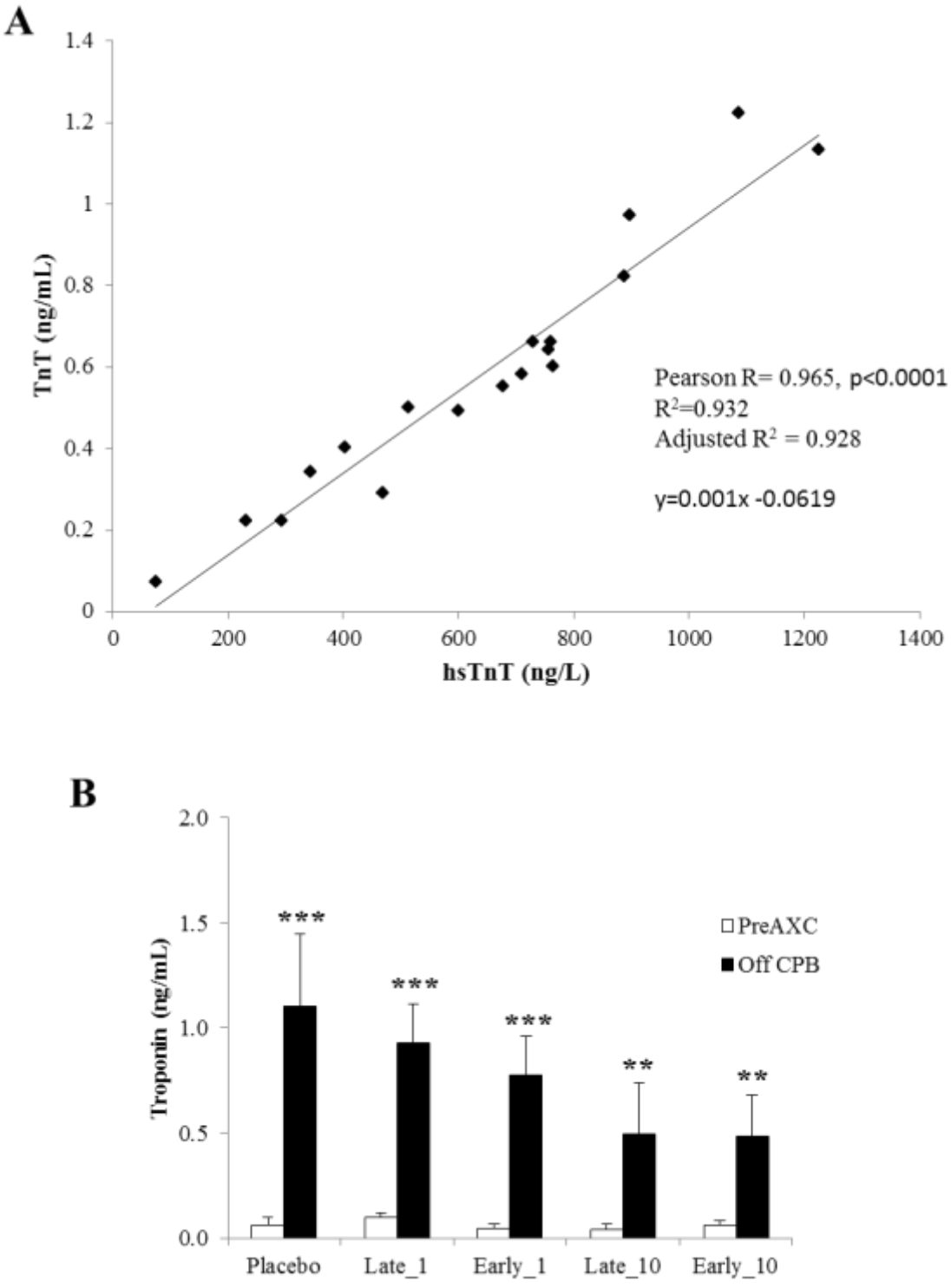


Figure 5.9: A) Regression model used for calculating TnT from hsTnT, n=18; B) Intra-operative troponin T levels. **p<0.01 and ***p<0.0001 comparing pre AXC with post CPB levels. N=5-9, Data is expressed as mean±SEM.

5.4.8 Plasma 8-isoprostane levels

Plasma 8-isoprostane levels were measured in the placebo group and in those subjects receiving 10µg/kg/min of nitrite the day before surgery or intra-operatively. In the Placebo group and the Late_10 group there was a significant increase in plasma 8-isoprostane concentration from pre-ischemia levels to after discontinuation of CPB (**figure 2.10**). Pre-ischaemia 8-isoprostane were 33.1±3.0pg/mL at baseline and increased to 45.6±7.1pg/mL after discontinuation of CPB, in the Placebo group (*p<0.05, n=7). A similar increase was noted in those subjects receiving nitrite the day before surgery from 34.2±3.0pg/mL to 51.5±7.1pg/mL pre-ischaemia to post-CPB levels, respectively (*p<0.05, n=7). In the Early_10 group, no significant increase in plasma isoprostane concentration was observed.

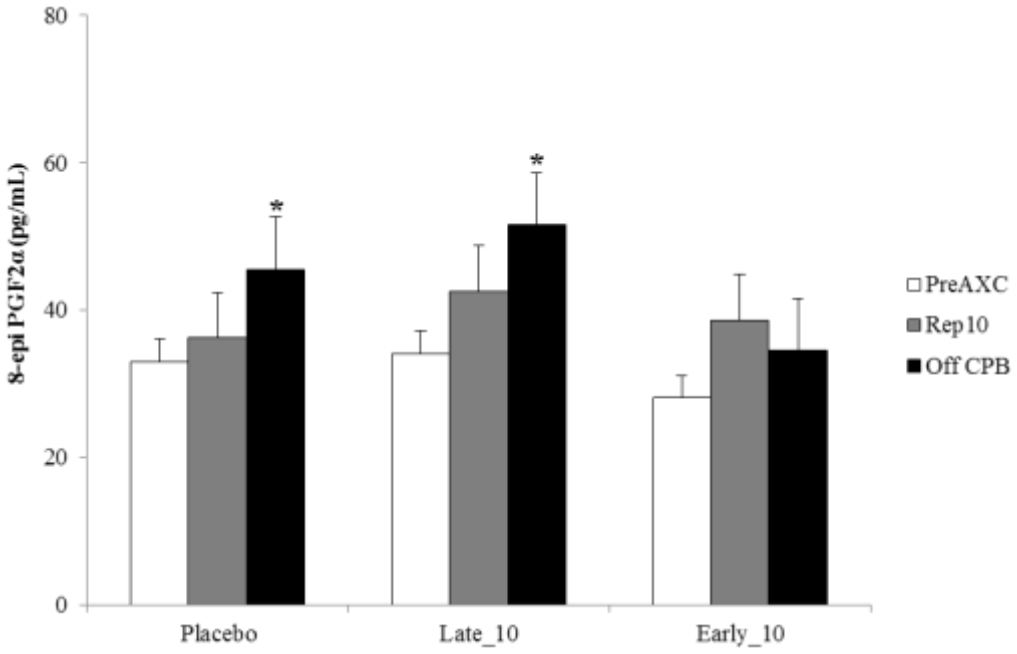


Figure 2.10: Intra-operative plasma isoprostane. Data is expressed as mean±SEM. *p<0.05 compared to pre AXC; n=7.

5.4.9 Myocardial expression of eNOS and Akt

Figures 5.11 and **5.12** depict myocardial expression of eNOS phosphorylation at ser1177 (p-eNOS) and pAKT phosphorylation at ser473 (p-Akt)and total Akt and eNOS expression, at three time-points – pre-ischaemia (LV1), pre-reperfusion (LV2) and 10minutes after onset of reperfusion (LV3).

Nitrite treatment did not enhance Akt phosphorylation in human myocardial cells compared to placebo, during ischaemia or 10-minutes after onset of reperfusion (**figure 5.11 B**, $p>0.05$). Although there was a trend towards increased eNOS phosphorylation with nitrite, particularly immediately prior to ischaemia and before onset of reperfusion that appeared to reduce after onset of reperfusion, this was not significant (**figure 5.11 C**, $p>0.05$).

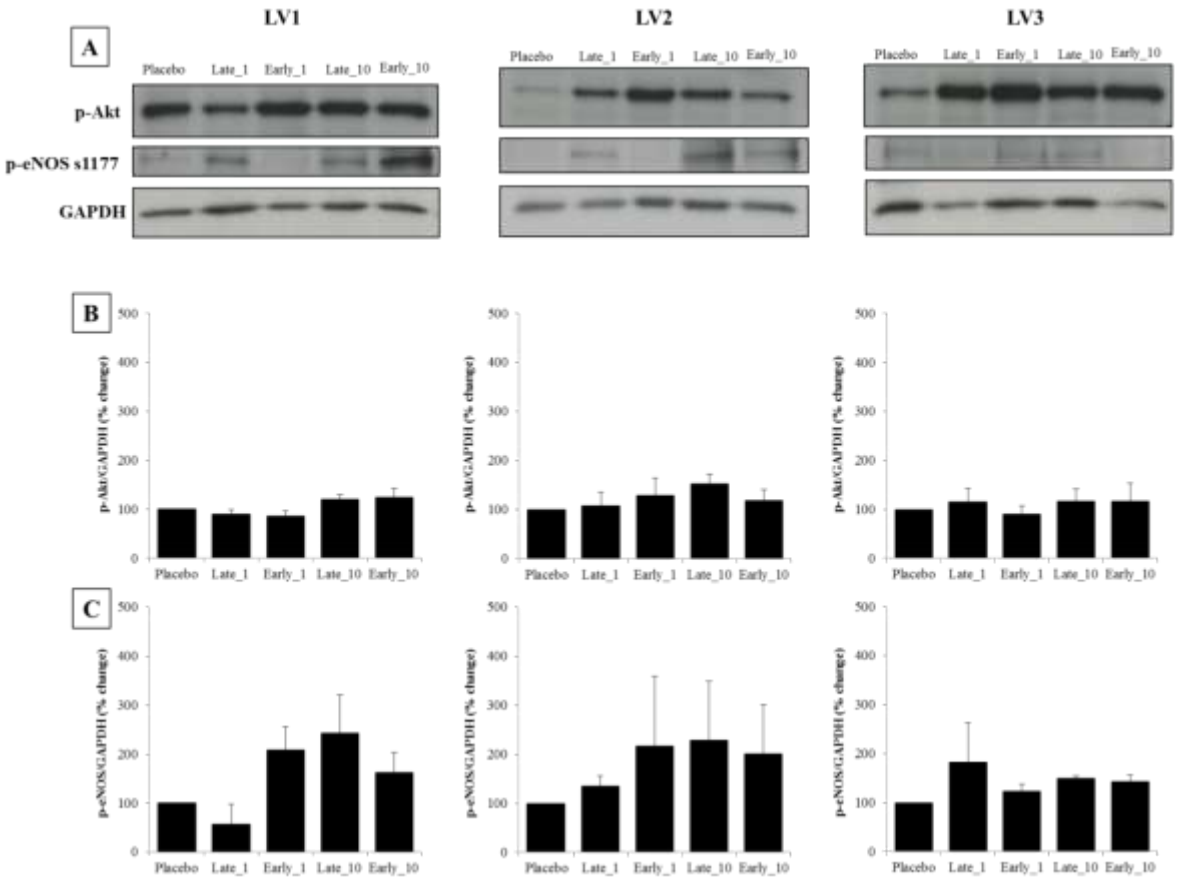


Figure 5.11: **A**) Western blots showing p-Akt(ser473), p-eNOS(ser1177) and GAPDH protein bands in human myocardial tissue from patients undergoing CABG surgery, following i.v. administration of sodium nitrite to patients; **B**) p-Akt protein expression in subjects receiving placebo or nitrite; **C**) p-eNOS protein expression in subjects receiving placebo or nitrite. Data is expressed as mean±SEM; n=4-8 in each group.

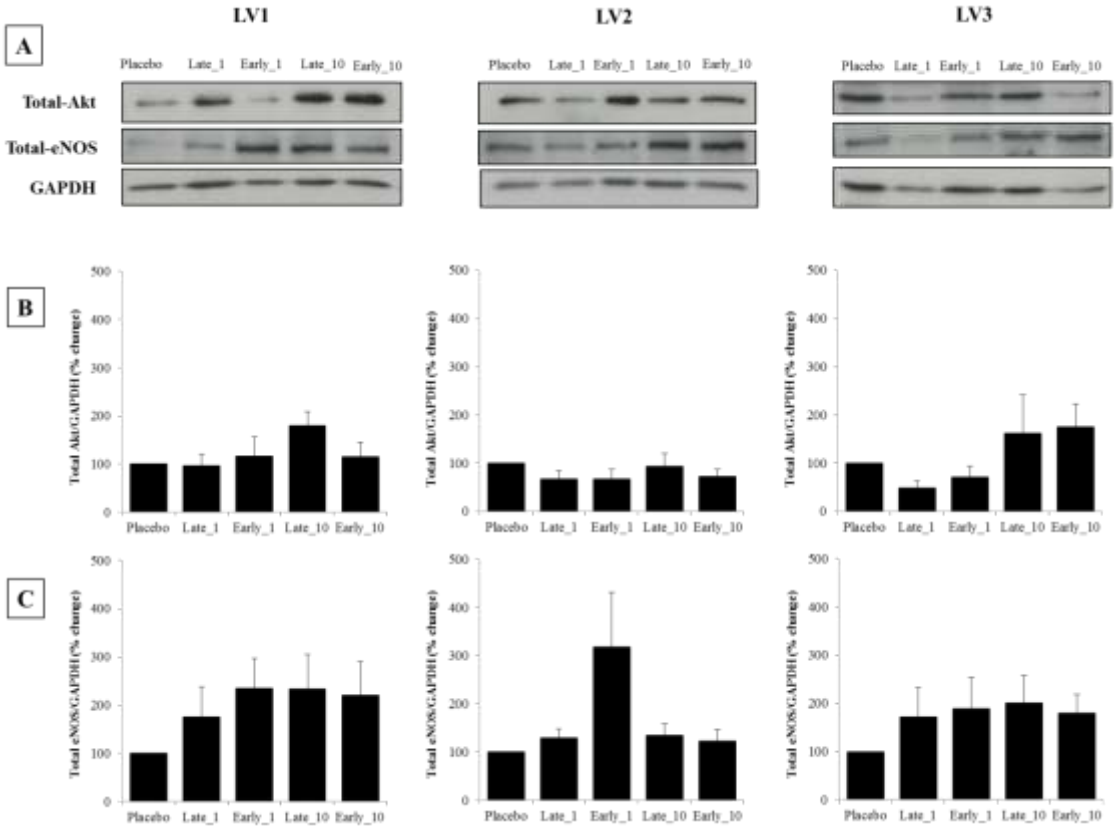


Figure 5.12: **A**) Western blots showing total Akt, total eNOS and GAPDH protein bands in human myocardial tissue from patients undergoing CABG surgery, following i.v. administration of sodium nitrite to patients; **B**) Total Akt protein expression in subjects receiving placebo or nitrite; **C**) Total eNOS protein expression in subjects receiving placebo or nitrite. Data is expressed as mean±SEM; n=4-8 in each group.

5.5 Discussion

To the best of our knowledge this is the first human study investigating the role of nitrite-mediated cardioprotection in humans in the setting of CABG surgery. Administration of 10µg/kg/min of i.v. sodium nitrite immediately prior to ischaemia, attenuated oxidative stress and myocardial injury. Oxidative stress following cardiac surgery and CPB is well recognised (479-481). During CABG, there is an increase in markers of oxidative stress which remain elevated for several hours after discontinuation of CPB and positively correlates with ischaemia time (482, 486). As anticipated, our findings in the Placebo group reproduce the intra-operative plasma 8-isoprostane profile observed by others (486). 10µg/kg/min sodium nitrite administered immediately prior to ischaemia significantly attenuated the increase in plasma isoprostane, suggesting that nitrite may abrogate oxidative stress in patients undergoing CABG (**figure 5.10**). Intra-operative myocardial injury, as assessed by an increase in troponin, demonstrated a trend towards attenuation with increasing nitrite dose (**figure 5.9**). Greater troponin release following cardiac surgery is associated with a worse clinical prognosis in several studies (473, 474, 476). The lack of statistical significance in this study may be explained in part due to change in the troponin assay partway through the study and therefore requirement of a regression model to calculate the troponin values for the latter 20 subjects and/or due to under-powering of the study for this end-point. Nonetheless, these findings are encouraging and favour a role of nitrite in cardio-protection.

Despite mounting evidence postulating a role for nitrite in affording protection against myocardial IRI, the exact mechanisms of nitrite-mediated cardioprotection remain to be fully elucidated. Cardioprotection is thought to be mediated via several molecular signalling cascades, including the RISK pathway (102, 296). Herein, the importance of the RISK

pathway in nitrite-mediated cardioprotection was explored. Whether administered the day before surgery or within 30minutes of ischaemia onset, nitrite did not induce phosphorylation of Akt (**figure 5.11**). However, phosphorylation of eNOS (which occurs downstream to Akt activation in the RISK pathway) demonstrated a non-significant trend towards accentuated protein levels with nitrite. eNOS uncoupling has been implicated in reducing NO bioavailability and augmenting oxidative stress, thereby facilitating IRI (487, 488). Greater phosphorylation of eNOS with nitrite may be a consequence of reduced oxidative stress seen with the larger nitrite infusion rate, and whether this was associated with abrogation of eNOS uncoupling and/or tetrahydrobiopterin (BH_4) depletion was not explored in this study. Numerous upstream activators of eNOS exist, and include protein kinase A (PKA), AMP-activated protein kinase (AMPK) and protein kinase G (PKG), particularly in endothelial cells (489). A recent *in vitro* study in culture rat cardiomyocytes and the intact rat heart demonstrated nitrite-mediated cytoprotection mediated through activation of PKA with consequent alteration in mitochondrion structure and function resulting in generation of reactive oxygen species (ROS) (490). In the intact heart, nitrite-associated protection occurred through up-regulation of AMPK (a downstream target of mitochondrial ROS). This protection was afforded with nitrite delivery up to 6 hours prior to ischaemia and demonstrated a bi-phasic response, similar to that with ischaemic preconditioning. With the complexity of mechanisms involved in conditioning, the observed trend towards eNOS activation with nitrite may be associated with a non-RISK signalling pathway and requires further exploration.

CPB is associated with elevated lactate levels and persistent hyperlactatemia on admission to the intensive care unit following cardiac surgery is thought to be associated with poorer clinical outcomes (491-493). In the present study, lactate levels pre-ischaemia were high as

expected and decreased significantly immediately following onset of reperfusion in all groups (**figure 5.6**). Nitrite administration did not appear to have a significant effect on alterations in lactate levels at any time-point.

As anticipated, nitrite concentration increased in a dose–dependent manner with i.v. nitrite infusion (**figure 5.7**). We observed a significant arterio-venous difference in plasma nitrite concentration with higher arterial concentrations as compared to venous levels, pre and intra-operatively. This is consistent with findings by others where a significant baseline arterio-venous gradient of nitrite was detected in the human forearm vasculature (194). Following nitrite infusion, peak nitrite concentrations were higher with both nitrite infusion rates in arterial blood compared to peripheral venous blood. A similar pattern of higher arterial nitrite concentration was found in CS and arterial blood, intra-operatively. This observation supports occurrence of greater nitrite bioactivity at lower oxygen tension, as reported by others (46, 194).

Nitrite infusion per se did not cause any alteration in HR or MABP (**figure 5.4**). Observed differences in intra-operative cardiac and peripheral haemodynamic parameters could not be solely attributed to nitrite infusion. This study was not designed to assess such clinical variables, but has demonstrated that no major adverse effects were noted in patients receiving nitrite compared to placebo, providing further support for the safety of nitrite use in humans at the present doses for a short period of time. Furthermore, venous peak venous metHb levels achieved with the higher nitrite infusion of 10µg/kg/min was 0.98±0.07% suggestive of clinically insignificant metHb accumulation with exogenous nitrite administration at potentially protective infusion rates.

Study limitations

A significant limitation of this study is underpowering. A scientifically improved approach would have been to simplify the study design to include less limbs e.g. comparison of one study dose rather than two, investigation of early or late window not both or comparison of two nitrite doses without assessment of time window of protection, or alternatively to perform an adequately powered study which would have required a greater number of subjects to be recruited. Although cardiac surgery lends itself well to investigating ischaemic pre-conditioning agents, several potential confounders do exists in these groups of patients which may not allow further conditioning to take place such as volatile anaesthetic agents (494). A standardised anaesthetic and surgical protocol was adhered to but this cannot take account of the differing needs of individual patients in terms of dosages/quantities of anaesthetic agents, inotropes, and/or fluid volume requirements. This study focussed on limited mechanistic/molecular outcomes, whereas several potential pathways of myocardial protection exist (as summarised in Chapter 1, section 1.7.2.4). The importance of the RISK pathway compared to these others remains to be fully elucidated. Moreover, whether the current cardio-protection is afforded through release of NO was not explored in this study.

Conclusion

The importance of nitrite in cytoprotection is being increasingly recognised. Despite probable underpowering, this study provides important mechanistic insights into myocardial protection afforded by nitrite in patients undergoing cardiac surgery at doses that are safe to administer in humans. We have demonstrated that nitrite at 10µg/kg/min reduces oxidative stress and may attenuate myocardial injury. This protection does not appear to be elicited through activation of Akt and may be elicited through eNOS up-regulation, suggesting that the RISK pathway may not be implicated in nitrite-mediated ischaemic pre-conditioning. Numerous

intra-cellular signalling processes have been implicated in protection against IRI. The importance of these pathways in nitrite-mediated cytoprotection remains to be fully determined. However, these data support larger placebo-controlled clinical trials of nitrite in patients undergoing cardiac surgery. There are currently on-going trials to investigate the clinical effect of nitrite-mediated cardioprotection in humans and these will provide further useful insight into the potential clinical role of nitrite in man (495, 496).

Chapter 6: The Role of ALDH2 in Nitrite-mediated Vascular Relaxation

6.1 Introduction

Nitrite reduction to NO under conditions of hypoxia and/or acidosis is well established (117). Numerous potential mechanisms of nitrite conversion to NO have been explored in a variety of experimental conditions from *in vitro* to *in vivo* animal and some human models, as detailed in section 1.6. One of the first mechanisms of nitrite reduction to be discovered was acid disproportionation, which occurs under profound hypoxia and/or acidosis (124). In less extreme conditions, nitrite reduction to NO has been observed with several endogenous substances. These include haemoglobin (26, 139, 497), myoglobin (141, 146, 147), neuroglobin (149), cytoglobin (151), XOR (160) and eNOS (160, 166). Thus far, it remains unclear which, if any one of these mechanism(s) is predominantly responsible for bio-activation of nitrite, particularly in humans.

A further important potential nitrite reductase enzyme is mitochondrial aldehyde dehydrogenase (ALDH2). ALDH2 is closely linked to alcohol dehydrogenase, which is a cytoplasmic protein that is found in the stomach and liver and breaksdowns ethanol to acetylaldehyde, which in turn is further metabolized by ALDH2 (mostly found within mitochondria of hepatic cells) to form an acetic acid radical and H^+. Both this enzymes are important in alcohol metabolism (498). 50% of the Far East Asian population is thought to lack intrinsic ALDH2 activity due to a genetic mutation of the ALDH2*2 gene (499). This commonly manifests as facial flushing following administration of alcohol, as ALDH2 is crucial for alcohol metabolism.

ALDH2 was identified by Chen and colleagues as an important enzyme involved in the bioconversion of GTN to 1,2-GDN within mitochondria (67). Firstly, *in vitro* experiments

using mouse macrophages, demonstrated the biotransformation of GTN to 1,2-GDN and nitrite (NO^{2-}). They proposed the following stoichiometric reaction for this:-

$$GTN + E_{red} \rightarrow 1, 2\ GDN + NO^{2-} + E_{ox}$$

(E_{red} = reductase enzyme; E_{ox} = oxidising enzyme)

Secondly, there was an attenuation of GTN-mediated vascular relaxation in rat thoracic aorta following delivery of the ALDH inhibitors cyanamide and chloral hydrate. Moreover, in anaesthetised rats receiving systemic infusions of i.v. GTN, administration of cyanamide and chloral hydrate attenuated the hypotensive effect of GTN, suggesting that nitrate tolerance may be caused by ALDH inhibition (predominantly the mitochondrial ALDH2 isoform). Further work undertaken by the same group, confirmed these findings in ALDH2-knockout mice (170). Others have also confirmed the role of ALDH2 in the bio-activation of organic nitrates in subsequent *in vitro* and *in vivo* animal studies (168-170). In humans, Mackenzie and colleagues verified these findings in the forearm vasculature of healthy volunteers. They found that ALDH inhibition (through administration of oral disulfiram or through more specific intrinsic ALDH2 inactivity in subjects with the glu504lys ALDH2 polymorphism) resulted in decreased GTN-mediated forearm vasodilatation (171). This study was particularly pertinent as up to 50% of the Far East Asian population is thought to lack intrinsic ALDH2 activity due to a genetic mutation of the ALDH2*2 gene (499).

It has been established that mitochondria may provide an important platform for the reduction of nitrite to NO (173, 174). As described above, nitrite is a major product of GTN bio-activation. Recently, Perlman and colleagues demonstrated that nitrite-mediated cardioprotection was associated with phosphorylation of ALDH2 in the rat myocardium

(176). Thus, ALDH may potentially be an important source of nitrite-derived NO. As nitrite has been discovered to be an important source of NO during hypoxia and/or ischaemia, ALDH may be an important nitrite reductase during these conditions. However, the exact role of ALDH2 as a nitrite reductase and its importance in nitrite-mediated vasodilatation remains unknown. Therefore, this mechanism and how this translates into physiological effects requires further investigation.

An additional important finding highlighted by Chen and colleagues was that prolonged exposure to low dose GTN diminished ALDH activity (168, 170). Other investigators have proposed that ALDH inactivation (primarily the mitochondrial ALDH2 isoform) is the principal mechanism responsible for the development of nitrate tolerance (169, 500, 501). These experimental studies have been performed in animal models. In contrast, in a human study in patients undergoing coronary artery bypass grafting surgery, Philpot and colleagues demonstrated abrogation of vascular ALDH activity with GTN administration (502). They administered low dose i.v. GTN to subjects for 18 hours prior to surgery. In vascular rings harvested from these subjects they found that there was no attenuation of vascular responsiveness with GTN (suggesting lack of tolerance) but there was significant inactivation of vascular ALDH activity. Chen and colleagues confirmed this *ex vivo* in rodent myocardium, where administration of GTN attenuated ALDH activity (503). Recently, D'Souza et al. demonstrated discrepancy between the onset/offset of ALDH inactivation by GTN and the time course of GTN tolerance (504). This pattern of activity potentially permits the utilisation of GTN pre-exposure as an ALDH-inactivating manoeuvre, without the induction of GTN tolerance.

6.1.1 Preliminary data

The study presented herein was based on preliminary *in vitro* experiments undertaken by Dr Melanie Madhani, utilising rat thoracic aortae, exploring (1) the effect of ALDH inhibition on nitrite-mediated vascular relaxation during normoxic and hypoxic conditions; (2) determination of the effect of GTN on nitrite responsiveness (presumably via inactivation of ALDH) and (3) determination of the effects of nitrite treatment in the presence or absence of GTN on ALDH2 expression and on the phosphorylation status of vasodilator-stimulated phosphoprotein (VASP) (Madhani M, unpublished data). Therefore, the findings from these *in vitro* experiments are summarised herein:-

(1) Using the technique of wire myography, vasorelaxation in rat thoracic aorta was observed following administration of sodium nitrite during normoxic and hypoxic conditions, with and without cyanamide (ALDH inhibitor). As shown in **figure 6.1**, no statistically significant difference was observed between the degree of nitrite-induced relaxation in the absence and presence of cyanamide during normoxic conditions. Hypoxia enhanced nitrite-induced vasorelaxation, as expected. However, following ALDH inhibition, there was a significant rightward shift of the concentration-response to nitrite, suggesting that nitrite responsiveness is attenuated with ALDH inhibition

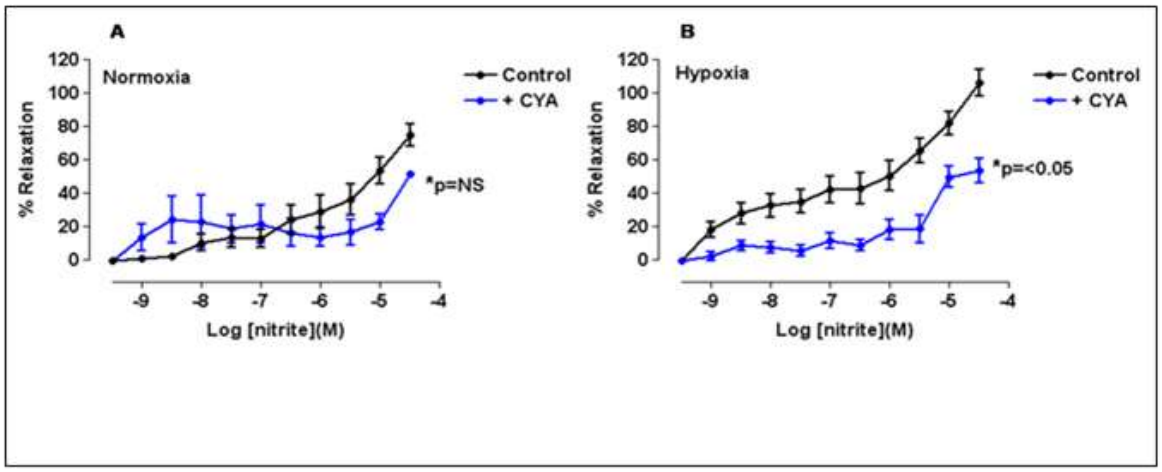


Figure 6.1: Concentration response curves to sodium nitrite in rat aortic thoracic rings in the absence and presence of ALDH inhibitor, cyanamide during (A) normoxia and (B) hypoxia, respectively. Relaxation is expressed as mean±SEM % reversal of phenylephrine -induced tone. NS=not significant; *P<0.05, control versus cyanamide for nitrite only (n=3-4).

(2) To determine whether GTN pre-exposure leads to attenuation of nitrite response (presumably via inactivation of ALDH), rat thoracic aortae were exposed to hypoxic conditions and then incubated in the presence or absence of 30 µM or 100 µM of GTN for a period of 60 minutes. A concentration response curve to sodium nitrite was then constructed under hypoxic conditions. Pre-treatment with 30 µM or 100 µM GTN significantly attenuated the response to sodium nitrite compared to controls during hypoxia (P<0.01 for 30µM GTN and P<0.001 for 100µM GTN; **figure 6.2**).

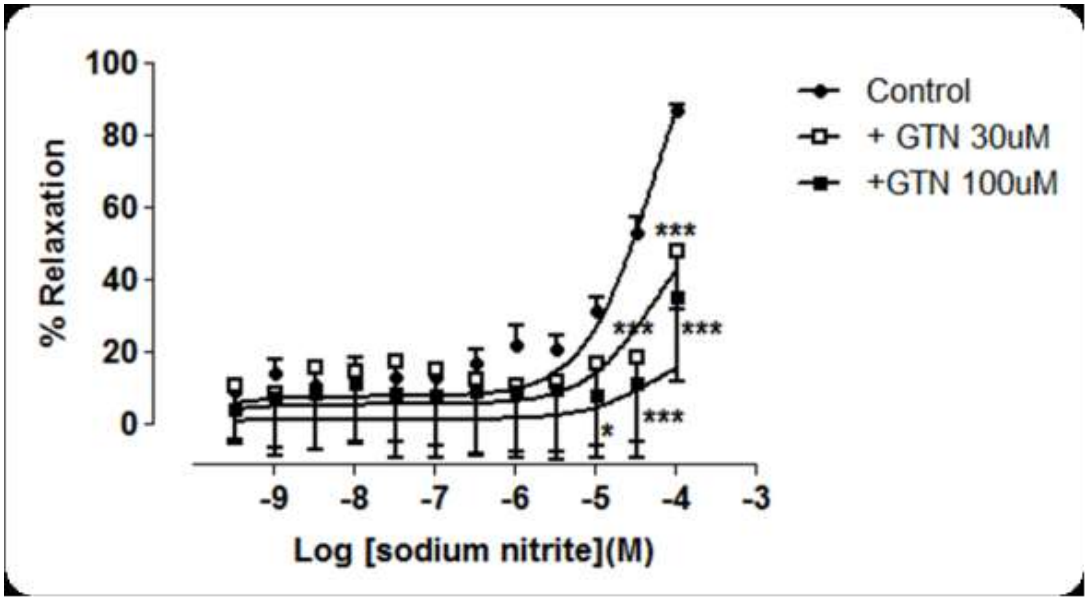


Figure 6.2: Concentration-response curve to sodium nitrite in isolated rat aortas in the absence or presence of GTN (30 or 100μM) during hypoxic conditions. Relaxation is expressed as mean ± SEM percentage reversal of phenylephrine-induced tone (n=12); *p<0.05, ***P< 0.001 versus control.

(3) To determine the effects of nitrite treatment in the presence or absence of GTN on ALDH2 expression and on the phosphorylation status of vasodilator-stimulated phosphoprotein, VASP-ser^{239} (a specific PKG substrate to determine whether ALDH-induced activation of nitrite involved the NO-sGC-cGMP-PKG-VASP pathway.), rat thoracic aortic vessels from the above myograph experiments were immediately snap frozen at the end of the defined protocol. Using Western Blot analysis, it was demonstrated that ALDH2 expression was significantly attenuated following pre-treatment with 100μM GTN and nitrite ($p<0.05$ when comparing to controls; **figure 6.3A**). In contrast, nitrite treatment significantly enhanced the phosphorylation of VASP ser^{239} ($P<0.05$; 2.6 fold over control; **figure 6.3B**). Following pre-treatment

with GTN (100 μM, but not 30μM), the effects of nitrite in stimulating VASPser^{239} phosphorylation was attenuated when compared to nitrite alone (**figure 6.3B**).

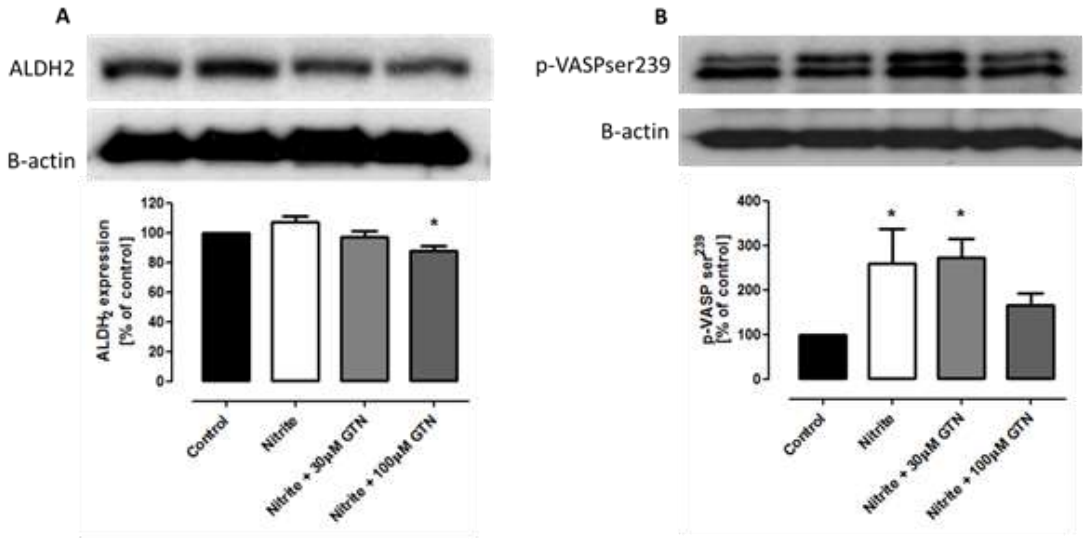


Figure 6.3: The effect of sodium nitrite in the presence or of GTN (30 or 100μM) during hypoxic conditions on (A) ALDH2 expression and (B) phosphorylation VASP ser239 in thoracic aorta. Bars represent mean ± SEM from n=6-9 animals. *p<0.05 versus control .

These findings in rat thoracic aortae demonstrate an attenuation of nitrite-mediated vasorelaxation following pre-treatment with GTN during hypoxic conditions. This attenuation in vascular response is associated with reduced ALDH2 expression. Furthermore, phosphorylation of VASP at ser^{239} site (a reliable biochemical biomarker of vascular PKG activity) demonstrates that nitrite significantly phosphorylates VASP-ser^{239} during hypoxia, thereby supporting the concept that nitrite mediates vasorelaxation through the NO-sGC-cGMP-PKG-VASP pathway. Additionally, nitrite-mediated vasorelaxation following pre-treatment with GTN treatment (100μM) is associated with decrease in VASP ser^{239}

phosphorylation. Thus, demonstrating that nitrite-mediated vasorelaxation involves both ALDH2 and VASP-ser[239].

6.2 Objectives

The aim of this study is to explore the putative role of ALDH as a contributor to nitrite-mediated vasorelaxation in humans, during normoxia and hypoxia. Both e*x vivo* vascular ring studies (wire myography) and *in vivo* forearm vasodilatory experiments (venous occlusion plethysmography) were performed.

Work detailed in chapter 3 (Systemic Nitrite in Heart failure) and further work undertaken by our group has demonstrated that patients with chronic heart failure (HF), exhibit accentuated vascular responses to nitrite and that nitrite should be investigated further in patients with heart failure (505). Given the potential therapeutic implications in heart failure, we felt it important to understand the role of ALDH2 in nitrite bioactivation in this disorder. Therefore, this study was undertaken in patients with HF rather than healthy volunteers.

The objectives of the study were as follows:-

(1) To determine the role of ALDH in nitrite mediated vasorelaxation during normoxia and hypoxia, using isolated resistance vessels from patients with HF.

(2) To determine whether ALDH activity is decreased in patients with heart failure, following GTN exposure, prior to development of nitrate tolerance, using isolated resistance vessels from patients with HF.

(3) To determine the role of ALDH in nitrite mediated vasorelaxation during normoxia and hypoxia, in intact human vasculature in patients with HF.

6.3 Power calculation

Based on the study by Maher et al (46) in healthy volunteers, during hypoxia FBF was 2.37±0.40 (mean±SD) at the 7.84micromol/min dose. To detect a 20% difference in nitrite-mediated vasorelaxation (as assessed by venous occlusion plethysmography) with 1-β of 0.8 and α of 0.05, 14 patients were recruited for the GTN group of the study. A further 8 patients were recruited for the saline group and 23 patients for the Biopsy only group. Therefore a total of 45 patients were required to accommodate this study.

6.4 Methods

The study was approved by the South Birmingham Research Ethics Committee, 10/H1207/50 and conforms to the principles outlined in the Declaration of Helsinki. All subjects gave written, informed consent.

6.4.1 Participants

Subjects were recruited from outpatient clinics at the University Hospitals Birmingham NHS Foundation Trust. The inclusion criteria included patients with systolic heart failure (left ventricular ejection fraction ≤ 40%) of any cause, aged between 40-80 years with no active history of smoking. Patients treated with long-acting nitrates, or with past histories of adverse reactions to organic nitrates, hypotension (systolic BP<110mmHg), concomitant warfarin/clopidrogel therapy or with bleeding diathesis were excluded from the study.

Because of the use of hypoxia in the *in vivo* arm of the study, patients with a BMI >30kg/m^2 and those with obstructive sleep apnoea, were excluded from this limb of the study. All subjects had a light breakfast and were requested to abstain from alcohol and caffeine-containing drinks and follow a low nitrate/nitrite diet (**table 2.1**) for 24 hours for before the study.

6.4.2 Study protocol

Figure 6.4 depicts the study protocol. The patients were grouped as follows: (1) Biopsy group (patients who participated in the *ex vivo* myography experiments only; n=22), (2) Saline group plethysmography study (n=8) and (3) GTN group plethysmography study (n=13).

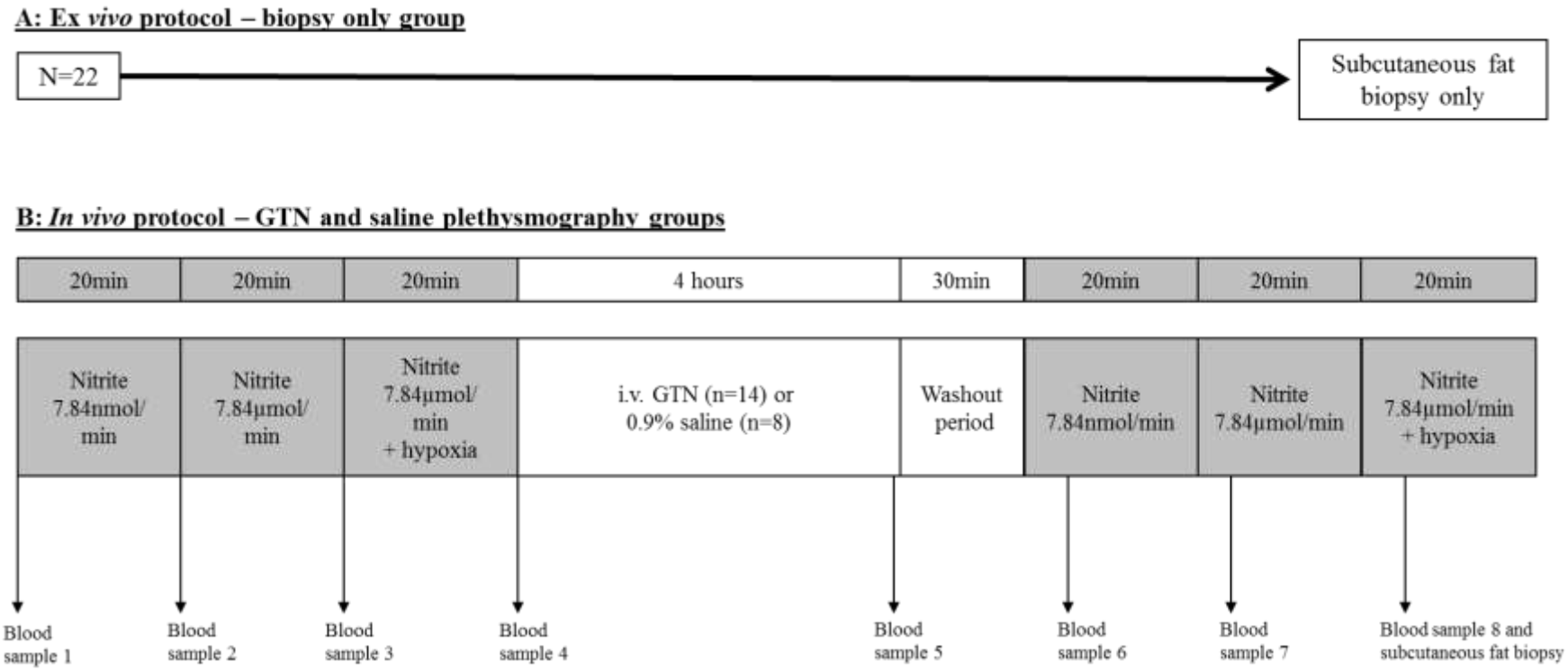


Figure 6.4: Study protocol

(1) Biopsy-only Group

To determine the impact of ALDH inhibition on nitrite bioactivity in resistance vessels, these vessels were obtained from a group of HF patients who were nitrite/nitrate free. A sample of subcutaneous gluteal fat (approximately 2cm x 2 cm x 2cm; **figure 6.5A**) was obtained under local anaesthetic (2% lidocaine). The harvested tissue was placed immediately in ice-cold Krebs-Hensleleit buffer solution (composition in mM: NaCl 119; KCl 4.7, KH_2PO_4 1.18; $NaHCO_3$ 25; $MgSO_4$ 1.2; Glucose 11; $CaCL_2$ 2.5). Subcutaneous small arteries (~250µM internal diameter and 2mm segment length) were dissected from the adipose tissue, cleaned of adherent fat and connective tissue, and mounted onto an automated tension wire myograph (AD Instruments, Denmark). In most cases, two vessels from each biopsy were studied. Vessels were processed using a well-described technique (506). Firstly, they were maintained in Krebs-Hensleleit buffer kept at 37°C under normoxic conditions with a 95% O_2/5% CO_2 gas mixture. After an equilibration period of 45minutes (with regular replacement of Krebs-Hensleleit buffer after every 15 minutes), vessels were normalised to obtain an internal circumference with a transmural pressure of 100mHg (13.3kPa). This allowed the vessels to be standardised prior to subsequent comparisons. Furthermore, the internal circumference that allows maximal response (both constriction and dilatation) is defined as the circumference when the transmural pressure is at 100mmHg (507). Following normalisation, each vessel was primed with potassium chloride solution (KCl; 4.8 x 10^{-2}M) to assess viability (KCl depolarises and therefore activates the vascular smooth muscle, thus causing vasoconstriction in an endothelium-dependent manner). This was proceeded by the addition of supra-maximal concentration of phenylephrine (PE; 10^{-6}M) to pre-constrict the vessels. Once this response had stabilised, acetylcholine (ACh; 10^{-6}M) was added to the bath to assess

the integrity of the vascular endothelium. If the constrictor responses to PE were not maintained, or relaxations greater than 50% of the PE-induced tone to ACh were not observed, the vascular rings were discarded. The vascular rings were then washed for 30 minutes (by addition of fresh Krebs-Hensleleit buffer at 15 minute intervals) after which cumulative concentrations of PE (10^{-9}, $3x10^{-9}$, 10^{-8}, $3x10^{-8}$, 10^{-7}, $3x10^{-7}$, 10^{-6}, $3x10^{-6}$ and 10^{-5}M) were added to the organ bath to constrict the vessels. Each subsequent concentration was added after the response to the present PE concentration had reached a plateau. 80% of the maximal contraction (EC80) of each vessel was calculated using the log dose-response curve. The vessels were then washed over 60 minutes to restore basal tone before contracting to EC80 of the PE-induced response. Once a stable response to PE was achieved, a cumulative concentration response curve to sodium nitrite was constructed (10^{-9}, $3x10^{-9}$, 10^{-8}, $3x10^{-8}$, 10^{-7}, $3x10^{-7}$, 10^{-6}, $3x10^{-6}$ and 10^{-5} and $3x10^{-5}$M) under normoxic conditions (95% O_2/5% CO_2). For the vessels tested under hypoxic conditions (95% N_2/5% CO_2; resulting in ≈1% organ bath O_2), the tissue was first incubated for 30 minutes before exposure to EC80 PE (to achieve similar tension observed at 95% O_2) before administration of sodium nitrite.

To investigate whether attenuation of nitrite efficacy during hypoxia was caused by the effects of ALDH inhibition on downstream signalling in the NO cascade, concentration response curves to the NO donor, spermine-NONOate (SPNO) were constructed in the absence and presence of the ALDH inhibitor, cyanamide, under hypoxic conditions.

(2) In vivo Plethysmography Group

To determine the effect of ALDH inactivation by GTN on arteriolar responsiveness in patients with HF, forearm vascular responsiveness was assessed using the technique of

venous occlusion (strain-gauge) plethysmography. The study was performed at the University of Birmingham, in a quiet and temperature controlled (22 - 24°C) laboratory. All subjects were placed in a semi-recumbent position enabling the administration of hypoxia to participants. Intravenous cannulae (20-gauge) were inserted into a vein in each antecubital fossa. This technique is described in detail in Chapter 4, section 4.3.3. Briefly, changes in blood flow, using mercury in-silastic strain gauges were recorded in both arms (DE Hokanson, Bellevue, Wash). Sodium nitrite was administered intra-arterially via a 27-gauge arterial needle (Coopers Engineering, UK) mounted onto a 16-gauge epidural catheter and sealed with dental wax, as depicted in **figure 6.5B**. This was inserted aseptically into the brachial artery of the non- dominant arm under local anaesthesia (1% lidocaine) and was kept patent by intra-arterial (i.a) 0.9% saline infused at 1ml/min before initiation of sodium nitrite. Each subject underwent placement of a finger cuff for continuous blood pressure recording, an upper arm cuff for the oscillating blood pressure measurement, and chest electrodes for continuous electrocardiogram monitoring using the TaskForce Monitor® (CN Systems, Graz, Austria). Oxygen saturation levels were monitored continuously with pulse oximetry (Nellcor, Pleasanton, CA, USA). Measurement of forearm blood flow (FBF) is described in more detail in Chapter 4 section 4.3.3. FBF was calculated in mL/100mL of tissue per minute and expressed as a ratio of FBF from infused:control arm (FBF_R). The experimental set-up is depicted in **figure 6.5**.

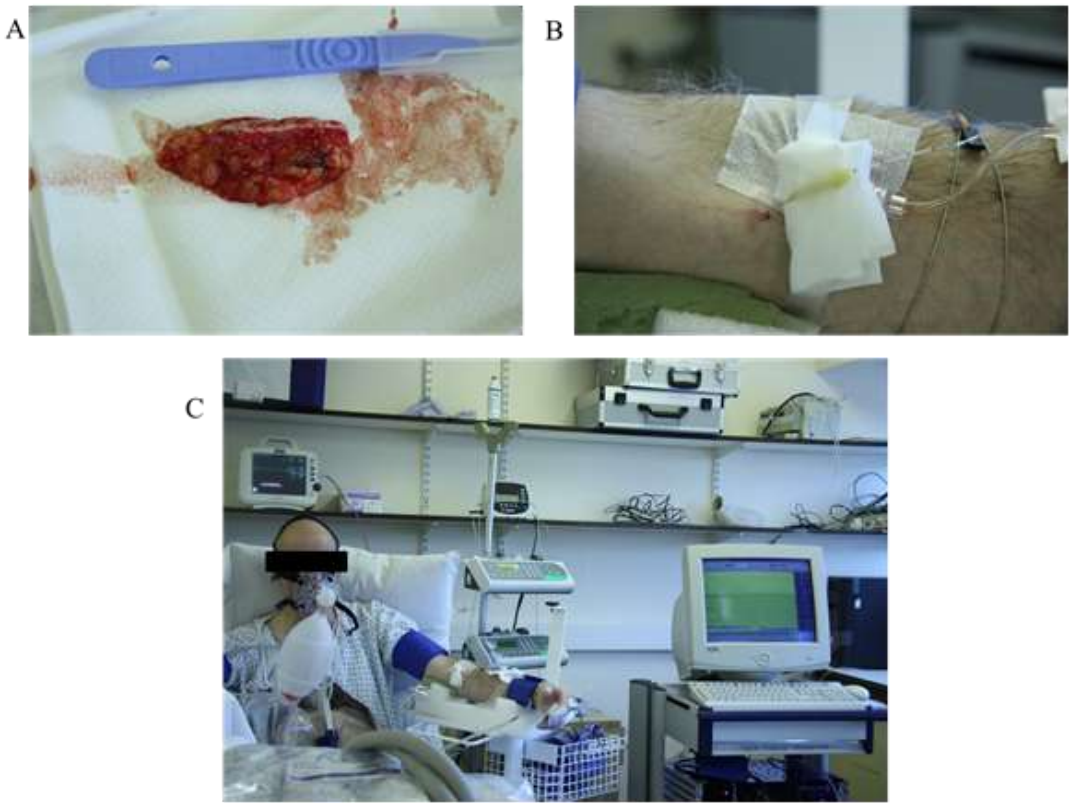


Figure 6.5: (**A**): Picture of typical gluteal fat biopsy obtained from participants, (**B**) Coopers's needle in situ, for administration of intra-arterial sodium nitrite; (**C**) Typical experimental set-up with administration of hypoxia.

As depicted in **Figure 6.4B**, following 10 minutes of stabilisation, CHF patients received two doses of intra-arterial sodium nitrite (784nmol/min then 7.84µmol/min for 20 min; infusion rate 1ml/min) as employed by Maher et al (46). Nitrite was infused into the brachial artery of the non-dominant arm and FBF was measured in both arms. Following the second dose of intra-arterial sodium nitrite (7.84µmol/min), the patients were rendered hypoxic by inspiring 12% oxygen *via* a Hudson facemask. Target oxygen saturation as measured by pulse oximetry was 83-88% (as described in Chapter 2, section 2.4.3). Upon achieving target oxygen saturation the study proceeded with the 7.84µmol/min infusion for a further 10 minutes. Thereafter, patients were randomised (in a double-blind fashion) to receive either an intravenous (i.v.) infusion of low-dose (10µg/min) GTN (to decrease ALDH activity) or 0.9% saline for 4 hours, at a rate of 15ml/hour. This was followed by a 30 minute washout period (i.v. 0.9% saline at 1ml/min). During the 4-hour intravenous saline/GTN infusion, the arterial needle was removed for subject comfort and re-inserted during the 30-minute washout period. Repeat infusions of sodium nitrite (i.a) were administered during normoxia and hypoxia, as described above. Heart rate (HR), mean arterial blood pressure (MABP) and arterial oxygen saturations were recorded at baseline and following each dose of nitrite during normoxia and following the administration of hypoxia.

Upon completion of the plethysmography protocol, the intra-arterial needle was removed. Participants were allowed to rest for 10 minutes following which a subcutaneous fat biopsy was obtained, as described above. The resistance vessels obtained were used to determine whether infusion of GTN had induced significant nitrate tolerance. GTN vasodilator concentration response curves were compared in resistance vessels obtained from

patients with and without prior GTN infusion using the technique of wire myography. As described above, the vascular rings were assessed for endothelial integrity and then contracted sub-maximally with phenylephrine (EC80) and cumulative concentration response curves were obtained for GTN (10^{-9}, $3x10^{-9}$, 10^{-8}, $3x10^{-8}$, 10^{-7}, $3x10^{-7}$ and 10^{-6}M).

6.4.3 Study medication

Sodium nitrite for the human studies was purchased from Martindale Pharmaceuticals (Brentwood, UK). 0.9% sodium chloride (Baxter Healthcare, USA) was used for the placebo (saline) infusion. GTN was purchased from Lipha Pharmacuticals Ltd, UK.

6.4.4 Blood sampling

Venous blood samples were obtained at eight time points during the *in vivo* plethysmography study, as depicted in **figure 6.4B**. These samples were analysed for the following:-

(1) <u>*Methaemoglobin and pH:*</u> Venous whole blood was analysed immediately using a blood gas analyser for methaemoglobin, lactate and pH levels (Bayer Rapidlab 865, Siemens, Tarrytown, USA).

(2) <u>*Assessment of total 8-iso Prostaglandin F2α:*</u> Total plasma 8-iso Prostaglandin F2α (8-isoprostane) was measured by using an 8-Isoprostane EIA assay kit protocol (Cayman Chemical, Ann Arbor, MI) as a marker of oxidative stress.

6.3.5 Statistical analysis

For the myography analysis, relaxations are expressed as percent reversal of PE-induced tone (mean±SEM). Curves were fitted to all the data using a nonlinear regression and the –log [M] of each drug giving a half maximal response (pEC_{50}) were used to compare potency. Concentration response curves were analysed using 2-way ANOVA. For the *in vivo* FBF analysis, 1-way ANOVA repeated measures coupled with a Bonferroni post hoc test was used to compare the change from baseline in FBF_R in each of the saline and GTN groups. An unpaired two- tailed Student t test was used to compare FBF_R following hypoxia between GTN and saline group. Data are given as mean ± SEM, and significance was accepted with $P<0.05$. Statistical analysis was undertaken using SPSS software (version 21.0).

6.5 Results

6.5.1 Baseline characteristics

Patient demographics are depicted in **table 6.1**. A total of 45 patients were recruited into the study. Following recruitment, one patient was excluded from the *in vivo* plethysmography GTN Group due to an episode of hypotension associated with pre-syncope after onset of the first infusion of sodium nitrite (784nmol/min). This subject was withdrawn from the study at that time point with no acquisition of data. One subject was excluded from data analysis from the Biopsy-only Group as no vessels were found in the gluteal fat excised from this subject.

The subjects were well matched between the groups. The major difference was between the body mass index between the Biopsy-only group ($31\pm8 kg/m^2$, n=22) compared to the plethysmography study groups ($25\pm2 kg/m^2$ in the Saline group and $27\pm8 kg/m^2$ in the GTN group). This difference was statistically significant ($p<0.05$).

	Ex vivo analysis	*In vivo* analysis	
	Biopsy only Group (n=22)	SALINE group (n=8)	GTN group (n=13)
Age, years (mean ±SD)	64±13	62±11	66±12
Male gender, n (%)	16 (80)	7(88)	12(92)
Mean weight (kg)	86±5	75±3	82±3
Body mass index (kg/m2)	31±2	25±2	27±1
Ejection fraction (%)	25±2	25±3	29±2
NYHA class, n (%)			
I	2	1	1
II	6	4	10
III	14	3	2
HR (bpm)	72±2	62±4	62±2
MABP (mmHg)	97±34	89±3	88±2
Aetiology, n (%)			
Dilated cardiomyopathy	13 (59)	5 (62)	3 (38)
Ischaemic cardiomyopathy	7 (32)	3 (38)	6 (46)
Other	2 (9)	0 (0)	1 (8)
Medication, n (%)			
ACEI/ARB antagonists	20 (91)	8 (100)	12 (92)
Beta-blockers	14 (64)	5 (63)	10 (77)
Spironolactone/eplerenone	12 (55)	3 (38)	3 (23)
Loop diuretic	15 (68)	4 (50)	8 (62)
Aspirin	15 (68)	4 (50)	10 (77)
Statin	17 (77)	5 (63)	9 (69)

Table 6.1: Baseline characteristics of study subjects. Data are expressed as mean±SEM, unless otherwise stated. NYHA, New York Heart Association class; ACEI, angiotensin-converting enzyme inhibitors; AT2, angiotensin 2 receptor blockers.

6.5.2 Effect of ALDH inhibition on nitrite-induced vasorelaxtion in resistance vessels from heart failure patients

Under both normoxic and hypoxic conditions, sodium nitrite caused concentration-dependent relaxation of resistance vessels obtained from HF patients (**figures 6.6A and 6.6B**). During normoxic conditions, pre-treatment with cyanamide tended to shift the nitrite concentration response curve to the right but this was not significant (n=7; **figure 6.6A**). However under hypoxic conditions, cyanamide potently inhibited nitrite-induced relaxation in a concentration-dependent manner. There was a significant difference between cyanamide and control; $P<0.01$ between 3-10 μM and at 100 μM; $P<0.05$ 30 μM; **figure 6.6B**; n=9). To assess whether this effect was nitrite-specific, concentration-response curves to the NO donor SPNO, were constructed in the presence or absence of cyanamide. Cyanamide had no inhibitory activity on the vasorelaxant responses to SPNO in isolated resistance vessels from HF patients under hypoxic conditions ($P>0.05$; n=6-10; **figure 6.6C**).

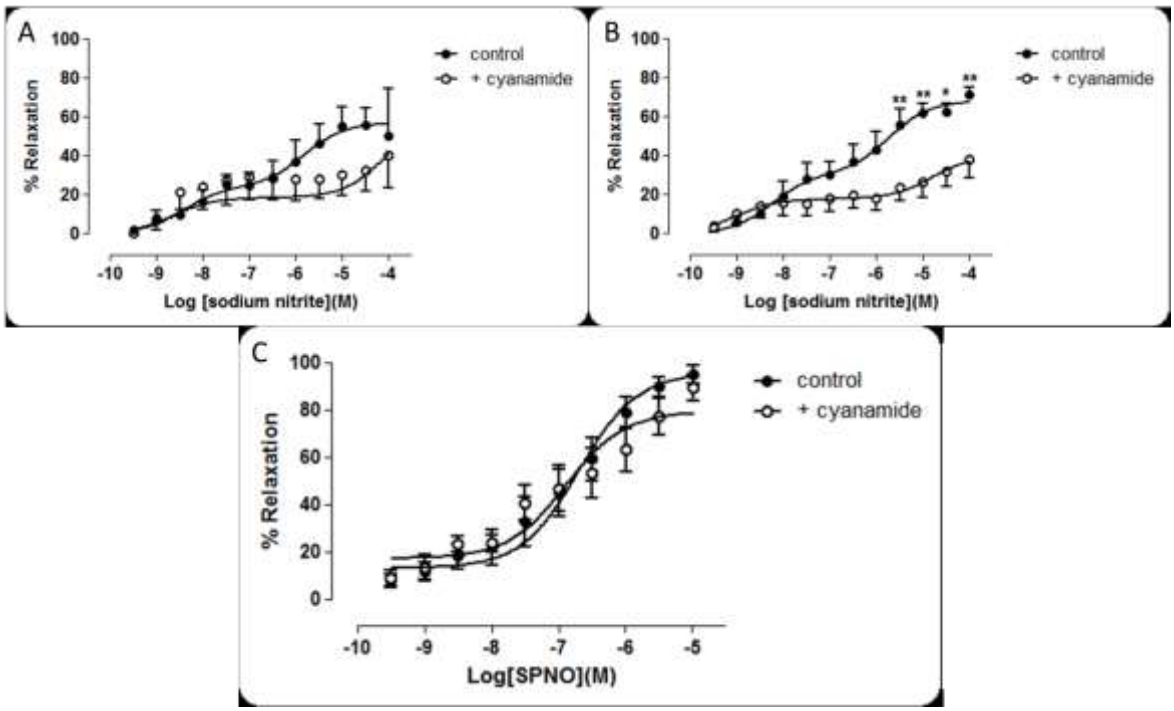


Figure 6.6: Concentration-response curve to sodium nitrite in resistance vessels obtained from HF subjects in the presence and absence of ALDH2 inhibitor, cyanamide (1mM) during (A) normoxia and (B) hypoxia. (C) Concentration-response curve to NO donor, SPNO, in the absence and presence of cyanamide during hypoxic conditions, resepectively Relaxation is expressed as mean±s.e.m. percentage reversal of PE-induced tone. *P< 0.05 and **P<0.01 control versus cyanamide (n=6-10).

6.5.3 Effect of ALDH inactivation by GTN on intact forearm vasculature

Forearm vasodilator response increased dose-dependently with nitrite in both the saline and GTN groups (**figure 6.7A and B**). FBF_R increased from 1.0±0.1 at baseline pre-saline infusion to 1.8±0.2 ($p<0.05$) during normoxia and 2.3±0.3 ($p<0.05$) during hypoxia. Post-saline infusion, FBF_R increased from 1.1±0.1 at baseline to 2.1±0.2 ($p<0.05$) during normoxia and 1.8±0.1 ($p<0.01$) during hypoxia. Similarly, prior to administration of i.v. GTN, FBF_R increased from 1.2±0.1 at baseline to 2.3±0.4 ($p<0.05$) during normoxia and 2.4±0.4 ($p<0.05$) during hypoxia. FBF_R increased from 1.2±0.1 at baseline post-GTN infusion to 2.0±0.2 ($p<0.01$) during normoxia and 1.7±0.1 ($p<0.05$) following hypoxia. Likewise, rendering subjects hypoxic did not result in a significant attenuation of vascular response (**figure 6.8**) following exposure to low=dose i.v. GTN treatment compared to saline (FBF_R is 1.8±0.1 post-saline and 1.7±0.1 post-GTN, p=NS).

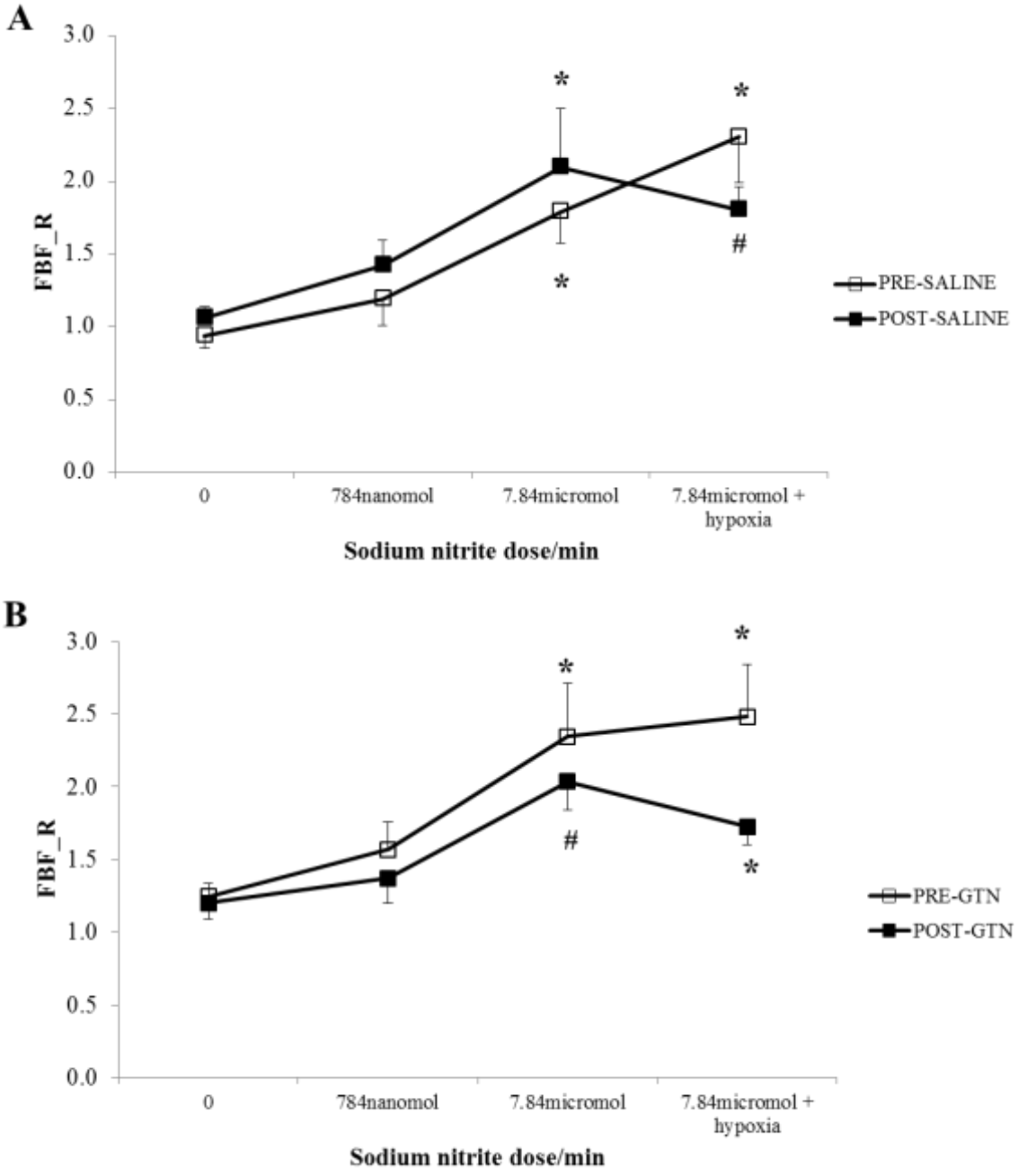


Figure 6.7: Dose-response forearm blood flow (expressed as FBF_R) in subjects receiving incremental doses of sodium nitrite either (A) pre- and post- i.v. saline (n=8) or (B) pre- and post i.v. GTN (n=13). Data is expressed as mean±SEM. *p=<0.05 and #p<0.01 compared to baseline.

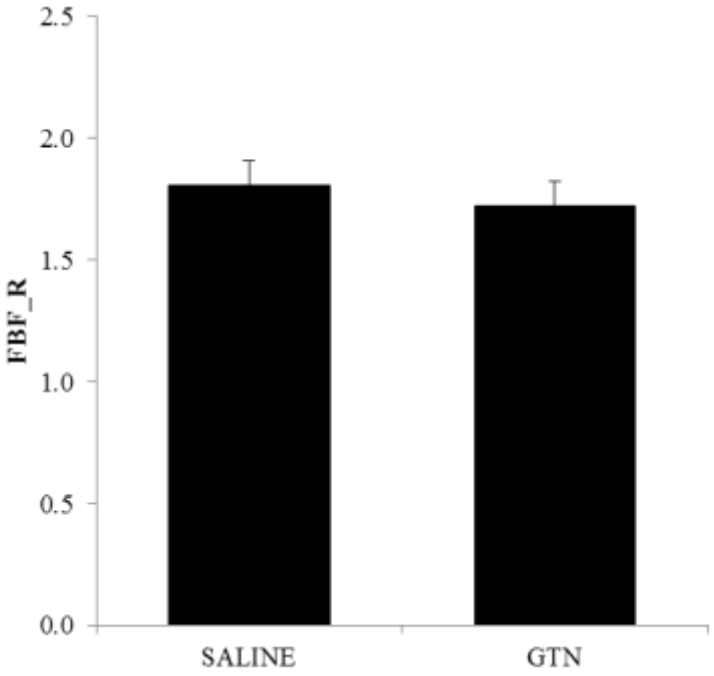


Figure 6.8: Forearm blood flow (expressed as FBF_R) following 7.84μmol/min of intra-arterial sodium nitrite during hypoxic conditions post saline/GTN infusion. Data is expressed as mean±SEM. N=6 in the saline group and n=11 in the GTN group.

	PRE-SALINE/GTN			
	Baseline	7.84nmol/min	7.84µmol/min	Hypoxia + 7.84µmol/min
SALINE group (n=8)				
HR (bpm)	59±5	60±5	60±4	63±4
MABP (mmHg)	87±4	89±5	83±2	77±3
Arterial 0_2 sats (%)	97.0±0.6	97.0±0.5	96.0±0.6	87.0±1.9**
GTN group (n=13)				
HR (bpm)	60±2	59±1	60±2	62±2
MABP (mmHg)	80±3	78±2	85±2	88±3*
Arterial 0_2 sats (%)	98.0±0.4	98.0±0.7	98.0±0.4	86.0±0.6**
	POST-SALINE/GTN			
	Baseline	7.84nmol/min	7.84µmol/min	Hypoxia + 7.84µmol/min
SALINE group (n=8)				
HR (bpm)	61±3	64±3	64±3	64±4
MABP (mmHg)	80±6	79±5	79±5	86±5
Arterial 0_2 sats (%)	97.0±0.6	97.0±0.3	97.0±0.6	86.0±0.8**
GTN group (n=13)				
HR (bpm)	60±2	60±2	60±2	64±42
MABP (mmHg)	85±4	84±4	82±2	863±3
Arterial 0_2 sats (%)	98.0±0.4	98.0±0.3	98.0±0.4	87.0±0.6**

Table 6.2: Haemodynamic data from HF subjects in the *in vivo* plethysmography study.

Data is expressed as mean±SEM, *$p<0.05$ and **$p\leq0.001$ compared to baseline.

6.5.4 Effect of nitrite on haemodynamic parameters before and after saline/GTN infusion

As shown in **table 6.2**, HR and MABP did not alter significantly from baseline following administration of sodium nitrite in the saline and GTN groups, both before and after the 4-hour i.v. saline/GTN infusions. Furthermore, there was no significant difference in baseline haemodynamic parameters before and after the saline/GTN infusions. Arterial oxygen saturations remained stable throughout the normoxia period in the saline group, but fell significantly following administration of 12% oxygen. Mean oxygen saturations were significantly lower than baseline following administration of hypoxia (from 97±0.5% to 87±1.1%, baseline and hypoxia 7.84μmol/min nitrite, respectively. n=8; $p<0.001$). Similarly, the arterial oxygen saturations in the GTN group remained stable throughout the normoxia period, and fell significantly following administration of 12% oxygen (from 97±0.3 to 87±0.6, baseline and hypoxia 7.84μmol/min nitrite, respectively; n= 12; $P<0.001$).

6.5.5 Effect of nitrite on venous pH, isoprostanes and methaemoglobin before and after saline/ GTN infusion

As shown in **table 6.3**, There was no significant change in venous pH in the control arm at any nitrite dose both pre and post i.v. 0.9% saline infusion. However, venous pH increased significantly from baseline following administration of hypoxia in the infused arms from 7.37±0.01 to 7.41±0.01 prior to the 4-hour saline infusion (n=7, $p<0.05$) and from 7.38±0.01 to 7.42±0.01 after the 4-hour saline infusion ($p<0.0001$, n=7).

In the GTN Group, venous pH remained unchanged in the control arm prior to the 4-hour i.v. GTN infusion. There was a significant increase from baseline with escalating nitrite dose post 4-hour i.v. GTN infusion in the control arm, from 7.38±0.01 to 7.40±0.01 following 784μmol/min during hypoxia ($p<0.01$, n=13). In the infused arm there was a significant change from baseline in venous pH in both limbs, both pre- and post- 4-hour i.v. GTN infusion. Prior to GTN infusion, venous pH increased from 7.37±0.01 at baseline to 7.40±0.01 (normoxia) and 7.41±0.01 (hypoxia) following peak nitrite dose ($p<0.001$, n=13, respectively). A similar change in pH was noted in the infused arm post-GTN infusion from7.37±0.01 at baseline to 7.40±0.01 (784μmol/min nitrite, normoxia; $p<0.001$, n=13) and 7.43±0.01 (784μmol/min nitrite, hypoxia; $p<0.001$, n=13). There was no significant change in pH at any stage between at peak nitrite dose between normoxia and hypoxia.

No change in plasma 8-isoprostane levels (saline group n=6; GTN group n=11; $P>0.05$) or lactate levels was detected (saline group n=7; GTN group n=13; $P>0.05$) in both the saline and GTN groups following nitrite infusions (**table 6.3**).

PRE-SALINE/GTN								
	BL		784nM		7.84µM		Hypoxia + 7.84µM	
	CON	INF	CON	INF	CON	INF	CON	INF
SALINE group (n=7)								
pH	7.38±0.13	7.37±0.01	7.38±0.11	7.40.±0.01	7.39±0.11	7.40±0.01	7.40±0.16	7.41±0.01*
lactate (mmol/L)	1.55±0.07	1.59±0.08	1.60±0.07	1.54±0.06**	1.42±0.05	1.52±0.04	1.53±0.05	1.51±0.05*
K^+ (mmol/L)	4.37±0.10	4.28±0.12	4.36±0.11	4.24±0.11	4.29±0.16	4.30±0.13	4.51±0.15	4.43±0.13
Isoprostane (ng/L)	10.8±1.6	6.0±1.0	6.8±1.6	6.8±0.9	7.7±1.0	13.1±4.7	11.0±2.8	4.7±1.8
GTN group (n=13)								
pH	7.38±0.01	7.37±0.01	7.38±0.01	7.39±0.01	7.39±0.01	7.40±0.01***	7.39±0.01	7.41±0.01***
lactate (mmol/L)	1.43±0.07	1.38±0.07	1.40±0.08	1.40±0.09	1.42±0.07	1.31±0.06	1.50±0.08	1.38±0.05
K^+ (mmol/L)	4.69±0.13	4.60±0.14	4.63±0.12	4.57±0.13	4.64±0.10	4.57±0.11***	4.56±0.09***	4.53±0.10***
Isoprostane (ng/L)	8.7±1.4	11.0±2.4	6.6±0.9	8.6±1.9	7.4±1.1	10.1±1.5	8.7±0.8	13.5±3.7

POST SALINE/GTN								
	BL		784nM		7.84µM		Hypoxia + 7.84µM	
	CON	INF	CON	INF	CON	INF	CON	INF
SALINE group (n=7)								
pH	7.38±0.01	7.38±0.01	7.39±0.01	7.40±0.01	7.40±0.01	7.42±0.01	7.40±0.01	7.42±0.01***
lactate (mmol/L)	1.59±0.08	1.58±0.06	1.54±0.06	1.32±0.04	1.52±0.04	1.60±0.06	1.51±0.05	1.50±0.02
K^+ (mmol/L)	4.74±0.14	4.77±0.10	4.85±0.12	4.53±0.10	4.78±0.11	4.58±0.07	4.74±0.07	4.49±0.08
Isoprostane (ng/L)	7.8±0.8	6.0±0.3	8.8±1.2	7.5±3.4	5.6±4.0	10.2±2.0	5.0±1.1	7.3±0.8
GTN group (n=13)								
pH	7.38±0.01	7.37±0.01	7.37±0.01	7.39±0.01**	7.37±0.01	7.40±0.01**	7.40±0.01**	7.43±0.01***
lactate (mmol/L)	1.70±0.08	1.70±0.12	1.42±0.08	1.48±0.09^	1.43±0.07	1.43±0.07	1.51±0.08	1.44±0.06*
K^+ (mmol/L)	4.79±0.09	4.84±0.16	4.70±0.14	4.64±0.12	5.01±0.19	4.59±0.12**	4.62±0.13***	4.60±0.16***
Isoprostane (ng/L)	7.6±1.8	7.7±0.9	5.2±1.7	8.6±1.6	6.5±0.8	7.3±0.7	7.7±0.9	12.1±3.5

Table 6.3: Venous blood data from HF subjects in the *in vivo* plethysmography study.

CON, control arm bloods, INF, infused arm bloods. For plasma isoprostane measurements, n=6 in the SALINE group and n=9 in the GTN group. Data are expressed as mean±SEM. *$p<0.05$, **$p\leq0.01$ and ***$p\leq0.001$ compared to baseline in the same limb.

Whole blood venous methaemoglobin (metHb) levels (**table 6.3**) increased significantly with escalating nitrite dose infusions in both the saline and GTN groups, in both control and infused arms. The greatest increases, as expected were observed in the infused arms as compared to the control arms. In the pre-saline group, metHb levels increased from 0.36±0.02% (baseline) to 1.31±0.18% (7.84μmol/min nitrite, normoxia; $p<0.001$, n=7) and 1.79±0.10% (7.84μmol/min nitrite, hypoxia; $p<0.001$, n=7) in the infused arm. Post-saline there was an increase from 0.19±0.04 (baseline) to 1.54±0.18 (7.84μmol/min nitrite, hypoxia; $p<0.001$, n=7).

In the GTN group, a similar trend was observed as metHb levels increased from 0.39±0.05% to 1.49±0.10% (baseline and 7.84μmol/min normoxia nitrite, respectively; $p<0.001$, n=13) with the highest levels of metHb (1.58±0.09%; $P<0.001$; n=13) reached following 7.84μmol/min nitrite infusion during hypoxic conditions, prior to i.v. GTN infusion. Post-GTN infusion a similar trend was observed in the infused arm. Venous metHb increased from 0.37±0.04 (baseline) to 1.54±0.17 (7.84μmol/min nitrite, normoxia; $p<0.001$, n=13) and 1.55±0.11 (7.84μmol/min nitrite, hypoxia; $p<0.001$, n=13).

6.5.6 GTN - induced tolerance in human resistance vessels

Vascular response of isolated resistance vessels (obtained at the end of the plethysmography protocol) to GTN was determined. There was no significant attenuation of GTN response relative to those in the control vessels (Saline group) and the GTN group (pEC50: 7.94 ± 0.14 and 8.72 ± 0.25 in the saline group (n=7) and GTN group (n=11), respectively; P=0>0.05; **figure 6.9**).

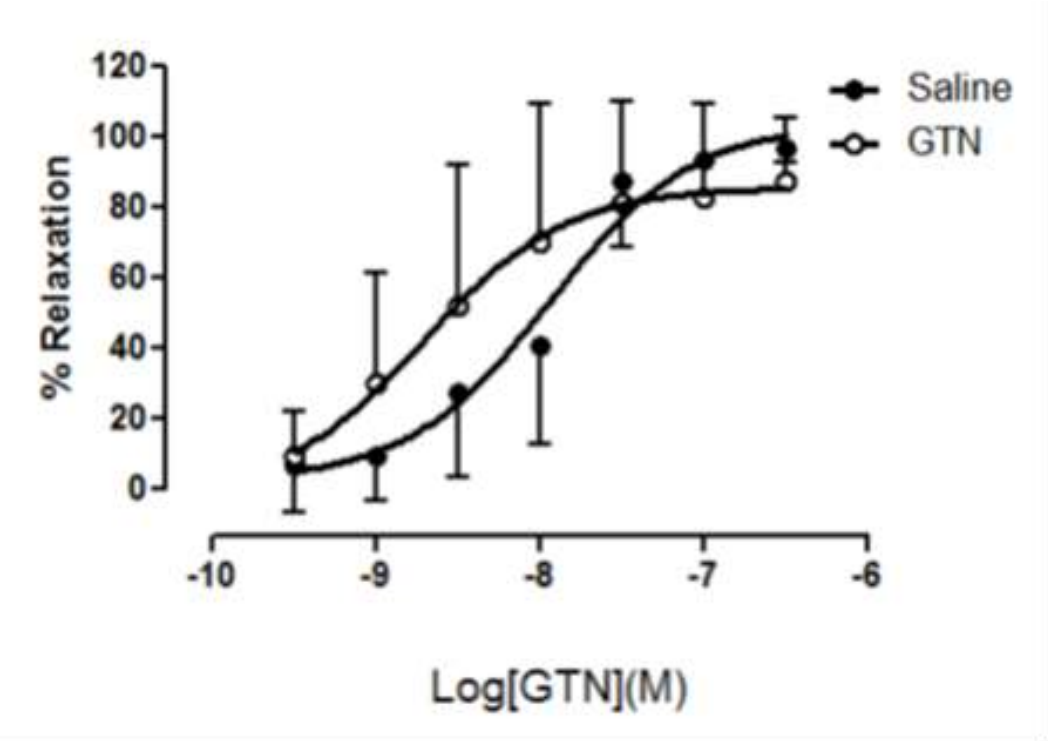


Figure 6.9: Concentration-response curve to GTN in isolated resistance vessels from CHF patients who were subjected to saline (n=7) or GTN (n=11) infusion.

6.6 Discussion

It has been previously observed that intra-arterial nitrite causes forearm vasodilatation in both healthy subjects and in patients with heart failure, with greater potency in the latter (505). However, the mechanisms underlying these effects remain to be fully established Numerous nitrite-reductases have been identified, including eNOS, XOR, haem proteins which can bio-activate nitrite under hypoxia (508). However, whilst all of these agents alone and/or in combination convert nitrite to NO, their exact physiological role remains incompletely understood. Additionally, inhibition of eNOS or XOR does not attenuate nitrite-induced vasodilatation in healthy volunteers, implying either that these mechanisms are unimportant in man or that there is considerable redundancy (26, 161). Despite data suggesting that ALDH may be an important nitrite reductase, the effects of ALDH inhibition during normoxia and hypoxia remain unknown (176).

Preliminary animal data from *in vitro* studies demonstrated that ALDH inhibition (by inhibitors/inactivators and tolerance-independent inactivation of ALDH by GTN), abrogated the vascular response to nitrite in isolated rat thoracic aortae (Madhani M, unpublished data). These *in vitro* experiments demonstrated that administration of an ALDH inhibitor (cyanamide) and substrate (propionaldehyde) reduced vascular relaxation to sodium nitrite significantly under hypoxic conditions when compared to normoxia (**figure 6.1**). These data indicated the potential for ALDH to be involved in nitrite-mediated hypoxic vasodilatation.

In this study, the ex vivo vascular rings studies confirmed that ALDH inhibition substantially attenuated nitrite-mediated vasorelaxation during hypoxia and further supported the results obtained from the rat thoracic aortae experiments (**figure 6.6**). Cyanamide did not alter the

potency of the NO donor SPNO, which ruled out non-specific effects on NO bioactivity. These observations are in keeping with functional data obtained by Huellner and co-workers, where pharmacological inhibition of ALDH did not alter the potency to the NO donor diethylamine NONOate in isolated human veins (509).

In vivo, GTN pre-treatment tended to decrease the forearm resistance vascular dilatation to nitrite infusion in HF patients, but this was not significant (**figure 6.7**). This was in contrast to the animal data from rat thoracic aortae and the response of the resistance vessels to sodium nitrite *ex vivo*. It may be that ALDH does plays a role in nitrite-mediated vasorelaxation although, this effect was less marked *in vivo*. This could be due to the presence of multiple *in vivo* mechanisms which are easier to elicit in isolation in the laboratory where the confounding effect of other nitrite reductases is absent.

Previous studies have reported that prior GTN exposure induces nitrate tolerance in association with attenuated ALDH activity in the vasculature (500, 504, 509). The preliminary studies by Madhani (Madhani M, unpublished data) utilised a well described model of nitrate tolerance in the isolated rat aortae, as described previously (510, 511). In these experiments there was reduction of nitrite-mediated vasorelaxation following pre-treatment with GTN during hypoxia (**figure 6.2**). Therefore, attenuation in vascular response associated with reduced ALDH2 expression was demonstrated, to confirm the role of ALDH as an important effector of nitrite-mediated vascular dilatation (**figure 6.3**). Furthermore, to establish whether ALDH-induced stimulation of nitrite involved the NO-sGC-cGMP-PKG-VASP pathway, expression of VASP-ser^{239} site (a reliable biochemical biomarker of vascular PKG activity) was evaluated, as described previously (512, 513). These results

demonstrated that nitrite substantially increases VASP-ser^{239} phosphorylation during hypoxia, thereby supporting the theory that nitrite mediates vasorelaxation via the NO-sGC-cGMP-PKG-VASP pathway. Moreover, it was shown that a reduction in nitrite-mediated vasorelaxation following GTN pre-treatment (100µM) is associated with reduction in VASP-ser^{239} phosphorylation. This demonstrates that nitrite-mediated vasorelaxation comprises both ALDH2 and VASP-ser^{239}. Therefore, in the current study low dose GTN infusion was used to inactivate ALDH. 10µg/min of i.v. GTN over 4 hours (as employed in the current study) has previously been utilised by Philpott and co-workers who have reported that it rapidly inactivates vascular ALDH. They reported that this inactivation occurs before significant nitrate tolerance develops and before impairment of GTN bioconversion is detected (502). In human clinical studies, disulfiram is commonly used as the ALDH inhibitor of choice however, its use in patients with heart failure is contra-indicated and it was therefore not employed in the current study (514).

Endothelial dysfunction and NO resistance are well established in HF (515). In this study, sufficient vascular activity in response to nitrite was observed. During normoxia, a doubling of forearm vasodilatory response was seen, following 7.84µmol/min of sodium nitrite in both the saline and GTN group when comparing to their baseline responses (**figure 6.7**). However, during hypoxia, the inhibition of ALDH with GTN marginally inhibited forearm vasodilatory response when compared to the saline group (**figure 6.8**). Nonetheless, to establish whether the trend to decreased forearm vasodilataion to nitrite was associated with GTN tolerance, resistance vessels were obtained from HF patients (both saline and GTN groups) at the end of the *in vivo* study protocol. These resistance vessels were then used to assess the vascular response to GTN. There was no significant difference in vascular response between the saline

and GTN groups, suggesting the observed trend to attenuation of FBF observed from the GTN group was not due to GTN tolerance, but potentially due to inactivation of ALDH (**figure 6.9**).

Study limitations

Despite strict inclusion/exclusion criteria to standardise experimental procedures, a degree of heterogeneity will exist in the population studied in terms of cause and severity of HF. Although, the majority of baseline characteristics were similar there was a discrepancy in the body mass index of subjects within the biopsy group (31±8kg/m^2, n=22) compared to the *in vivo* plethysmography study group (25±2kg/m^2 in the saline group and 27±8kg/m^2 in the GTN group) which was statistically significant ($p<0.05$). This could be a possible confounder for the vascular responses seen, as described previously (516). Limited options exist for clinical ALDH inhibition in humans; we were unable to use Disulfiram (ALDH inhibitor in clinical studies), as it is contra-indicated in patients with cardiovascular disease, as described above. Furthermore, plasma levels of nitrite/nitrate were not measured as the results are likely to have been affected greatly by the metabolism of GTN to nitrite/nitrate, thus masking any changes to endogenous nitrite/nitrate levels. Finally, we were unable to confirm in our *in vivo* studies whether ALDH activity was actually attenuated by GTN.

Conclusions

The major finding of this study is that ALDH is involved in nitrite-mediated vasorelaxation during hypoxia, as demonstrated in human resistance vessels from patients with HF. The *in vivo* results demonstrate a (non significant) trend towards attenuation of intact forearm vasodilatory response to nitrite following GTN treatment, which may be due to a reduction in ALDH activity. However, no firm conclusion can be made about this effect

in vivo. The non-significance of this effect may reflect the presence of multiple *in vivo* mechanisms of nitrite –mediated vasorelaxation. Therefore, the relative contribution to the hypoxic vasodilatory effect of the various nitrite reductases *in vivo* remains unsolved. Furthermore, these observations in the present study suggest that pre-treatment with GTN (even without inducing tolerance) might abrogate nitrite-mediated hypoxic vasodilatation. Further studies to elucidate the extent to which ALDH is involved in nitrite reduction to NO *in vivo* in man may be required.

Chapter 7: General Discussion

In recent years, there has been an explosion of studies investigating the role of nitrite in both physiological and pathological states. The work presented herein provides important new insights into this re-emergence of nitrite through both physiological and molecular/mechanistic studies.

Firstly the role of nitrite in healthy volunteers was explored. Our group had previously evaluated the role of nitrite in the human forearm vasculature under both normoxic and hypoxic conditions, reporting hypoxic augmentation of vascular effects during intra-arterial administration of nitrite (46). Others have corroborated these findings in humans with greater observed vasodilatation in the hypoxic pulmonary vasculature (199). In contrast to these findings, we observed that in healthy volunteers (**Chapter 2 – the role of systemic nitrite in healthy volunteers**) systemic nitrite administration did not reproduce enhanced vasodilatation during hypoxia, in respect relative to normoxia, despite substantial increase in plasma nitrite concentration. Importantly, this study established the safety profile of sodium nitrite at doses of up to 50µg/kg/min in humans. Therefore, nitrite may not have an enhanced hypoxic vasodilatory effect in hypoxia that could be potentially utilised in disease states such as heart failure or pulmonary artrterial hypertension. Furthermore, the safety issue of methaemoglobin accumulation with nitrite therapy has been addressed. The findings indicate that sustained infusions at rates higher than 10µg/kg/min, would require close monitoring of methaemoglobin and is likely to provide a limitation to continuous nitrite therapy in patients. Peak methaemoglobinaemia following a 5minute infusion of 50µg/kg/min was approximately 1%. Data from the preliminary healthy volunteer study (figure 2.3) showed that 60µg/kg/min i.v. nitrite was associated with a methaemoglobinaemia of 10% after 2.5 hours.

Furthermore, the pre-clinical and earlier human studies as mentioned above are reasonably convincing in demonstrating nitrite-mediated hypoxic vasodilatation. Therefore, further studies to assess reproducibility of our data with a longer period of hypoxia stabilisation may prove useful prior to labelling nitrite as an ineffective hypoxic vasodilator when administered intravenously. Particularly as more marked effects were seen in the HF group in **Chapter 3 – the effects of systemic nitrite in heart failure**. In HF, i.v.sodium nitrite was associated with a favourable haemodynamic profile during short term administration, with an increase in cardiac output in these patients, albeit at the peak infusion rate of 50µg/kg/min only. There was a substantial reduction in PVR (30%) but only a modest reduction in SVR (11%) at this dose. Since CO increased and SVR fell only modestly, the fall in blood pressure was very small. Despite the differing haemodynamic effects, plasma nitrite increased to a similar degree in heart failure patients as compared to the healthy volunteers. Likewise, in keeping with the findings in healthy subjects, sustained infusion of 10µg/kg/min of i.v. sodium nitrite in heart failure patients did not demonstrate any significant effect on cardiac or peripheral haemodynamic measurements.

Despite providing important mechanistic insights into nitrite infusion in heart failure (i.e. relief of diastolic ventricular interaction), the vasodilatory effect of nitrite may not be as beneficial as previously suggested, particularly at doses deemed to be safe and tolerable in humans. Nitrite therapy was deemed to be potentially attractive in patients with congestive heart failure due to its relative venoselectivity, however continuous therapy at efficacious doses may be limited by clinically significant methaemoglobinaemia. Ingram and colleagues reported that infusion of sodium nitrite prevented hypoxia induced vasoconstriction in healthy subjects an hour after ceasing infusion at a time when plasma nitrite had returned to baseline (199). These findings support the exploration of an alternative nitrite administration

regimen, such as pulsed therapy (i.e. interrupted infusions of nitrite rather than a continuous infusion) prior to complete rejection of nitrite as a potential therapeutic option in heart failure. A placebo-controlled trial with greater number of patients may provide more insight into the haemodynamic effects of nitrite in this patients group.

Alternatively, this study does not rule out the potential for nitrite therapy in subjects with pulmonary hypertension, without left ventricular systolic dysfunction. Current treats for pulmonary arterial hypertension include selective pulmonary vasodilators such as phosphodiesterase V inhibitors (which inhibit breakdown of cGMP and therefore increase NO bioavailability, endothelin antagonists and prostacyclins (517). Nitrite, particularly if targeted to the pulmonary vasculature e.g. inhaled preparations, could be used to enhance NO bioavailability in the relatively hypoxic pulmonary vasculature, without significant clinical methaemoglobinaemia. Future studies in this patient group may provide favourable results to a greater degree than that seen in our heart failure patients. Animal models of pulmonary hypertension (e.g. monocrotaline model of pulmonary hypertension in rats) could also be utilised to investigate whether nitrite has the potential to reverse adverse vascular remodeling seen in pulmonary hypertension, through enhanced NO generation or directly through other unspecified mechanisms.

Alternatively, the well-established organic nitrates are in widespread clinical use for treatment of heart failure. Whether GTN exhibits hypoxia-specific vasodilatation is unknown, and was investigated in **Chapter 4** (**Effects of glyceryl trinitrate on human resistance vessels during normoxia and hypoxia**). This may be of importance as patients presenting in acute, decompensated heart failure often are hypoxic due to acute pulmonary oedema, and there is no data to guide differing nitrate therapy in these patients, in respect to those with

chronic congestive heart failure. GTN did not display an accentuated vasodilatory response during hypoxia, suggesting that GTN vasodilatation is independent of ambient oxygen tension. Interestingly, the forearm vasculature tended to dilate to a lesser degree in response to intra-arterial GTN during hypoxia as compared to normoxia, likely through activation of sympathetic vasoconstrictor activity during hypoxia (140, 450, 451). Future studies of GTN *vs* nitrite in heart failure subjects may provide further insight into the comparative efficacy of these agents. These studies could initially be conducted in the forearm vasculature of HF subjects to determine the relative potency of GTN *vs* nitrite, with further studies involving systemic infusions of GTN *vs* nitrite in a larger number of heart failure subjects.

Protection against ischaemia-reperfusion injury (IRI) has been proposed as a further potential application of nitrite in clinical practice. Numerous *in vitro* and animal studies exist to support this notion, with limited human data (as reviewed in **Chapter 5 - Mechanisms of nitrite-mediated cardioprotection in patients undergoing cardiac surgery**). We sought to explore this further in patients undergoing cardiac surgery, a clinical model of IRI with an associated mortality and morbidity. Nitrite appears to abrogate myocardial injury and oxidative stress associated with cardio-pulmonary bypass. This appears to be mediated via eNOS conceivably through a RISK-independent pathway, which requires further investigation. The nitrite administration regimen required for cardioprotection is not associated with clinically significant methaemoglobinaemia. These promising findings lend further support to exploration of nitrite in human models of IRI. One of the major limitations of this study was the likely underpowering for the multiple arms of this study i.e. early *vs* late window and two differeng nitrite doses. A simpler study would have been more likely to determine the role of nitrite-mediated cardioprotection, for the given number of recruited patients. Therefore clinical trials powered to end-points of myocardial injury e.g. troponin,

myocardial area at risk on cardiac magnetic resonance imaging and low cardiac output syndromes will provide useful insight into this expanding field. Furthermore, larger-scale trials in cardiac surgery patients will provide a larger bank of human myocardial tissue which could be utilised to investigate other (several) mechanisms of nitrite-mediated cardioprotection, as depicted in figure 1.4. Moreover, the downstream effects of nitrite on the mitochondrial transition permeability pore could be explored, although this requires 'fresh' tissue samples which need to be analysed immediately. The results of two on-going trials investigating nitrite in the setting of acute myocardial infarction are eagerly awaited and should help determine the clinical utility of nitrite in cardioprotection (196, 518).

Despite some controversy, nitrite-mediated biological actions are thought to be largely mediated through NO (117). Several reported mechanisms of nitrite reduction to NO exist e.g. haem proteins, xanthine oxireductase, aldehyde oxidase, mitochondrial aldehyde dehydrogenase and acid disproportionation. Numerous experimental studies imply the significance of individual mechanisms in generating nitrite-derived NO. However, the relative importance of these mechanisms *in vivo* remains to be fully determined. In experiments presented herein, isolation of molecular mechanisms in rat thoracic aortae and human resistance vessels demonstrated that ALDH causes significant reduction in nitrite-mediated vasorelaxation, particularly during hypoxia (**Chapter 6 – the role of ALDH2 in nitrite-mediated vascular relaxation during normoxia and hypoxia**). However, these findings were not reproduced in the intact forearm vasculature of patients with heart failure. This is likely to be representative of the importance of an array of complex mechanisms which underly this process *in vivo* with no one mechanism predominating. Nonetheless, no firm conclusion can be drawn about the role of ALDH in nitrite bioactivity *in vivo*. Pharmacological methods of ALDH inhibition e.g. disulfiram or GTN, utilised for research

purposes tend to provide non-specific ALDH inactivation. Future human studies should recruit subjects with the Glu504Lys mutation, as they specifically lack ALDH2 function. This population is likely to possess reliable and consistent ALDH2 inactivity which can be utilised to explore the role of ALDH2 in nitrite-mediated bioactivity.

References

1. Moncada S, Higgs A. The L-arginine-nitric oxide pathway. N Engl J Med. 1993 Dec 30;329(27):2002-12.
2. Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature. 1980 Nov 27;288(5789):373-6.
3. Ignarro LJ, Buga GM, Wood KS, Byrns RE, Chaudhuri G. Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. Proc Natl Acad Sci U S A. 1987 Dec;84(24):9265-9.
4. Palmer RM, Ferrige AG, Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature. 1987 Jun 11-17;327(6122):524-6.
5. Arnold WP, Mittal CK, Katsuki S, Murad F. Nitric oxide activates guanylate cyclase and increases guanosine 3':5'-cyclic monophosphate levels in various tissue preparations. Proc Natl Acad Sci U S A. 1977 Aug;74(8):3203-7.
6. Hibbs JB, Jr., Taintor RR, Vavrin Z. Macrophage cytotoxicity: role for L-arginine deiminase and imino nitrogen oxidation to nitrite. Science. 1987 Jan 23;235(4787):473-6.
7. Hill BG, Dranka BP, Bailey SM, Lancaster JR, Jr., Darley-Usmar VM. What part of NO don't you understand? Some answers to the cardinal questions in nitric oxide biology. J Biol Chem. 2010 Jun 25;285(26):19699-704.
8. Schulz R, Rassaf T, Massion PB, Kelm M, Balligand JL. Recent advances in the understanding of the role of nitric oxide in cardiovascular homeostasis. Pharmacol Ther. 2005 Dec;108(3):225-56.
9. Balligand JL, Cannon PJ. Nitric oxide synthases and cardiac muscle. Autocrine and paracrine influences. Arterioscler Thromb Vasc Biol. 1997 Oct;17(10):1846-58.
10. Alderton WK, Cooper CE, Knowles RG. Nitric oxide synthases: structure, function and inhibition. Biochem J. 2001 Aug 1;357(Pt 3):593-615.
11. Kanai AJ, Pearce LL, Clemens PR, Birder LA, VanBibber MM, Choi SY, et al. Identification of a neuronal nitric oxide synthase in isolated cardiac mitochondria using electrochemical detection. Proc Natl Acad Sci U S A. 2001 Nov 20;98(24):14126-31.
12. Bates TE, Loesch A, Burnstock G, Clark JB. Immunocytochemical evidence for a mitochondrially located nitric oxide synthase in brain and liver. Biochem Biophys Res Commun. 1995 Aug 24;213(3):896-900.
13. Lacza Z, Pankotai E, Csordas A, Gero D, Kiss L, Horvath EM, et al. Mitochondrial NO and reactive nitrogen species production: does mtNOS exist? Nitric Oxide. 2006 Mar;14(2):162-8.
14. Carreras MC, Poderoso JJ. Mitochondrial nitric oxide in the signaling of cell integrated responses. Am J Physiol Cell Physiol. 2007 May;292(5):C1569-80.
15. Stevenson TH, Gutierrez AF, Alderton WK, Lian L, Scrutton NS. Kinetics of CO binding to the haem domain of murine inducible nitric oxide synthase: differential effects of haem domain ligands. Biochem J. 2001 Aug 15;358(Pt 1):201-8.
16. Gladwin MT, Crawford JH, Patel RP. The biochemistry of nitric oxide, nitrite, and hemoglobin: role in blood flow regulation. Free Radic Biol Med. 2004 Mar 15;36(6):707-17.
17. Stuehr DJ. Mammalian nitric oxide synthases. Biochim Biophys Acta. 1999 May 5;1411(2-3):217-30.
18. Knowles RG, Moncada S. Nitric oxide synthases in mammals. Biochem J. 1994 Mar 1;298 (Pt 2):249-58.
19. Munzel T, Daiber A, Ullrich V, Mulsch A. Vascular consequences of endothelial nitric oxide synthase uncoupling for the activity and expression of the soluble guanylyl

cyclase and the cGMP-dependent protein kinase. Arterioscler Thromb Vasc Biol. 2005 Aug;25(8):1551-7.
20. Roe ND, Ren J. Nitric oxide synthase uncoupling: a therapeutic target in cardiovascular diseases. Vascul Pharmacol. 2012 Nov-Dec;57(5-6):168-72.
21. Alp NJ, Channon KM. Regulation of endothelial nitric oxide synthase by tetrahydrobiopterin in vascular disease. Arterioscler Thromb Vasc Biol. 2004 Mar;24(3):413-20.
22. Kim-Shapiro DB, Schechter AN, Gladwin MT. Unraveling the reactions of nitric oxide, nitrite, and hemoglobin in physiology and therapeutics. Arterioscler Thromb Vasc Biol. 2006 Apr;26(4):697-705.
23. Jia L, Bonaventura C, Bonaventura J, Stamler JS. S-nitrosohaemoglobin: a dynamic activity of blood involved in vascular control. Nature. 1996 Mar 21;380(6571):221-6.
24. Stamler JS, Jaraki O, Osborne J, Simon DI, Keaney J, Vita J, et al. Nitric oxide circulates in mammalian plasma primarily as an S-nitroso adduct of serum albumin. Proc Natl Acad Sci U S A. 1992 Aug 15;89(16):7674-7.
25. Zweier JL, Wang P, Samouilov A, Kuppusamy P. Enzyme-independent formation of nitric oxide in biological tissues. Nat Med. 1995 Aug;1(8):804-9.
26. Cosby K, Partovi KS, Crawford JH, Patel RP, Reiter CD, Martyr S, et al. Nitrite reduction to nitric oxide by deoxyhemoglobin vasodilates the human circulation. Nat Med. 2003 Dec;9(12):1498-505.
27. Webb A, Bond R, McLean P, Uppal R, Benjamin N, Ahluwalia A. Reduction of nitrite to nitric oxide during ischemia protects against myocardial ischemia-reperfusion damage. Proc Natl Acad Sci U S A. 2004 Sep 14;101(37):13683-8.
28. Nathan C, Xie QW. Nitric oxide synthases: roles, tolls, and controls. Cell. 1994 Sep 23;78(6):915-8.
29. Murad F. Shattuck Lecture. Nitric oxide and cyclic GMP in cell signaling and drug development. N Engl J Med. 2006 Nov 9;355(19):2003-11.
30. Francis SH, Busch JL, Corbin JD, Sibley D. cGMP-dependent protein kinases and cGMP phosphodiesterases in nitric oxide and cGMP action. Pharmacol Rev. 2010 Sep;62(3):525-63.
31. Taylor CT, Moncada S. Nitric oxide, cytochrome C oxidase, and the cellular response to hypoxia. Arterioscler Thromb Vasc Biol. 2010 Apr;30(4):643-7.
32. Victor VM, Nunez C, D'Ocon P, Taylor CT, Esplugues JV, Moncada S. Regulation of oxygen distribution in tissues by endothelial nitric oxide. Circ Res. 2009 May 22;104(10):1178-83.
33. Gladwin MT, Shiva S. The ligand binding battle at cytochrome c oxidase: how NO regulates oxygen gradients in tissue. Circ Res. 2009 May 22;104(10):1136-8.
34. Thomas DD, Liu X, Kantrow SP, Lancaster JR, Jr. The biological lifetime of nitric oxide: implications for the perivascular dynamics of NO and O2. Proc Natl Acad Sci U S A. 2001 Jan 2;98(1):355-60.
35. Jensen FB. The role of nitrite in nitric oxide homeostasis: a comparative perspective. Biochim Biophys Acta. 2009 Jul;1787(7):841-8.
36. Lima B, Forrester MT, Hess DT, Stamler JS. S-nitrosylation in cardiovascular signaling. Circ Res. 2010 Mar 5;106(4):633-46.
37. Singel DJ, Stamler JS. Chemical physiology of blood flow regulation by red blood cells: the role of nitric oxide and S-nitrosohemoglobin. Annu Rev Physiol. 2005;67:99-145.
38. Bryan NS, Rassaf T, Maloney RE, Rodriguez CM, Saijo F, Rodriguez JR, et al. Cellular targets and mechanisms of nitros(yl)ation: an insight into their nature and kinetics in vivo. Proc Natl Acad Sci U S A. 2004 Mar 23;101(12):4308-13.

39. McMahon TJ, Doctor A. Extrapulmonary effects of inhaled nitric oxide: role of reversible S-nitrosylation of erythrocytic hemoglobin. Proc Am Thorac Soc. 2006 Apr;3(2):153-60.
40. Diesen DL, Hess DT, Stamler JS. Hypoxic vasodilation by red blood cells: evidence for an s-nitrosothiol-based signal. Circ Res. 2008 Aug 29;103(5):545-53.
41. Allen BW, Stamler JS, Piantadosi CA. Hemoglobin, nitric oxide and molecular mechanisms of hypoxic vasodilation. Trends Mol Med. 2009 Oct;15(10):452-60.
42. Shiva S, Gladwin MT. Nitrite therapeutics: back to the future. Crit Care Med. 2005 Aug;33(8):1865-7.
43. Kleinbongard P, Dejam A, Lauer T, Rassaf T, Schindler A, Picker O, et al. Plasma nitrite reflects constitutive nitric oxide synthase activity in mammals. Free Radic Biol Med. 2003 Oct 1;35(7):790-6.
44. Gladwin MT. Nitrite as an intrinsic signaling molecule. Nat Chem Biol. 2005 Oct;1(5):245-6.
45. Crawford JH, Isbell TS, Huang Z, Shiva S, Chacko BK, Schechter AN, et al. Hypoxia, red blood cells, and nitrite regulate NO-dependent hypoxic vasodilation. Blood. 2006 Jan 15;107(2):566-74.
46. Maher AR, Milsom AB, Gunaruwan P, Abozguia K, Ahmed I, Weaver RA, et al. Hypoxic modulation of exogenous nitrite-induced vasodilation in humans. Circulation. 2008 Feb 5;117(5):670-7.
47. Beckman JS, Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. Am J Physiol. 1996 Nov;271(5 Pt 1):C1424-37.
48. Pacher P, Beckman JS, Liaudet L. Nitric oxide and peroxynitrite in health and disease. Physiol Rev. 2007 Jan;87(1):315-424.
49. Turko IV, Murad F. Protein nitration in cardiovascular diseases. Pharmacol Rev. 2002 Dec;54(4):619-34.
50. Moncada S, Higgs EA. The discovery of nitric oxide and its role in vascular biology. Br J Pharmacol. 2006 Jan;147 Suppl 1:S193-201.
51. Moncada S, Higgs EA. Nitric oxide and the vascular endothelium. Handb Exp Pharmacol. 2006(176 Pt 1):213-54.
52. Corson MA, James NL, Latta SE, Nerem RM, Berk BC, Harrison DG. Phosphorylation of endothelial nitric oxide synthase in response to fluid shear stress. Circ Res. 1996 Nov;79(5):984-91.
53. Govers R, Rabelink TJ. Cellular regulation of endothelial nitric oxide synthase. Am J Physiol Renal Physiol. 2001 Feb;280(2):F193-206.
54. Gao Y. The multiple actions of NO. Pflugers Arch. 2010 May;459(6):829-39.
55. Rees DD, Palmer RM, Moncada S. Role of endothelium-derived nitric oxide in the regulation of blood pressure. Proc Natl Acad Sci U S A. 1989 May;86(9):3375-8.
56. Huang PL. Lessons learned from nitric oxide synthase knockout animals. Semin Perinatol. 2000 Feb;24(1):87-90.
57. Flammer AJ, Luscher TF. Three decades of endothelium research: from the detection of nitric oxide to the everyday implementation of endothelial function measurements in cardiovascular diseases. Swiss Med Wkly. 2010;140:w13122.
58. Villalobo A. Nitric oxide and cell proliferation. FEBS J. 2006 Jun;273(11):2329-44.
59. Radomski MW, Palmer RM, Moncada S. Endogenous nitric oxide inhibits human platelet adhesion to vascular endothelium. Lancet. 1987 Nov 7;2(8567):1057-8.
60. Furlong B, Henderson AH, Lewis MJ, Smith JA. Endothelium-derived relaxing factor inhibits in vitro platelet aggregation. Br J Pharmacol. 1987 Apr;90(4):687-92.

61. Radomski MW, Palmer RM, Moncada S. Characterization of the L-arginine:nitric oxide pathway in human platelets. Br J Pharmacol. 1990 Oct;101(2):325-8.
62. Radomski MW, Palmer RM, Moncada S. An L-arginine/nitric oxide pathway present in human platelets regulates aggregation. Proc Natl Acad Sci U S A. 1990 Jul;87(13):5193-7.
63. Malinski T, Taha Z, Grunfeld S, Patton S, Kapturczak M, Tomboulian P. Diffusion of nitric oxide in the aorta wall monitored in situ by porphyrinic microsensors. Biochem Biophys Res Commun. 1993 Jun 30;193(3):1076-82.
64. Gkaliagkousi E, Ritter J, Ferro A. Platelet-derived nitric oxide signaling and regulation. Circ Res. 2007 Sep 28;101(7):654-62.
65. Irwin C, Roberts W, Naseem KM. Nitric oxide inhibits platelet adhesion to collagen through cGMP-dependent and independent mechanisms: the potential role for S-nitrosylation. Platelets. 2009 Nov;20(7):478-86.
66. Ziolo MT, Kohr MJ, Wang H. Nitric oxide signaling and the regulation of myocardial function. J Mol Cell Cardiol. 2008 Nov;45(5):625-32.
67. Chen Y, Traverse JH, Du R, Hou M, Bache RJ. Nitric oxide modulates myocardial oxygen consumption in the failing heart. Circulation. 2002 Jul 9;106(2):273-9.
68. Rastaldo R, Pagliaro P, Cappello S, Penna C, Mancardi D, Westerhof N, et al. Nitric oxide and cardiac function. Life Sci. 2007 Aug 16;81(10):779-93.
69. Kojda G, Kottenberg K, Nix P, Schluter KD, Piper HM, Noack E. Low increase in cGMP induced by organic nitrates and nitrovasodilators improves contractile response of rat ventricular myocytes. Circ Res. 1996 Jan;78(1):91-101.
70. Mohan P, Brutsaert DL, Paulus WJ, Sys SU. Myocardial contractile response to nitric oxide and cGMP. Circulation. 1996 Mar 15;93(6):1223-9.
71. Pagliaro P, Dalla Valle R, Gattullo D, Merletti A, Marsh NA. The heart rate after inhibition of nitric oxide release in the anaesthetized dog. Gen Pharmacol. 1996 Jun;27(4):695-9.
72. Barouch LA, Harrison RW, Skaf MW, Rosas GO, Cappola TP, Kobeissi ZA, et al. Nitric oxide regulates the heart by spatial confinement of nitric oxide synthase isoforms. Nature. 2002 Mar 21;416(6878):337-9.
73. Shinke T, Takaoka H, Takeuchi M, Hata K, Kawai H, Okubo H, et al. [Nitric oxide spares myocardial oxygen consumption through attenuation of contractile response to beta-adrenergic stimulation in patients with idiopathic dilated cardiomyopathy]. J Cardiol. 2001 Feb;37(2):119-20.
74. Recchia FA, McConnell PI, Bernstein RD, Vogel TR, Xu X, Hintze TH. Reduced nitric oxide production and altered myocardial metabolism during the decompensation of pacing-induced heart failure in the conscious dog. Circ Res. 1998 Nov 16;83(10):969-79.
75. Ferreira-Melo SE, Yugar-Toledo JC, Coelho OR, De Luca IM, Tanus-Santos JE, Hyslop S, et al. Sildenafil reduces cardiovascular remodeling associated with hypertensive cardiomyopathy in NOS inhibitor-treated rats. Eur J Pharmacol. 2006 Aug 7;542(1-3):141-7.
76. Scherrer-Crosbie M, Ullrich R, Bloch KD, Nakajima H, Nasseri B, Aretz HT, et al. Endothelial nitric oxide synthase limits left ventricular remodeling after myocardial infarction in mice. Circulation. 2001 Sep 11;104(11):1286-91.
77. Dawson D, Lygate CA, Zhang MH, Hulbert K, Neubauer S, Casadei B. nNOS gene deletion exacerbates pathological left ventricular remodeling and functional deterioration after myocardial infarction. Circulation. 2005 Dec 13;112(24):3729-37.
78. Nisoli E, Clementi E, Paolucci C, Cozzi V, Tonello C, Sciorati C, et al. Mitochondrial biogenesis in mammals: the role of endogenous nitric oxide. Science. 2003 Feb 7;299(5608):896-9.

79. Nisoli E, Falcone S, Tonello C, Cozzi V, Palomba L, Fiorani M, et al. Mitochondrial biogenesis by NO yields functionally active mitochondria in mammals. Proc Natl Acad Sci U S A. 2004 Nov 23;101(47):16507-12.
80. Finocchietto PV, Franco MC, Holod S, Gonzalez AS, Converso DP, Antico Arciuch VG, et al. Mitochondrial nitric oxide synthase: a masterpiece of metabolic adaptation, cell growth, transformation, and death. Exp Biol Med (Maywood). 2009 Sep;234(9):1020-8.
81. Smeitink J, van den Heuvel L, DiMauro S. The genetics and pathology of oxidative phosphorylation. Nat Rev Genet. 2001 May;2(5):342-52.
82. Elston T, Wang H, Oster G. Energy transduction in ATP synthase. Nature. 1998 Jan 29;391(6666):510-3.
83. Schon EA, DiMauro S, Hirano M, Gilkerson RW. Therapeutic prospects for mitochondrial disease. Trends Mol Med. 2010 Jun;16(6):268-76.
84. Brown GC, Cooper CE. Nanomolar concentrations of nitric oxide reversibly inhibit synaptosomal respiration by competing with oxygen at cytochrome oxidase. FEBS Lett. 1994 Dec 19;356(2-3):295-8.
85. Shen W, Xu X, Ochoa M, Zhao G, Wolin MS, Hintze TH. Role of nitric oxide in the regulation of oxygen consumption in conscious dogs. Circ Res. 1994 Dec;75(6):1086-95.
86. Heinonen I, Saltin B, Kemppainen J, Sipila HT, Oikonen V, Nuutila P, et al. Skeletal muscle blood flow and oxygen uptake at rest and during exercise in humans: a pet study with nitric oxide and cyclooxygenase inhibition. Am J Physiol Heart Circ Physiol. 2011 Apr;300(4):H1510-7.
87. Antunes F, Boveris A, Cadenas E. On the mechanism and biology of cytochrome oxidase inhibition by nitric oxide. Proc Natl Acad Sci U S A. 2004 Nov 30;101(48):16774-9.
88. Erusalimsky JD, Moncada S. Nitric oxide and mitochondrial signaling: from physiology to pathophysiology. Arterioscler Thromb Vasc Biol. 2007 Dec;27(12):2524-31.
89. Sarkar S, Korolchuk VI, Renna M, Imarisio S, Fleming A, Williams A, et al. Complex inhibitory effects of nitric oxide on autophagy. Mol Cell. 2011 Jul 8;43(1):19-32.
90. Feelisch M. [Nitrogen monoxide (NO)--the active principle of organic nitrates]. Schweiz Rundsch Med Prax. 1993 Oct 19;82(42):1167-71.
91. Habib S, Ali A. Biochemistry of nitric oxide. Indian J Clin Biochem. 2011 Jan;26(1):3-17.
92. Rakhit RD, Mojet MH, Marber MS, Duchen MR. Mitochondria as targets for nitric oxide-induced protection during simulated ischemia and reoxygenation in isolated neonatal cardiomyocytes. Circulation. 2001 May 29;103(21):2617-23.
93. Crouser ED. Mitochondrial dysfunction in septic shock and multiple organ dysfunction syndrome. Mitochondrion. 2004 Sep;4(5-6):729-41.
94. Jennings RB, Sommers HM, Smyth GA, Flack HA, Linn H. Myocardial necrosis induced by temporary occlusion of a coronary artery in the dog. Arch Pathol. 1960 Jul;70:68-78.
95. Baines CP. How and when do myocytes die during ischemia and reperfusion: the late phase. J Cardiovasc Pharmacol Ther. 2011 Sep-Dec;16(3-4):239-43.
96. Bolli R. Cardioprotective function of inducible nitric oxide synthase and role of nitric oxide in myocardial ischemia and preconditioning: an overview of a decade of research. J Mol Cell Cardiol. 2001 Nov;33(11):1897-918.
97. Jones SP, Bolli R. The ubiquitous role of nitric oxide in cardioprotection. J Mol Cell Cardiol. 2006 Jan;40(1):16-23.
98. Vegh A, Szekeres L, Parratt J. Preconditioning of the ischaemic myocardium; involvement of the L-arginine nitric oxide pathway. Br J Pharmacol. 1992 Nov;107(3):648-52.

99. Obata T. Adenosine production and its interaction with protection of ischemic and reperfusion injury of the myocardium. Life Sci. 2002 Sep 20;71(18):2083-103.
100. Wang Y, Hirai K, Ashraf M. Activation of mitochondrial ATP-sensitive K(+) channel for cardiac protection against ischemic injury is dependent on protein kinase C activity. Circ Res. 1999 Oct 15;85(8):731-41.
101. Javadov SA, Clarke S, Das M, Griffiths EJ, Lim KH, Halestrap AP. Ischaemic preconditioning inhibits opening of mitochondrial permeability transition pores in the reperfused rat heart. J Physiol. 2003 Jun 1;549(Pt 2):513-24.
102. Hausenloy DJ, Yellon DM. New directions for protecting the heart against ischaemia-reperfusion injury: targeting the Reperfusion Injury Salvage Kinase (RISK)-pathway. Cardiovasc Res. 2004 Feb 15;61(3):448-60.
103. Yellon DM, Downey JM. Preconditioning the myocardium: from cellular physiology to clinical cardiology. Physiol Rev. 2003 Oct;83(4):1113-51.
104. Tsang A, Hausenloy DJ, Mocanu MM, Yellon DM. Postconditioning: a form of "modified reperfusion" protects the myocardium by activating the phosphatidylinositol 3-kinase-Akt pathway. Circ Res. 2004 Aug 6;95(3):230-2.
105. Leaf CD, Wishnok JS, Tannenbaum SR. L-arginine is a precursor for nitrate biosynthesis in humans. Biochem Biophys Res Commun. 1989 Sep 15;163(2):1032-7.
106. Ignarro LJ, Fukuto JM, Griscavage JM, Rogers NE, Byrns RE. Oxidation of nitric oxide in aqueous solution to nitrite but not nitrate: comparison with enzymatically formed nitric oxide from L-arginine. Proc Natl Acad Sci U S A. 1993 Sep 1;90(17):8103-7.
107. Rhodes P, Leone AM, Francis PL, Struthers AD, Moncada S, Rhodes PM. The L-arginine:nitric oxide pathway is the major source of plasma nitrite in fasted humans. Biochem Biophys Res Commun. 1995 Apr 17;209(2):590-6.
108. Eich RF, Li T, Lemon DD, Doherty DH, Curry SR, Aitken JF, et al. Mechanism of NO-induced oxidation of myoglobin and hemoglobin. Biochemistry. 1996 Jun 4;35(22):6976-83.
109. Shiva S, Wang X, Ringwood LA, Xu X, Yuditskaya S, Annavajjhala V, et al. Ceruloplasmin is a NO oxidase and nitrite synthase that determines endocrine NO homeostasis. Nat Chem Biol. 2006 Sep;2(9):486-93.
110. Shi Y, Li HQ, Shen CK, Wang JH, Qin SW, Liu R, et al. Plasma nitric oxide levels in newborn infants with sepsis. J Pediatr. 1993 Sep;123(3):435-8.
111. Kirkeboen KA, Strand OA. The role of nitric oxide in sepsis--an overview. Acta Anaesthesiol Scand. 1999 Mar;43(3):275-88.
112. Pannala AS, Mani AR, Spencer JP, Skinner V, Bruckdorfer KR, Moore KP, et al. The effect of dietary nitrate on salivary, plasma, and urinary nitrate metabolism in humans. Free Radic Biol Med. 2003 Mar 1;34(5):576-84.
113. Qin L, Liu X, Sun Q, Fan Z, Xia D, Ding G, et al. Sialin (SLC17A5) functions as a nitrate transporter in the plasma membrane. Proc Natl Acad Sci U S A. 2012 Aug 14;109(33):13434-9.
114. Spiegelhalder B, Eisenbrand G, Preussmann R. Influence of dietary nitrate on nitrite content of human saliva: possible relevance to in vivo formation of N-nitroso compounds. Food Cosmet Toxicol. 1976 Dec;14(6):545-8.
115. van Maanen JM, van Geel AA, Kleinjans JC. Modulation of nitrate-nitrite conversion in the oral cavity. Cancer Detect Prev. 1996;20(6):590-6.
116. Lundberg JO, Weitzberg E, Cole JA, Benjamin N. Nitrate, bacteria and human health. Nat Rev Microbiol. 2004 Jul;2(7):593-602.
117. Lundberg JO, Weitzberg E, Gladwin MT. The nitrate-nitrite-nitric oxide pathway in physiology and therapeutics. Nat Rev Drug Discov. 2008 Feb;7(2):156-67.

118. Gladwin MT. Haldane, hot dogs, halitosis, and hypoxic vasodilation: the emerging biology of the nitrite anion. J Clin Invest. 2004 Jan;113(1):19-21.
119. Rassaf T, Feelisch M, Kelm M. Circulating NO pool: assessment of nitrite and nitroso species in blood and tissues. Free Radic Biol Med. 2004 Feb 15;36(4):413-22.
120. Rodriguez J, Maloney RE, Rassaf T, Bryan NS, Feelisch M. Chemical nature of nitric oxide storage forms in rat vascular tissue. Proc Natl Acad Sci U S A. 2003 Jan 7;100(1):336-41.
121. Lundberg JO, Feelisch M, Bjorne H, Jansson EA, Weitzberg E. Cardioprotective effects of vegetables: is nitrate the answer? Nitric Oxide. 2006 Dec;15(4):359-62.
122. Kim HJ, Conca KR. Determination of nitrite in cured meats by ion-exclusion chromatography with electrochemical detection. J Assoc Off Anal Chem. 1990 Jul-Aug;73(4):561-4.
123. Kevil CG, Kolluru GK, Pattillo CB, Giordano T. Inorganic nitrite therapy: historical perspective and future directions. Free Radic Biol Med. 2011 Aug 1;51(3):576-93.
124. Benjamin N, O'Driscoll F, Dougall H, Duncan C, Smith L, Golden M, et al. Stomach NO synthesis. Nature. 1994 Apr 7;368(6471):502.
125. Gago B, Lundberg JO, Barbosa RM, Laranjinha J. Red wine-dependent reduction of nitrite to nitric oxide in the stomach. Free Radic Biol Med. 2007 Nov 1;43(9):1233-42.
126. Weitzberg E, Lundberg JO. Nonenzymatic nitric oxide production in humans. Nitric Oxide. 1998;2(1):1-7.
127. Lundberg JO, Weitzberg E, Lundberg JM, Alving K. Intragastric nitric oxide production in humans: measurements in expelled air. Gut. 1994 Nov;35(11):1543-6.
128. Zweier JL, Li H, Samouilov A, Liu X. Mechanisms of nitrite reduction to nitric oxide in the heart and vessel wall. Nitric Oxide. 2010 Feb 15;22(2):83-90.
129. Ignarro LJ, Lippton H, Edwards JC, Baricos WH, Hyman AL, Kadowitz PJ, et al. Mechanism of vascular smooth muscle relaxation by organic nitrates, nitrites, nitroprusside and nitric oxide: evidence for the involvement of S-nitrosothiols as active intermediates. J Pharmacol Exp Ther. 1981 Sep;218(3):739-49.
130. Matsunaga K, Furchgott RF. Interactions of light and sodium nitrite in producing relaxation of rabbit aorta. J Pharmacol Exp Ther. 1989 Feb;248(2):687-95.
131. Demoncheaux EA, Higenbottam TW, Foster PJ, Borland CD, Smith AP, Marriott HM, et al. Circulating nitrite anions are a directly acting vasodilator and are donors for nitric oxide. Clin Sci (Lond). 2002 Jan;102(1):77-83.
132. Ferrari R, Cargnoni A, Bernocchi P, Pasini E, Curello S, Ceconi C, et al. Metabolic adaptation during a sequence of no-flow and low-flow ischemia. A possible trigger for hibernation. Circulation. 1996 Nov 15;94(10):2587-96.
133. Samouilov A, Kuppusamy P, Zweier JL. Evaluation of the magnitude and rate of nitric oxide production from nitrite in biological systems. Arch Biochem Biophys. 1998 Sep 1;357(1):1-7.
134. Haldane J. The Red Colour of Salted Meat. J Hyg (Lond). 1901 Jan;1(1):115-22.
135. brooks J. The Action of Nitrite on Haemoglobin in the Absence of Oxygen. Proceedings of the Royal Society of Medicine. 1937;123:368-82.
136. Doyle MP, LePoire DM, Pickering RA. Oxidation of hemoglobin and myoglobin by alkyl nitrites inhibition by oxygen. J Biol Chem. 1981 Dec 10;256(23):12399-404.
137. Gladwin MT, Schechter AN, Kim-Shapiro DB, Patel RP, Hogg N, Shiva S, et al. The emerging biology of the nitrite anion. Nat Chem Biol. 2005 Nov;1(6):308-14.
138. Grubina R, Huang Z, Shiva S, Joshi MS, Azarov I, Basu S, et al. Concerted nitric oxide formation and release from the simultaneous reactions of nitrite with deoxy- and oxyhemoglobin. J Biol Chem. 2007 Apr 27;282(17):12916-27.

139. Gladwin MT, Kim-Shapiro DB. The functional nitrite reductase activity of the heme-globins. Blood. 2008 Oct 1;112(7):2636-47.
140. Blood AB, Power GG. In vitro and in vivo kinetic handling of nitrite in blood: effects of varying hemoglobin oxygen saturation. Am J Physiol Heart Circ Physiol. 2007 Sep;293(3):H1508-17.
141. Shiva S, Huang Z, Grubina R, Sun J, Ringwood LA, MacArthur PH, et al. Deoxymyoglobin is a nitrite reductase that generates nitric oxide and regulates mitochondrial respiration. Circ Res. 2007 Mar 16;100(5):654-61.
142. Wittenberg JB, Wittenberg BA. Myoglobin function reassessed. J Exp Biol. 2003 Jun;206(Pt 12):2011-20.
143. Bekedam MA, van Beek-Harmsen BJ, van Mechelen W, Boonstra A, van der Laarse WJ. Myoglobin concentration in skeletal muscle fibers of chronic heart failure patients. J Appl Physiol (1985). 2009 Oct;107(4):1138-43.
144. Huang Z, Shiva S, Kim-Shapiro DB, Patel RP, Ringwood LA, Irby CE, et al. Enzymatic function of hemoglobin as a nitrite reductase that produces NO under allosteric control. J Clin Invest. 2005 Aug;115(8):2099-107.
145. Dejam A, Hunter CJ, Pelletier MM, Hsu LL, Machado RF, Shiva S, et al. Erythrocytes are the major intravascular storage sites of nitrite in human blood. Blood. 2005 Jul 15;106(2):734-9.
146. Ormerod JO, Ashrafian H, Maher AR, Arif S, Steeples V, Born GV, et al. The role of vascular myoglobin in nitrite-mediated blood vessel relaxation. Cardiovasc Res. [Research Support, Non-U.S. Gov't]. 2011 Feb 15;89(3):560-5.
147. Totzeck M, Hendgen-Cotta UB, Luedike P, Berenbrink M, Klare JP, Steinhoff HJ, et al. Nitrite regulates hypoxic vasodilation via myoglobin-dependent nitric oxide generation. Circulation. 2012 Jul 17;126(3):325-34.
148. Burmester T, Hankeln T. What is the function of neuroglobin? J Exp Biol. 2009 May;212(Pt 10):1423-8.
149. Tiso M, Tejero J, Basu S, Azarov I, Wang X, Simplaceanu V, et al. Human neuroglobin functions as a redox-regulated nitrite reductase. J Biol Chem. 2011 May 20;286(20):18277-89.
150. Burmester T, Ebner B, Weich B, Hankeln T. Cytoglobin: a novel globin type ubiquitously expressed in vertebrate tissues. Mol Biol Evol. 2002 Apr;19(4):416-21.
151. Li H, Hemann C, Abdelghany TM, El-Mahdy MA, Zweier JL. Characterization of the mechanism and magnitude of cytoglobin-mediated nitrite reduction and nitric oxide generation under anaerobic conditions. J Biol Chem. 2012 Oct 19;287(43):36623-33.
152. Kooij A, Bosch KS, Frederiks WM, Van Noorden CJ. High levels of xanthine oxidoreductase in rat endothelial, epithelial and connective tissue cells. A relation between localization and function? Virchows Arch B Cell Pathol Incl Mol Pathol. 1992;62(3):143-50.
153. Dixon M, Thurlow S. Studies on Xanthine Oxidase: The Reduction of Nitrates. Biochem J. 1924;18(5):989-92.
154. Westerfeld WW, Richert DA, Higgins ES. Further studies with xanthine oxidase inhibitors. J Biol Chem. 1959 Jul;234(7):1897-900.
155. Sergeev NS, Ananiadi LI, L'Vov N P, Kretovich WL. The nitrate reductase activity of milk xanthine oxidase. J Appl Biochem. 1985 Apr;7(2):86-92.
156. Millar TM, Stevens CR, Benjamin N, Eisenthal R, Harrison R, Blake DR. Xanthine oxidoreductase catalyses the reduction of nitrates and nitrite to nitric oxide under hypoxic conditions. FEBS Lett. 1998 May 8;427(2):225-8.

157. Godber BL, Doel JJ, Sapkota GP, Blake DR, Stevens CR, Eisenthal R, et al. Reduction of nitrite to nitric oxide catalyzed by xanthine oxidoreductase. J Biol Chem. 2000 Mar 17;275(11):7757-63.
158. Li H, Samouilov A, Liu X, Zweier JL. Characterization of the magnitude and kinetics of xanthine oxidase-catalyzed nitrite reduction. Evaluation of its role in nitric oxide generation in anoxic tissues. J Biol Chem. 2001 Jul 6;276(27):24482-9.
159. Tripatara P, Patel NS, Webb A, Rathod K, Lecomte FM, Mazzon E, et al. Nitrite-derived nitric oxide protects the rat kidney against ischemia/reperfusion injury in vivo: role for xanthine oxidoreductase. J Am Soc Nephrol. 2007 Feb;18(2):570-80.
160. Webb AJ, Milsom AB, Rathod KS, Chu WL, Qureshi S, Lovell MJ, et al. Mechanisms underlying erythrocyte and endothelial nitrite reduction to nitric oxide in hypoxia: role for xanthine oxidoreductase and endothelial nitric oxide synthase. Circ Res. 2008 Oct 24;103(9):957-64.
161. Dejam A, Hunter CJ, Tremonti C, Pluta RM, Hon YY, Grimes G, et al. Nitrite infusion in humans and nonhuman primates: endocrine effects, pharmacokinetics, and tolerance formation. Circulation. 2007 Oct 16;116(16):1821-31.
162. Ghosh SM, Kapil V, Fuentes-Calvo I, Bubb KJ, Pearl V, Milsom AB, et al. Enhanced vasodilator activity of nitrite in hypertension: critical role for erythrocytic xanthine oxidoreductase and translational potential. Hypertension. [Randomized Controlled Trial Research Support, Non-U.S. Gov't]. 2013 May;61(5):1091-102.
163. Calzi ML, Raviolo C, Ghibaudi E, de Gioia L, Salmona M, Cazzaniga G, et al. Purification, cDNA cloning, and tissue distribution of bovine liver aldehyde oxidase. J Biol Chem. 1995 Dec 29;270(52):31037-45.
164. Li H, Cui H, Kundu TK, Alzawahra W, Zweier JL. Nitric oxide production from nitrite occurs primarily in tissues not in the blood: critical role of xanthine oxidase and aldehyde oxidase. J Biol Chem. 2008 Jun 27;283(26):17855-63.
165. Li H, Kundu TK, Zweier JL. Characterization of the magnitude and mechanism of aldehyde oxidase-mediated nitric oxide production from nitrite. J Biol Chem. 2009 Dec 4;284(49):33850-8.
166. Vanin AF, Bevers LM, Slama-Schwok A, van Faassen EE. Nitric oxide synthase reduces nitrite to NO under anoxia. Cell Mol Life Sci. 2007 Jan;64(1):96-103.
167. Milsom AB, Patel NS, Mazzon E, Tripatara P, Storey A, Mota-Filipe H, et al. Role for endothelial nitric oxide synthase in nitrite-induced protection against renal ischemia-reperfusion injury in mice. Nitric Oxide. 2010 Feb 15;22(2):141-8.
168. Sydow K, Daiber A, Oelze M, Chen Z, August M, Wendt M, et al. Central role of mitochondrial aldehyde dehydrogenase and reactive oxygen species in nitroglycerin tolerance and cross-tolerance. J Clin Invest. 2004 Feb;113(3):482-9.
169. de la Lande IS, Stepien JM, Philpott AC, Hughes PA, Stafford I, Horowitz JD. Aldehyde dehydrogenase, nitric oxide synthase and superoxide in ex vivo nitrate tolerance in rat aorta. Eur J Pharmacol. 2004 Aug 2;496(1-3):141-9.
170. Kollau A, Hofer A, Russwurm M, Koesling D, Keung WM, Schmidt K, et al. Contribution of aldehyde dehydrogenase to mitochondrial bioactivation of nitroglycerin: evidence for the activation of purified soluble guanylate cyclase through direct formation of nitric oxide. Biochem J. 2005 Feb 1;385(Pt 3):769-77.
171. Mackenzie IS, Maki-Petaja KM, McEniery CM, Bao YP, Wallace SM, Cheriyan J, et al. Aldehyde dehydrogenase 2 plays a role in the bioactivation of nitroglycerin in humans. Arterioscler Thromb Vasc Biol. 2005 Sep;25(9):1891-5.
172. Mayer B, Beretta M. The enigma of nitroglycerin bioactivation and nitrate tolerance: news, views and troubles. Br J Pharmacol. 2008 Sep;155(2):170-84.

173. Kozlov AV, Staniek K, Nohl H. Nitrite reductase activity is a novel function of mammalian mitochondria. FEBS Lett. 1999 Jul 2;454(1-2):127-30.
174. Castello PR, David PS, McClure T, Crook Z, Poyton RO. Mitochondrial cytochrome oxidase produces nitric oxide under hypoxic conditions: implications for oxygen sensing and hypoxic signaling in eukaryotes. Cell Metab. 2006 Apr;3(4):277-87.
175. Philpott A. Dissociation of Aldehyde Dehydrogenase Activity from Nitrate Effect, Bioconversion, and Tolerance in Humans. The Journal of the American College of Cardiology; 06/03/2007: The Journal of the American College of Cardiology; 2007. p. 420A.
176. Perlman DH, Bauer SM, Ashrafian H, Bryan NS, Garcia-Saura MF, Lim CC, et al. Mechanistic insights into nitrite-induced cardioprotection using an integrated metabolomic/proteomic approach. Circ Res. 2009 Mar 27;104(6):796-804.
177. Nohl H, Staniek K, Kozlov AV. The existence and significance of a mitochondrial nitrite reductase. Redox Rep. [Review]. 2005;10(6):281-6.
178. Poyton RO, Ball KA, Castello PR. Mitochondrial generation of free radicals and hypoxic signaling. Trends Endocrinol Metab. 2009 Sep;20(7):332-40.
179. Heistad DD, Abboud FM. Dickinson W. Richards Lecture: Circulatory adjustments to hypoxia. Circulation. 1980 Mar;61(3):463-70.
180. Gui Y, Zheng XL, Zheng J, Walsh MP. Inhibition of rat aortic smooth muscle contraction by 2-methoxyestradiol. Am J Physiol Heart Circ Physiol. 2008 Nov;295(5):H1935-42.
181. Ouchi N, Oshima Y, Ohashi K, Higuchi A, Ikegami C, Izumiya Y, et al. Follistatin-like 1, a secreted muscle protein, promotes endothelial cell function and revascularization in ischemic tissue through a nitric-oxide synthase-dependent mechanism. J Biol Chem. 2008 Nov 21;283(47):32802-11.
182. Stamler JS, Jia L, Eu JP, McMahon TJ, Demchenko IT, Bonaventura J, et al. Blood flow regulation by S-nitrosohemoglobin in the physiological oxygen gradient. Science. 1997 Jun 27;276(5321):2034-7.
183. Huang KT, Keszler A, Patel N, Patel RP, Gladwin MT, Kim-Shapiro DB, et al. The reaction between nitrite and deoxyhemoglobin. Reassessment of reaction kinetics and stoichiometry. J Biol Chem. 2005 Sep 2;280(35):31126-31.
184. Gladwin MT, Schechter AN. NO contest: nitrite versus S-nitroso-hemoglobin. Circ Res. 2004 Apr 16;94(7):851-5.
185. Walsh S, Borgese F, Gabillat N, Unwin R, Guizouarn H. Cation transport activity of anion exchanger 1 mutations found in inherited distal renal tubular acidosis. Am J Physiol Renal Physiol. 2008 Aug;295(2):F343-50.
186. Kock K, Grube M, Jedlitschky G, Oevermann L, Siegmund W, Ritter CA, et al. Expression of adenosine triphosphate-binding cassette (ABC) drug transporters in peripheral blood cells: relevance for physiology and pharmacotherapy. Clin Pharmacokinet. 2007;46(6):449-70.
187. Rassaf T, Bryan NS, Maloney RE, Specian V, Kelm M, Kalyanaraman B, et al. NO adducts in mammalian red blood cells: too much or too little? Nat Med. 2003 May;9(5):481-2; author reply 2-3.
188. Gladwin MT, Wang X, Reiter CD, Yang BK, Vivas EX, Bonaventura C, et al. S-Nitrosohemoglobin is unstable in the reductive erythrocyte environment and lacks O2/NO-linked allosteric function. J Biol Chem. 2002 Aug 2;277(31):27818-28.
189. Ng ES, Jourd'heuil D, McCord JM, Hernandez D, Yasui M, Knight D, et al. Enhanced S-nitroso-albumin formation from inhaled NO during ischemia/reperfusion. Circ Res. 2004 Mar 5;94(4):559-65.

190. Wiley KE, Davenport AP. Comparison of vasodilators in human internal mammary artery: ghrelin is a potent physiological antagonist of endothelin-1. Br J Pharmacol. 2002 Aug;136(8):1146-52.
191. Fye WB. T. Lauder Brunton and amyl nitrite: a Victorian vasodilator. Circulation. 1986 Aug;74(2):222-9.
192. Weiss S, Wilkins RW, Haynes FW. The Nature of Circulatory Collapse Induced by Sodium Nitrite. J Clin Invest. 1937 Jan;16(1):73-84.
193. Furchgott RF, Bhadrakom S. Reactions of strips of rabbit aorta to epinephrine, isopropylarterenol, sodium nitrite and other drugs. J Pharmacol Exp Ther. 1953 Jun;108(2):129-43.
194. Gladwin MT, Shelhamer JH, Schechter AN, Pease-Fye ME, Waclawiw MA, Panza JA, et al. Role of circulating nitrite and S-nitrosohemoglobin in the regulation of regional blood flow in humans. Proc Natl Acad Sci U S A. 2000 Oct 10;97(21):11482-7.
195. Lauer T, Preik M, Rassaf T, Strauer BE, Deussen A, Feelisch M, et al. Plasma nitrite rather than nitrate reflects regional endothelial nitric oxide synthase activity but lacks intrinsic vasodilator action. Proc Natl Acad Sci U S A. 2001 Oct 23;98(22):12814-9.
196. Nagababu E, Ramasamy S, Abernethy DR, Rifkind JM. Active nitric oxide produced in the red cell under hypoxic conditions by deoxyhemoglobin-mediated nitrite reduction. J Biol Chem. 2003 Nov 21;278(47):46349-56.
197. Isbell TS, Gladwin MT, Patel RP. Hemoglobin oxygen fractional saturation regulates nitrite-dependent vasodilation of aortic ring bioassays. Am J Physiol Heart Circ Physiol. 2007 Oct;293(4):H2565-72.
198. Ingram TE, Pinder AG, Milsom AB, Rogers SC, Thomas DE, James PE. Blood vessel specific vaso-activity to nitrite under normoxic and hypoxic conditions. Adv Exp Med Biol. 2009;645:21-5.
199. Ingram TE, Pinder AG, Bailey DM, Fraser AG, James PE. Low-dose sodium nitrite vasodilates hypoxic human pulmonary vasculature by a means that is not dependent on a simultaneous elevation in plasma nitrite. Am J Physiol Heart Circ Physiol. [Controlled Clinical Trial
Research Support, Non-U.S. Gov't]. 2010 Feb;298(2):H331-9.
200. Rifkind JM, Salgado MT, Cao Z. Regulation of oxygen delivery by the reaction of nitrite with RBCs under hypoxic conditions. Adv Exp Med Biol. 2012;737:183-9.
201. Jensen FB, Rohde S. Comparative analysis of nitrite uptake and hemoglobin-nitrite reactions in erythrocytes: sorting out uptake mechanisms and oxygenation dependencies. Am J Physiol Regul Integr Comp Physiol. 2010 Apr;298(4):R972-82.
202. Samouilov A, Woldman YY, Zweier JL, Khramtsov VV. Magnetic resonance study of the transmembrane nitrite diffusion. Nitric Oxide. 2007 May;16(3):362-70.
203. Kubli DA, Gustafsson AB. Mitochondria and mitophagy: the yin and yang of cell death control. Circ Res. 2012 Oct 12;111(9):1208-21.
204. Allen DG, Orchard CH. Myocardial contractile function during ischemia and hypoxia. Circ Res. 1987 Feb;60(2):153-68.
205. Marban E, Koretsune Y, Kusuoka H. Disruption of intracellular Ca2+ homeostasis in hearts reperfused after prolonged episodes of ischemia. Ann N Y Acad Sci. 1994 Jun 17;723:38-50.
206. Pike MM, Luo CS, Clark MD, Kirk KA, Kitakaze M, Madden MC, et al. NMR measurements of Na+ and cellular energy in ischemic rat heart: role of Na(+)-H+ exchange. Am J Physiol. 1993 Dec;265(6 Pt 2):H2017-26.
207. Duff HJ. Clinical and in vivo antiarrhythmic potential of sodium-hydrogen exchange inhibitors. Cardiovasc Res. 1995 Feb;29(2):189-93.

208. Marban E, Kitakaze M, Kusuoka H, Porterfield JK, Yue DT, Chacko VP. Intracellular free calcium concentration measured with 19F NMR spectroscopy in intact ferret hearts. Proc Natl Acad Sci U S A. 1987 Aug;84(16):6005-9.
209. Hausenloy DJ, Yellon DM. The evolving story of "conditioning" to protect against acute myocardial ischaemia-reperfusion injury. Heart. 2007 Jun;93(6):649-51.
210. Frank A, Bonney M, Bonney S, Weitzel L, Koeppen M, Eckle T. Myocardial ischemia reperfusion injury: from basic science to clinical bedside. Semin Cardiothorac Vasc Anesth. 2012 Sep;16(3):123-32.
211. Heusch G. Cardioprotection: chances and challenges of its translation to the clinic. Lancet. 2013 Jan 12;381(9861):166-75.
212. Zweier JL, Kuppusamy P, Lutty GA. Measurement of endothelial cell free radical generation: evidence for a central mechanism of free radical injury in postischemic tissues. Proc Natl Acad Sci U S A. 1988 Jun;85(11):4046-50.
213. Park JL, Lucchesi BR. Mechanisms of myocardial reperfusion injury. Ann Thorac Surg. 1999 Nov;68(5):1905-12.
214. Hearse DJ, Humphrey SM, Chain EB. Abrupt reoxygenation of the anoxic potassium-arrested perfused rat heart: a study of myocardial enzyme release. J Mol Cell Cardiol. 1973 Aug;5(4):395-407.
215. Andreadou I, Iliodromitis EK, Farmakis D, Kremastinos DT. To prevent, protect and save the ischemic heart: antioxidants revisited. Expert Opin Ther Targets. 2009 Aug;13(8):945-56.
216. Zweier JL, Talukder MA. The role of oxidants and free radicals in reperfusion injury. Cardiovasc Res. 2006 May 1;70(2):181-90.
217. Di Lisa F, Canton M, Menabo R, Kaludercic N, Bernardi P. Mitochondria and cardioprotection. Heart Fail Rev. 2007 Dec;12(3-4):249-60.
218. Zorov DB, Filburn CR, Klotz LO, Zweier JL, Sollott SJ. Reactive oxygen species (ROS)-induced ROS release: a new phenomenon accompanying induction of the mitochondrial permeability transition in cardiac myocytes. J Exp Med. 2000 Oct 2;192(7):1001-14.
219. Roberts MJ, Young IS, Trouton TG, Trimble ER, Khan MM, Webb SW, et al. Transient release of lipid peroxides after coronary artery balloon angioplasty. Lancet. 1990 Jul 21;336(8708):143-5.
220. Kim KB, Chung HH, Kim MS, Rho JR. Changes in the antioxidative defensive system during open heart operations in humans. Ann Thorac Surg. 1994 Jul;58(1):170-5.
221. Bond JM, Harper IS, Chacon E, Reece JM, Herman B, Lemasters JJ. The pH paradox in the pathophysiology of reperfusion injury to rat neonatal cardiac myocytes. Ann N Y Acad Sci. 1994 Jun 17;723:25-37.
222. Bond JM, Herman B, Lemasters JJ. Protection by acidotic pH against anoxia/reoxygenation injury to rat neonatal cardiac myocytes. Biochem Biophys Res Commun. 1991 Sep 16;179(2):798-803.
223. Lemasters JJ, Bond JM, Chacon E, Harper IS, Kaplan SH, Ohata H, et al. The pH paradox in ischemia-reperfusion injury to cardiac myocytes. EXS. 1996;76:99-114.
224. Shen AC, Jennings RB. Kinetics of calcium accumulation in acute myocardial ischemic injury. Am J Pathol. 1972 Jun;67(3):441-52.
225. Schafer C, Ladilov Y, Inserte J, Schafer M, Haffner S, Garcia-Dorado D, et al. Role of the reverse mode of the Na+/Ca2+ exchanger in reoxygenation-induced cardiomyocyte injury. Cardiovasc Res. 2001 Aug 1;51(2):241-50.

226. Moens AL, Claeys MJ, Timmermans JP, Vrints CJ. Myocardial ischemia/reperfusion-injury, a clinical view on a complex pathophysiological process. Int J Cardiol. 2005 Apr 20;100(2):179-90.
227. Piper HM, Garcia-Dorado D, Ovize M. A fresh look at reperfusion injury. Cardiovasc Res. 1998 May;38(2):291-300.
228. Rodriguez-Sinovas A, Abdallah Y, Piper HM, Garcia-Dorado D. Reperfusion injury as a therapeutic challenge in patients with acute myocardial infarction. Heart Fail Rev. 2007 Dec;12(3-4):207-16.
229. Halestrap AP, Clarke SJ, Javadov SA. Mitochondrial permeability transition pore opening during myocardial reperfusion--a target for cardioprotection. Cardiovasc Res. 2004 Feb 15;61(3):372-85.
230. Halestrap AP, Clarke SJ, Khaliulin I. The role of mitochondria in protection of the heart by preconditioning. Biochim Biophys Acta. 2007 Aug;1767(8):1007-31.
231. Griffiths EJ, Halestrap AP. Mitochondrial non-specific pores remain closed during cardiac ischaemia, but open upon reperfusion. Biochem J. 1995 Apr 1;307 (Pt 1):93-8.
232. Crompton M, Ellinger H, Costi A. Inhibition by cyclosporin A of a Ca2+-dependent pore in heart mitochondria activated by inorganic phosphate and oxidative stress. Biochem J. 1988 Oct 1;255(1):357-60.
233. Bernardi P, Krauskopf A, Basso E, Petronilli V, Blachly-Dyson E, Di Lisa F, et al. The mitochondrial permeability transition from in vitro artifact to disease target. FEBS J. 2006 May;273(10):2077-99.
234. Halestrap AP. What is the mitochondrial permeability transition pore? J Mol Cell Cardiol. 2009 Jun;46(6):821-31.
235. Hausenloy DJ, Maddock HL, Baxter GF, Yellon DM. Inhibiting mitochondrial permeability transition pore opening: a new paradigm for myocardial preconditioning? Cardiovasc Res. 2002 Aug 15;55(3):534-43.
236. Argaud L, Gateau-Roesch O, Raisky O, Loufouat J, Robert D, Ovize M. Postconditioning inhibits mitochondrial permeability transition. Circulation. 2005 Jan 18;111(2):194-7.
237. Kloner RA, Giacomelli F, Alker KJ, Hale SL, Matthews R, Bellows S. Influx of neutrophils into the walls of large epicardial coronary arteries in response to ischemia/reperfusion. Circulation. 1991 Oct;84(4):1758-72.
238. Dreyer WJ, Michael LH, West MS, Smith CW, Rothlein R, Rossen RD, et al. Neutrophil accumulation in ischemic canine myocardium. Insights into time course, distribution, and mechanism of localization during early reperfusion. Circulation. 1991 Jul;84(1):400-11.
239. Davies MJ, Thomas AC, Knapman PA, Hangartner JR. Intramyocardial platelet aggregation in patients with unstable angina suffering sudden ischemic cardiac death. Circulation. 1986 Mar;73(3):418-27.
240. Banz Y, Rieben R. Role of complement and perspectives for intervention in ischemia-reperfusion damage. Ann Med. 2012 May;44(3):205-17.
241. Dai W, Kloner RA. Potential role of renin-angiotensin system blockade for preventing myocardial ischemia/reperfusion injury and remodeling after myocardial infarction. Postgrad Med. 2011 Mar;123(2):49-55.
242. Wang QD, Pernow J, Sjoquist PO, Ryden L. Pharmacological possibilities for protection against myocardial reperfusion injury. Cardiovasc Res. 2002 Jul;55(1):25-37.
243. Braunwald E, Kloner RA. The stunned myocardium: prolonged, postischemic ventricular dysfunction. Circulation. 1982 Dec;66(6):1146-9.

244. Deakin CD, Ambler JJ. Post-shock myocardial stunning: a prospective randomised double-blind comparison of monophasic and biphasic waveforms. Resuscitation. 2006 Mar;68(3):329-33.
245. Heyndrickx GR, Millard RW, McRitchie RJ, Maroko PR, Vatner SF. Regional myocardial functional and electrophysiological alterations after brief coronary artery occlusion in conscious dogs. J Clin Invest. 1975 Oct;56(4):978-85.
246. Jeroudi MO, Cheirif J, Habib G, Bolli R. Prolonged wall motion abnormalities after chest pain at rest in patients with unstable angina: a possible manifestation of myocardial stunning. Am Heart J. 1994 May;127(5):1241-50.
247. Kloner RA, Jennings RB. Consequences of brief ischemia: stunning, preconditioning, and their clinical implications: part 2. Circulation. 2001 Dec 18;104(25):3158-67.
248. Kloner RA, Jennings RB. Consequences of brief ischemia: stunning, preconditioning, and their clinical implications: part 1. Circulation. 2001 Dec 11;104(24):2981-9.
249. Mentzer RM, Jr. Myocardial protection in heart surgery. J Cardiovasc Pharmacol Ther. 2011 Sep-Dec;16(3-4):290-7.
250. Bolli R, Zhu WX, Thornby JI, O'Neill PG, Roberts R. Time course and determinants of recovery of function after reversible ischemia in conscious dogs. Am J Physiol. 1988 Jan;254(1 Pt 2):H102-14.
251. Bax JJ, Visser FC, Poldermans D, Elhendy A, Cornel JH, Boersma E, et al. Time course of functional recovery of stunned and hibernating segments after surgical revascularization. Circulation. 2001 Sep 18;104(12 Suppl 1):I314-8.
252. Ellis SG, Henschke CI, Sandor T, Wynne J, Braunwald E, Kloner RA. Time course of functional and biochemical recovery of myocardium salvaged by reperfusion. J Am Coll Cardiol. 1983 Apr;1(4):1047-55.
253. Usui Y, Chikamori T, Nakajima K, Hida S, Yamashina A, Nishimura T. Prognostic value of post-ischemic stunning as assessed by gated myocardial perfusion single-photon emission computed tomography: a subanalysis of the J-ACCESS study. Circ J. 2010 Aug;74(8):1591-9.
254. Kloner RA, Arimie RB, Kay GL, Cannom D, Matthews R, Bhandari A, et al. Evidence for stunned myocardium in humans: a 2001 update. Coron Artery Dis. 2001 Aug;12(5):349-56.
255. Manning AS, Hearse DJ. Reperfusion-induced arrhythmias: mechanisms and prevention. J Mol Cell Cardiol. 1984 Jun;16(6):497-518.
256. Goldberg S, Greenspon AJ, Urban PL, Muza B, Berger B, Walinsky P, et al. Reperfusion arrhythmia: a marker of restoration of antegrade flow during intracoronary thrombolysis for acute myocardial infarction. Am Heart J. 1983 Jan;105(1):26-32.
257. Bernier M, Manning AS, Hearse DJ. Reperfusion arrhythmias: dose-related protection by anti-free radical interventions. Am J Physiol. 1989 May;256(5 Pt 2):H1344-52.
258. Ibuki C, Hearse DJ, Avkiran M. Mechanisms of antifibrillatory effect of acidic reperfusion: role of perfusate bicarbonate concentration. Am J Physiol. 1993 Mar;264(3 Pt 2):H783-90.
259. Ku DD. Coronary vascular reactivity after acute myocardial ischemia. Science. 1982 Nov 5;218(4572):576-8.
260. Zhao M, He X, Wier WG, Zhang HL, Yu XJ, Zang WJ. Endothelial dysfunction in rat mesenteric artery after regional cardiac ischaemia-reperfusion. Exp Physiol. 2012 Jan;97(1):70-9.
261. Armiger LC, Gavin JB. Changes in the microvasculature of ischemic and infarcted myocardium. Lab Invest. 1975 Jul;33(1):51-6.

262. Viehman GE, Ma XL, Lefer DJ, Lefer AM. Time course of endothelial dysfunction and myocardial injury during coronary arterial occlusion. Am J Physiol. 1991 Sep;261(3 Pt 2):H874-81.
263. Laude K, Richard V, Thuillez C. Coronary endothelial cells: a target of ischemia reperfusion and its treatment? Arch Mal Coeur Vaiss. 2004 Mar;97(3):250-4.
264. Kaeffer N, Richard V, Thuillez C. Delayed coronary endothelial protection 24 hours after preconditioning: role of free radicals. Circulation. 1997 Oct 7;96(7):2311-6.
265. Huet O, Dupic L, Batteux F, Matar C, Conti M, Chereau C, et al. Postresuscitation syndrome: potential role of hydroxyl radical-induced endothelial cell damage. Crit Care Med. 2011 Jul;39(7):1712-20.
266. Saitoh S, Matsumoto K, Kamioka M, Ohkawara H, Kaneshiro T, Ishibashi T, et al. Novel pathway of endothelin-1 and reactive oxygen species in coronary vasospasm with endothelial dysfunction. Coron Artery Dis. 2009 Sep;20(6):400-8.
267. Hu G, Salem MR, Crystal GJ. Isoflurane prevents platelets from enhancing neutrophil-induced coronary endothelial dysfunction. Anesth Analg. 2005 Nov;101(5):1261-8.
268. Pearson PJ, Schaff HV, Vanhoutte PM. Long-term impairment of endothelium-dependent relaxations to aggregating platelets after reperfusion injury in canine coronary arteries. Circulation. 1990 Jun;81(6):1921-7.
269. Ishihara M, Sato H, Tateishi H, Kawagoe T, Shimatani Y, Kurisu S, et al. Time course of impaired coronary flow reserve after reperfusion in patients with acute myocardial infarction. Am J Cardiol. 1996 Nov 15;78(10):1103-8.
270. Jennings RB, Schaper J, Hill ML, Steenbergen C, Jr., Reimer KA. Effect of reperfusion late in the phase of reversible ischemic injury. Changes in cell volume, electrolytes, metabolites, and ultrastructure. Circ Res. 1985 Feb;56(2):262-78.
271. Ganote CE. Contraction band necrosis and irreversible myocardial injury. J Mol Cell Cardiol. 1983 Feb;15(2):67-73.
272. Amini K, Simko E, Davies JL. Sudden death associated with myocardial contraction band necrosis in Boer goat kids. Vet Pathol. 2011 Nov;48(6):1212-5.
273. Kloner RA, Ganote CE, Jennings RB. The "no-reflow" phenomenon after temporary coronary occlusion in the dog. J Clin Invest. 1974 Dec;54(6):1496-508.
274. Abbo KM, Dooris M, Glazier S, O'Neill WW, Byrd D, Grines CL, et al. Features and outcome of no-reflow after percutaneous coronary intervention. Am J Cardiol. 1995 Apr 15;75(12):778-82.
275. Morishima I, Sone T, Mokuno S, Taga S, Shimauchi A, Oki Y, et al. Clinical significance of no-reflow phenomenon observed on angiography after successful treatment of acute myocardial infarction with percutaneous transluminal coronary angioplasty. Am Heart J. 1995 Aug;130(2):239-43.
276. Wu KC. CMR of microvascular obstruction and hemorrhage in myocardial infarction. J Cardiovasc Magn Reson. 2012;14:68.
277. Wu KC, Zerhouni EA, Judd RM, Lugo-Olivieri CH, Barouch LA, Schulman SP, et al. Prognostic significance of microvascular obstruction by magnetic resonance imaging in patients with acute myocardial infarction. Circulation. 1998 Mar 3;97(8):765-72.
278. Niccoli G, Burzotta F, Galiuto L, Crea F. Myocardial no-reflow in humans. J Am Coll Cardiol. 2009 Jul 21;54(4):281-92.
279. Bolognese L, Carrabba N, Parodi G, Santoro GM, Buonamici P, Cerisano G, et al. Impact of microvascular dysfunction on left ventricular remodeling and long-term clinical outcome after primary coronary angioplasty for acute myocardial infarction. Circulation. 2004 Mar 9;109(9):1121-6.

280. Ndrepepa G, Tiroch K, Fusaro M, Keta D, Seyfarth M, Byrne RA, et al. 5-year prognostic value of no-reflow phenomenon after percutaneous coronary intervention in patients with acute myocardial infarction. J Am Coll Cardiol. 2010 May 25;55(21):2383-9.
281. Jaffe MD, Quinn NK. Warm-up phenomenon in angina pectoris. Lancet. 1980 Nov 1;2(8201):934-6.
282. Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. Circulation. 1986 Nov;74(5):1124-36.
283. Marber MS, Latchman DS, Walker JM, Yellon DM. Cardiac stress protein elevation 24 hours after brief ischemia or heat stress is associated with resistance to myocardial infarction. Circulation. 1993 Sep;88(3):1264-72.
284. Kuzuya T, Hoshida S, Kim Y, Nishida M, Fuji H, Kitabatake A, et al. Detection of oxygen-derived free radical generation in the canine postischemic heart during late phase of reperfusion. Circ Res. 1990 Apr;66(4):1160-5.
285. Kuzuya T, Hoshida S, Yamashita N, Fuji H, Oe H, Hori M, et al. Delayed effects of sublethal ischemia on the acquisition of tolerance to ischemia. Circ Res. 1993 Jun;72(6):1293-9.
286. Baxter GF, Goma FM, Yellon DM. Characterisation of the infarct-limiting effect of delayed preconditioning: timecourse and dose-dependency studies in rabbit myocardium. Basic Res Cardiol. 1997 Jun;92(3):159-67.
287. Yellon DM, Alkhulaifi AM, Pugsley WB. Preconditioning the human myocardium. Lancet. 1993 Jul 31;342(8866):276-7.
288. Noda T, Minatoguchi S, Fujii K, Hori M, Ito T, Kanmatsuse K, et al. Evidence for the delayed effect in human ischemic preconditioning: prospective multicenter study for preconditioning in acute myocardial infarction. J Am Coll Cardiol. 1999 Dec;34(7):1966-74.
289. Yang X, Cohen MV, Downey JM. Mechanism of cardioprotection by early ischemic preconditioning. Cardiovasc Drugs Ther. 2010 Jun;24(3):225-34.
290. Bolli R. The late phase of preconditioning. Circ Res. 2000 Nov 24;87(11):972-83.
291. Hausenloy DJ, Yellon DM. The second window of preconditioning (SWOP) where are we now? Cardiovasc Drugs Ther. 2010 Jun;24(3):235-54.
292. Na HS, Kim YI, Yoon YW, Han HC, Nahm SH, Hong SK. Ventricular premature beat-driven intermittent restoration of coronary blood flow reduces the incidence of reperfusion-induced ventricular fibrillation in a cat model of regional ischemia. Am Heart J. 1996 Jul;132(1 Pt 1):78-83.
293. Zhao ZQ, Corvera JS, Halkos ME, Kerendi F, Wang NP, Guyton RA, et al. Inhibition of myocardial injury by ischemic postconditioning during reperfusion: comparison with ischemic preconditioning. Am J Physiol Heart Circ Physiol. 2003 Aug;285(2):H579-88.
294. Ovize M, Baxter GF, Di Lisa F, Ferdinandy P, Garcia-Dorado D, Hausenloy DJ, et al. Postconditioning and protection from reperfusion injury: where do we stand? Position paper from the Working Group of Cellular Biology of the Heart of the European Society of Cardiology. Cardiovasc Res. 2010 Aug 1;87(3):406-23.
295. Heusch G, Boengler K, Schulz R. Cardioprotection: nitric oxide, protein kinases, and mitochondria. Circulation. 2008 Nov 4;118(19):1915-9.
296. Hausenloy DJ, Tsang A, Mocanu MM, Yellon DM. Ischemic preconditioning protects by activating prosurvival kinases at reperfusion. Am J Physiol Heart Circ Physiol. 2005 Feb;288(2):H971-6.
297. Liu GS, Thornton J, Van Winkle DM, Stanley AW, Olsson RA, Downey JM. Protection against infarction afforded by preconditioning is mediated by A1 adenosine receptors in rabbit heart. Circulation. 1991 Jul;84(1):350-6.

298. Wall TM, Sheehy R, Hartman JC. Role of bradykinin in myocardial preconditioning. J Pharmacol Exp Ther. 1994 Aug;270(2):681-9.
299. Schultz JE, Rose E, Yao Z, Gross GJ. Evidence for involvement of opioid receptors in ischemic preconditioning in rat hearts. Am J Physiol. 1995 May;268(5 Pt 2):H2157-61.
300. Lecour S. Activation of the protective Survivor Activating Factor Enhancement (SAFE) pathway against reperfusion injury: Does it go beyond the RISK pathway? J Mol Cell Cardiol. 2009 Jul;47(1):32-40.
301. Lecour S. Multiple protective pathways against reperfusion injury: a SAFE path without Aktion? J Mol Cell Cardiol. 2009 May;46(5):607-9.
302. Juhaszova M, Zorov DB, Kim SH, Pepe S, Fu Q, Fishbein KW, et al. Glycogen synthase kinase-3beta mediates convergence of protection signaling to inhibit the mitochondrial permeability transition pore. J Clin Invest. 2004 Jun;113(11):1535-49.
303. Matsui T, Li L, del Monte F, Fukui Y, Franke TF, Hajjar RJ, et al. Adenoviral gene transfer of activated phosphatidylinositol 3'-kinase and Akt inhibits apoptosis of hypoxic cardiomyocytes in vitro. Circulation. 1999 Dec 7;100(23):2373-9.
304. Fujio Y, Nguyen T, Wencker D, Kitsis RN, Walsh K. Akt promotes survival of cardiomyocytes in vitro and protects against ischemia-reperfusion injury in mouse heart. Circulation. 2000 Feb 15;101(6):660-7.
305. Tong H, Chen W, Steenbergen C, Murphy E. Ischemic preconditioning activates phosphatidylinositol-3-kinase upstream of protein kinase C. Circ Res. 2000 Aug 18;87(4):309-15.
306. Shimizu N, Yoshiyama M, Omura T, Hanatani A, Kim S, Takeuchi K, et al. Activation of mitogen-activated protein kinases and activator protein-1 in myocardial infarction in rats. Cardiovasc Res. 1998 Apr;38(1):116-24.
307. Yue TL, Wang C, Gu JL, Ma XL, Kumar S, Lee JC, et al. Inhibition of extracellular signal-regulated kinase enhances Ischemia/Reoxygenation-induced apoptosis in cultured cardiac myocytes and exaggerates reperfusion injury in isolated perfused heart. Circ Res. 2000 Mar 31;86(6):692-9.
308. Cross TG, Scheel-Toellner D, Henriquez NV, Deacon E, Salmon M, Lord JM. Serine/threonine protein kinases and apoptosis. Exp Cell Res. 2000 Apr 10;256(1):34-41.
309. Bell RM, Yellon DM. Atorvastatin, administered at the onset of reperfusion, and independent of lipid lowering, protects the myocardium by up-regulating a pro-survival pathway. J Am Coll Cardiol. 2003 Feb 5;41(3):508-15.
310. Jonassen AK, Sack MN, Mjos OD, Yellon DM. Myocardial protection by insulin at reperfusion requires early administration and is mediated via Akt and p70s6 kinase cell-survival signaling. Circ Res. 2001 Dec 7;89(12):1191-8.
311. Murriel CL, Churchill E, Inagaki K, Szweda LI, Mochly-Rosen D. Protein kinase Cdelta activation induces apoptosis in response to cardiac ischemia and reperfusion damage: a mechanism involving BAD and the mitochondria. J Biol Chem. 2004 Nov 12;279(46):47985-91.
312. Le Good JA, Ziegler WH, Parekh DB, Alessi DR, Cohen P, Parker PJ. Protein kinase C isotypes controlled by phosphoinositide 3-kinase through the protein kinase PDK1. Science. 1998 Sep 25;281(5385):2042-5.
313. Gross ER, Hsu AK, Gross GJ. Opioid-induced cardioprotection occurs via glycogen synthase kinase beta inhibition during reperfusion in intact rat hearts. Circ Res. 2004 Apr 16;94(7):960-6.
314. Gomez L, Paillard M, Thibault H, Derumeaux G, Ovize M. Inhibition of GSK3beta by postconditioning is required to prevent opening of the mitochondrial permeability transition pore during reperfusion. Circulation. 2008 May 27;117(21):2761-8.

315. Deuchar GA, Opie LH, Lecour S. TNFalpha is required to confer protection in an in vivo model of classical ischaemic preconditioning. Life Sci. 2007 Apr 10;80(18):1686-91.
316. Belosjorow S, Schulz R, Dorge H, Schade FU, Heusch G. Endotoxin and ischemic preconditioning: TNF-alpha concentration and myocardial infarct development in rabbits. Am J Physiol. 1999 Dec;277(6 Pt 2):H2470-5.
317. Negoro S, Kunisada K, Fujio Y, Funamoto M, Darville MI, Eizirik DL, et al. Activation of signal transducer and activator of transcription 3 protects cardiomyocytes from hypoxia/reoxygenation-induced oxidative stress through the upregulation of manganese superoxide dismutase. Circulation. 2001 Aug 28;104(9):979-81.
318. Xuan YT, Guo Y, Han H, Zhu Y, Bolli R. An essential role of the JAK-STAT pathway in ischemic preconditioning. Proc Natl Acad Sci U S A. 2001 Jul 31;98(16):9050-5.
319. Heusch G, Musiolik J, Kottenberg E, Peters J, Jakob H, Thielmann M. STAT5 activation and cardioprotection by remote ischemic preconditioning in humans: short communication. Circ Res. 2012 Jan 6;110(1):111-5.
320. Gross ER, Hsu AK, Gross GJ. The JAK/STAT pathway is essential for opioid-induced cardioprotection: JAK2 as a mediator of STAT3, Akt, and GSK-3 beta. Am J Physiol Heart Circ Physiol. 2006 Aug;291(2):H827-34.
321. Boengler K, Buechert A, Heinen Y, Roeskes C, Hilfiker-Kleiner D, Heusch G, et al. Cardioprotection by ischemic postconditioning is lost in aged and STAT3-deficient mice. Circ Res. 2008 Jan 4;102(1):131-5.
322. Lacerda L, Somers S, Opie LH, Lecour S. Ischaemic postconditioning protects against reperfusion injury via the SAFE pathway. Cardiovasc Res. 2009 Nov 1;84(2):201-8.
323. Pain T, Yang XM, Critz SD, Yue Y, Nakano A, Liu GS, et al. Opening of mitochondrial K(ATP) channels triggers the preconditioned state by generating free radicals. Circ Res. 2000 Sep 15;87(6):460-6.
324. Chai Y, Zhang DM, Lin YF. Activation of cGMP-dependent protein kinase stimulates cardiac ATP-sensitive potassium channels via a ROS/calmodulin/CaMKII signaling cascade. PLoS One. 2011;6(3):e18191.
325. Hunter DR, Haworth RA, Southard JH. Relationship between configuration, function, and permeability in calcium-treated mitochondria. J Biol Chem. 1976 Aug 25;251(16):5069-77.
326. Piot C, Croisille P, Staat P, Thibault H, Rioufol G, Mewton N, et al. Effect of cyclosporine on reperfusion injury in acute myocardial infarction. N Engl J Med. 2008 Jul 31;359(5):473-81.
327. Mitocare. Rationale and design of the 'MITOCARE' Study: a phase II, multicenter, randomized, double-blind, placebo-controlled study to assess the safety and efficacy of TRO40303 for the reduction of reperfusion injury in patients undergoing percutaneous coronary intervention for acute myocardial infarction. Cardiology. 2012;123(4):201-7.
328. Dezfulian C, Raat N, Shiva S, Gladwin MT. Role of the anion nitrite in ischemia-reperfusion cytoprotection and therapeutics. Cardiovasc Res. 2007 Jul 15;75(2):327-38.
329. Johnson G, 3rd, Tsao PS, Mulloy D, Lefer AM. Cardioprotective effects of acidified sodium nitrite in myocardial ischemia with reperfusion. J Pharmacol Exp Ther. 1990 Jan;252(1):35-41.
330. Jones SP, Girod WG, Palazzo AJ, Granger DN, Grisham MB, Jourd'Heuil D, et al. Myocardial ischemia-reperfusion injury is exacerbated in absence of endothelial cell nitric oxide synthase. Am J Physiol. 1999 May;276(5 Pt 2):H1567-73.
331. Xuan YT, Tang XL, Qiu Y, Banerjee S, Takano H, Han H, et al. Biphasic response of cardiac NO synthase isoforms to ischemic preconditioning in conscious rabbits. Am J Physiol Heart Circ Physiol. 2000 Nov;279(5):H2360-71.

332. Duranski MR, Greer JJ, Dejam A, Jaganmohan S, Hogg N, Langston W, et al. Cytoprotective effects of nitrite during in vivo ischemia-reperfusion of the heart and liver. J Clin Invest. 2005 May;115(5):1232-40.
333. Shiva S, Sack MN, Greer JJ, Duranski M, Ringwood LA, Burwell L, et al. Nitrite augments tolerance to ischemia/reperfusion injury via the modulation of mitochondrial electron transfer. J Exp Med. 2007 Sep 3;204(9):2089-102.
334. Baker JE, Su J, Fu X, Hsu A, Gross GJ, Tweddell JS, et al. Nitrite confers protection against myocardial infarction: role of xanthine oxidoreductase, NADPH oxidase and K(ATP) channels. J Mol Cell Cardiol. 2007 Oct;43(4):437-44.
335. Bryan NS, Calvert JW, Elrod JW, Gundewar S, Ji SY, Lefer DJ. Dietary nitrite supplementation protects against myocardial ischemia-reperfusion injury. Proc Natl Acad Sci U S A. 2007 Nov 27;104(48):19144-9.
336. Gonzalez FM, Shiva S, Vincent PS, Ringwood LA, Hsu LY, Hon YY, et al. Nitrite anion provides potent cytoprotective and antiapoptotic effects as adjunctive therapy to reperfusion for acute myocardial infarction. Circulation. 2008 Jun 10;117(23):2986-94.
337. Neye N, Enigk F, Shiva S, Habazettl H, Plesnila N, Kuppe H, et al. Inhalation of NO during myocardial ischemia reduces infarct size and improves cardiac function. Intensive Care Med. 2012 Aug;38(8):1381-91.
338. Webb AJ, Patel N, Loukogeorgakis S, Okorie M, Aboud Z, Misra S, et al. Acute blood pressure lowering, vasoprotective, and antiplatelet properties of dietary nitrate via bioconversion to nitrite. Hypertension. 2008 Mar;51(3):784-90.
339. Dezfulian C, Shiva S, Alekseyenko A, Pendyal A, Beiser DG, Munasinghe JP, et al. Nitrite Therapy After Cardiac Arrest Reduces Reactive Oxygen Species Generation, Improves Cardiac and Neurological Function, and Enhances Survival via Reversible Inhibition of Mitochondrial Complex I. Circulation. 2009 Aug 24.
340. Dezfulian C, Alekseyenko A, Dave KR, Raval AP, Do R, Kim F, et al. Nitrite therapy is neuroprotective and safe in cardiac arrest survivors. Nitric Oxide. 2012 May 15;26(4):241-50.
341. Mo L, Wang Y, Geary L, Corey C, Alef MJ, Beer-Stolz D, et al. Nitrite activates AMP kinase to stimulate mitochondrial biogenesis independent of soluble guanylate cyclase. Free Radic Biol Med. 2012 Oct 1;53(7):1440-50.
342. Singh M, Arya A, Kumar R, Bhargava K, Sethy NK. Dietary nitrite attenuates oxidative stress and activates antioxidant genes in rat heart during hypobaric hypoxia. Nitric Oxide. 2012 Jan 1;26(1):61-73.
343. Nadtochiy SM, Baker PR, Freeman BA, Brookes PS. Mitochondrial nitroalkene formation and mild uncoupling in ischaemic preconditioning: implications for cardioprotection. Cardiovasc Res. 2009 May 1;82(2):333-40.
344. Nadtochiy SM, Zhu Q, Urciuoli W, Rafikov R, Black SM, Brookes PS. Nitroalkenes confer acute cardioprotection via adenine nucleotide translocase 1. J Biol Chem. 2012 Jan 27;287(5):3573-80.
345. Pluta RM, Dejam A, Grimes G, Gladwin MT, Oldfield EH. Nitrite infusions to prevent delayed cerebral vasospasm in a primate model of subarachnoid hemorrhage. JAMA. 2005 Mar 23;293(12):1477-84.
346. Jung KH, Chu K, Ko SY, Lee ST, Sinn DI, Park DK, et al. Early intravenous infusion of sodium nitrite protects brain against in vivo ischemia-reperfusion injury. Stroke. 2006 Nov;37(11):2744-50.
347. Tonelli AR, Haserodt S, Aytekin M, Dweik RA. Nitric oxide deficiency in pulmonary hypertension: Pathobiology and implications for therapy. Pulm Circ. 2013 Jan;3(1):20-30.

348. Farber HW, Loscalzo J. Pulmonary arterial hypertension. N Engl J Med. 2004 Oct 14;351(16):1655-65.
349. Coggins MP, Bloch KD. Nitric oxide in the pulmonary vasculature. Arterioscler Thromb Vasc Biol. 2007 Sep;27(9):1877-85.
350. Braunwald E. Braunwald's Heart Disease: A Textbook of Cardiovascular Medicine. 9th edition ed: Saunders; 2012.
351. Hunter CJ, Dejam A, Blood AB, Shields H, Kim-Shapiro DB, Machado RF, et al. Inhaled nebulized nitrite is a hypoxia-sensitive NO-dependent selective pulmonary vasodilator. Nat Med. 2004 Oct;10(10):1122-7.
352. Casey DB, Badejo AM, Jr., Dhaliwal JS, Murthy SN, Hyman AL, Nossaman BD, et al. Pulmonary vasodilator responses to sodium nitrite are mediated by an allopurinol-sensitive mechanism in the rat. Am J Physiol Heart Circ Physiol. 2009 Feb;296(2):H524-33.
353. Zuckerbraun BS, Shiva S, Ifedigbo E, Mathier MA, Mollen KP, Rao J, et al. Nitrite potently inhibits hypoxic and inflammatory pulmonary arterial hypertension and smooth muscle proliferation via xanthine oxidoreductase-dependent nitric oxide generation. Circulation. 2010 Jan 5;121(1):98-109.
354. Zuckerbraun BS, Stoyanovsky DA, Sengupta R, Shapiro RA, Ozanich BA, Rao J, et al. Nitric oxide-induced inhibition of smooth muscle cell proliferation involves S-nitrosation and inactivation of RhoA. Am J Physiol Cell Physiol. 2007 Feb;292(2):C824-31.
355. Baliga RS, Milsom AB, Ghosh SM, Trinder SL, Macallister RJ, Ahluwalia A, et al. Dietary nitrate ameliorates pulmonary hypertension: cytoprotective role for endothelial nitric oxide synthase and xanthine oxidoreductase. Circulation. [Research Support, Non-U.S. Gov't]. 2012 Jun 12;125(23):2922-32.
356. Jhund PS, Macintyre K, Simpson CR, Lewsey JD, Stewart S, Redpath A, et al. Long-term trends in first hospitalization for heart failure and subsequent survival between 1986 and 2003: a population study of 5.1 million people. Circulation. 2009 Feb 3;119(4):515-23.
357. Stewart S, MacIntyre K, Hole DJ, Capewell S, McMurray JJ. More 'malignant' than cancer? Five-year survival following a first admission for heart failure. Eur J Heart Fail. 2001 Jun;3(3):315-22.
358. Nossaman BD, Pankey EA, Badejo AR, Jr., Casey DB, Uppu S, Murthy SN, et al. Analysis of responses to glyceryl trinitrate and sodium nitrite in the intact chest rat. Nitric Oxide. 2012 May 15;26(4):223-8.
359. Atherton JJ, Moore TD, Lele SS, Thomson HL, Galbraith AJ, Belenkie I, et al. Diastolic ventricular interaction in chronic heart failure. Lancet. 1997 Jun 14;349(9067):1720-4.
360. Kearney PM, Whelton M, Reynolds K, Muntner P, Whelton PK, He J. Global burden of hypertension: analysis of worldwide data. Lancet. 2005 Jan 15-21;365(9455):217-23.
361. Boeing H, Bechthold A, Bub A, Ellinger S, Haller D, Kroke A, et al. Critical review: vegetables and fruit in the prevention of chronic diseases. Eur J Nutr. 2012 Sep;51(6):637-63.
362. Larsen FJ, Ekblom B, Sahlin K, Lundberg JO, Weitzberg E. Effects of dietary nitrate on blood pressure in healthy volunteers. N Engl J Med. 2006 Dec 28;355(26):2792-3.
363. Kapil V, Milsom AB, Okorie M, Maleki-Toyserkani S, Akram F, Rehman F, et al. Inorganic nitrate supplementation lowers blood pressure in humans: role for nitrite-derived NO. Hypertension. [Randomized Controlled Trial
Research Support, Non-U.S. Gov't]. 2010 Aug;56(2):274-81.
364. Gilchrist M, Winyard PG, Aizawa K, Anning C, Shore A, Benjamin N. Effect of dietary nitrate on blood pressure, endothelial function, and insulin sensitivity in type 2 diabetes. Free Radic Biol Med. 2013 Jul;60:89-97.

365. Murohara T, Tayama S, Tabuchi T, Sumida H, Honda T, Hayasaki K, et al. Effects of angiotensin-converting enzyme inhibitor alacepril in patients with stable effort angina during chronic isosorbide dinitrate treatment. Am J Cardiol. 1996 Jun 1;77(14):1159-63.
366. Kumar D, Branch BG, Pattillo CB, Hood J, Thoma S, Simpson S, et al. Chronic sodium nitrite therapy augments ischemia-induced angiogenesis and arteriogenesis. Proc Natl Acad Sci U S A. [Research Support, N.I.H., Extramural
Research Support, Non-U.S. Gov't]. 2008 May 27;105(21):7540-5.
367. Ferlito S, Gallina M, Catassi S, Bisicchia A, Di Salvo MM. Nitrite plasma levels in normolipemic and hypercholesterolemic patients with peripheral occlusive arteriopathy. Panminerva Med. 1999 Dec;41(4):307-9.
368. de Haro Miralles J, Martinez-Aguilar E, Florez A, Varela C, Bleda S, Acin F. Nitric oxide: link between endothelial dysfunction and inflammation in patients with peripheral arterial disease of the lower limbs. Interact Cardiovasc Thorac Surg. 2009 Jul;9(1):107-12.
369. Allen JD, Miller EM, Schwark E, Robbins JL, Duscha BD, Annex BH. Plasma nitrite response and arterial reactivity differentiate vascular health and performance. Nitric Oxide. 2009 Jun;20(4):231-7.
370. Ahanchi SS, Tsihlis ND, Kibbe MR. The role of nitric oxide in the pathophysiology of intimal hyperplasia. J Vasc Surg. 2007 Jun;45 Suppl A:A64-73.
371. Alef MJ, Vallabhaneni R, Carchman E, Morris SM, Jr., Shiva S, Wang Y, et al. Nitrite-generated NO circumvents dysregulated arginine/NOS signaling to protect against intimal hyperplasia in Sprague-Dawley rats. J Clin Invest. 2011 Apr;121(4):1646-56.
372. Kanoore Edul VS, Dubin A, Ince C. The microcirculation as a therapeutic target in the treatment of sepsis and shock. Semin Respir Crit Care Med. 2011 Oct;32(5):558-68.
373. Cauwels A, Buys ES, Thoonen R, Geary L, Delanghe J, Shiva S, et al. Nitrite protects against morbidity and mortality associated with TNF- or LPS-induced shock in a soluble guanylate cyclase-dependent manner. J Exp Med. 2009 Dec 21;206(13):2915-24.
374. Papp J, Kenyeres P, Toth K. Clinical importance of antiplatelet drugs in cardiovascular diseases. Clin Hemorheol Microcirc. 2013;53(1-2):81-96.
375. Velmurugan S, Kapil V, Ghosh SM, Davies S, McKnight A, Aboud Z, et al. Antiplatelet effects of dietary nitrate in healthy volunteers: Involvement of cGMP and influence of sex. Free Radic Biol Med. 2013 Jun 24.
376. Lefer DJ. Emerging role of nitrite in myocardial protection. Arch Pharm Res. [Research Support, N.I.H., Extramural
Research Support, Non-U.S. Gov't
Review]. 2009 Aug;32(8):1127-38.
377. Thomas DD, Jourd'heuil D. S-nitrosation: current concepts and new developments. Antioxid Redox Signal. 2012 Oct 1;17(7):934-6.
378. Cannon RO, 3rd, Schechter AN, Panza JA, Ognibene FP, Pease-Fye ME, Waclawiw MA, et al. Effects of inhaled nitric oxide on regional blood flow are consistent with intravascular nitric oxide delivery. J Clin Invest. 2001 Jul;108(2):279-87.
379. Gladwin MT, Raat NJ, Shiva S, Dezfulian C, Hogg N, Kim-Shapiro DB, et al. Nitrite as a vascular endocrine nitric oxide reservoir that contributes to hypoxic signaling, cytoprotection, and vasodilation. Am J Physiol Heart Circ Physiol. 2006 Nov;291(5):H2026-35.
380. Dias-Junior CA, Gladwin MT, Tanus-Santos JE. Low-dose intravenous nitrite improves hemodynamics in a canine model of acute pulmonary thromboembolism. Free Radic Biol Med. 2006 Dec 15;41(12):1764-70.

381. Pluta RM, Oldfield EH, Bakhtian KD, Fathi AR, Smith RK, Devroom HL, et al. Safety and feasibility of long-term intravenous sodium nitrite infusion in healthy volunteers. PLoS One. [Clinical Trial
Research Support, N.I.H., Intramural
Research Support, Non-U.S. Gov't]. 2011;6(1):e14504.
382. Rehman HU. Methemoglobinemia. West J Med. [Case Reports
Review]. 2001 Sep;175(3):193-6.
383. Gratze G, Rudnicki R, Urban W, Mayer H, Schlogl A, Skrabal F. Hemodynamic and autonomic changes induced by Ironman: prediction of competition time by blood pressure variability. J Appl Physiol (1985). 2005 Nov;99(5):1728-35.
384. Raaijmakers E, Faes TJ, Scholten RJ, Goovaerts HG, Heethaar RM. A meta-analysis of published studies concerning the validity of thoracic impedance cardiography. Ann N Y Acad Sci. 1999 Apr 20;873:121-7.
385. Severinghaus JW. Simple, accurate equations for human blood O2 dissociation computations. Journal of applied physiology: respiratory, environmental and exercise physiology. 1979 Mar;46(3):599-602.
386. Hausladen A, Rafikov R, Angelo M, Singel DJ, Nudler E, Stamler JS. Assessment of nitric oxide signals by triiodide chemiluminescence. Proc Natl Acad Sci U S A. [Evaluation Studies
Research Support, N.I.H., Extramural
Research Support, Non-U.S. Gov't
Research Support, U.S. Gov't, Non-P.H.S.]. 2007 Feb 13;104(7):2157-62.
387. Dyson A, Bryan NS, Fernandez BO, Garcia-Saura MF, Saijo F, Mongardon N, et al. An integrated approach to assessing nitroso-redox balance in systemic inflammation. Free Radic Biol Med. 2011 Sep 15;51(6):1137-45.
388. Chowdhary S, Vaile JC, Fletcher J, Ross HF, Coote JH, Townend JN. Nitric oxide and cardiac autonomic control in humans. Hypertension. [Clinical Trial
Randomized Controlled Trial
Research Support, Non-U.S. Gov't]. 2000 Aug;36(2):264-9.
389. Paton JF, Kasparov S, Paterson DJ. Nitric oxide and autonomic control of heart rate: a question of specificity. Trends Neurosci. [Research Support, Non-U.S. Gov't
Review]. 2002 Dec;25(12):626-31.
390. Hogan N, Casadei B, Paterson DJ. Nitric oxide donors can increase heart rate independent of autonomic activation. J Appl Physiol (1985). [In Vitro
Research Support, Non-U.S. Gov't]. 1999 Jul;87(1):97-103.
391. Hogan N, Kardos A, Paterson DJ, Casadei B. Effect of exogenous nitric oxide on baroreflex function in humans. Am J Physiol. [Clinical Trial
Randomized Controlled Trial
Research Support, Non-U.S. Gov't]. 1999 Jul;277(1 Pt 2):H221-7.
392. Horackova M, Armour JA, Hopkins DA, Huang MH. Nitric oxide modulates signaling between cultured adult peripheral cardiac neurons and cardiomyocytes. Am J Physiol. [Research Support, Non-U.S. Gov't]. 1995 Aug;269(2 Pt 1):C504-10.
393. Armour JA, Smith FM, Losier AM, Ellenberger HH, Hopkins DA. Modulation of intrinsic cardiac neuronal activity by nitric oxide donors induces cardiodynamic changes. Am J Physiol. [Research Support, Non-U.S. Gov't]. 1995 Feb;268(2 Pt 2):R403-13.
394. Stanley WC, Mazer CD, Neese RA, Wisneski JA, Cason BA, Demas KA, et al. Increased lactate appearance and reduced clearance during hypoxia in dogs. Horm Metab Res. 1990 Sep;22(9):478-84.

395. Sutherland K. Bridging the quality gap: Heart failure: The Health Foundation, London UK. March 2010.
396. Bonios MJ, Terrovitis JV, Kaldara E, Ntalianis A, Nanas JN. The challenge of treating congestion in advanced heart failure. Expert Rev Cardiovasc Ther. 2011 Sep;9(9):1181-91.
397. Caccamo MA, Eckman PM. Pharmacologic therapy for New York Heart Association class IV heart failure. Congest Heart Fail. 2011 Sep-Oct;17(5):213-9.
398. Yancy CW, Jessup M, Bozkurt B, Butler J, Casey DE, Jr., Drazner MH, et al. 2013 ACCF/AHA Guideline for the Management of Heart Failure: A Report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines. Circulation. 2013 Oct 15;128(16):e240-319.
399. Elkayam U, Janmohamed M, Habib M, Hatamizadeh P. Vasodilators in the management of acute heart failure. Crit Care Med. 2008 Jan;36(1 Suppl):S95-105.
400. Publication Committee for the VMAC Investigators. Intravenous nesiritide vs nitroglycerin for treatment of decompensated congestive heart failure: a randomized controlled trial. JAMA. 2002 Mar 27;287(12):1531-40.
401. Cotter G, Metzkor E, Kaluski E, Faigenberg Z, Miller R, Simovitz A, et al. Randomised trial of high-dose isosorbide dinitrate plus low-dose furosemide versus high-dose furosemide plus low-dose isosorbide dinitrate in severe pulmonary oedema. Lancet. 1998 Feb 7;351(9100):389-93.
402. Sharon A, Shpirer I, Kaluski E, Moshkovitz Y, Milovanov O, Polak R, et al. High-dose intravenous isosorbide-dinitrate is safer and better than Bi-PAP ventilation combined with conventional treatment for severe pulmonary edema. J Am Coll Cardiol. 2000 Sep;36(3):832-7.
403. Elkayam U, Karaalp IS, Wani OR, Tummala P, Akhter MW. The role of organic nitrates in the treatment of heart failure. Prog Cardiovasc Dis. 1999 Jan-Feb;41(4):255-64.
404. Hollenberg SM. Vasodilators in acute heart failure. Heart Fail Rev. 2007 Jun;12(2):143-7.
405. Atherton JJ, Thomson HL, Moore TD, Wright KN, Muehle GW, Fitzpatrick LE, et al. Diastolic ventricular interaction: a possible mechanism for abnormal vascular responses during volume unloading in heart failure. Circulation. 1997 Dec 16;96(12):4273-9.
406. Dejam A, Hunter CJ, Gladwin MT. Effects of dietary nitrate on blood pressure. N Engl J Med. 2007 Apr 12;356(15):1590; author reply
407. Pellegrino D, Shiva S, Angelone T, Gladwin MT, Tota B. Nitrite exerts potent negative inotropy in the isolated heart via eNOS-independent nitric oxide generation and cGMP-PKG pathway activation. Biochim Biophys Acta. 2009 Jul;1787(7):818-27.
408. van Grondelle A, Ditchey RV, Groves BM, Wagner WW, Jr., Reeves JT. Thermodilution method overestimates low cardiac output in humans. Am J Physiol. 1983 Oct;245(4):H690-2.
409. Fritts HW, Cournand A. The Application of the Fick Principle to the Measurement of Pulmonary Blood Flow. Proc Natl Acad Sci U S A. 1958 Oct 15;44(10):1079-87.
410. Davies GG, Jebson PJ, Glasgow BM, Hess DR. Continuous Fick cardiac output compared to thermodilution cardiac output. Crit Care Med. 1986 Oct;14(10):881-5.
411. Kingma I, Tyberg JV, Smith ER. Effects of diastolic transseptal pressure gradient on ventricular septal position and motion. Circulation. 1983 Dec;68(6):1304-14.
412. Pankey EA, Badejo AM, Casey DB, Lasker GF, Riehl RA, Murthy SN, et al. Effect of chronic sodium nitrite therapy on monocrotaline-induced pulmonary hypertension. Nitric Oxide. [Research Support, N.I.H., Extramural]. 2012 Jun 30;27(1):1-8.

413. Clark AL, Volterrani M, Swan JW, Coats AJ. Ventilation-perfusion matching in chronic heart failure. Int J Cardiol. [Comparative Study
Research Support, Non-U.S. Gov't]. 1995 Mar 3;48(3):259-70.
414. Anderson RA, Ellis GR, Chirkov YY, Holmes AS, Payne N, Blackman DJ, et al. Determinants of platelet responsiveness to nitric oxide in patients with chronic heart failure. Eur J Heart Fail. [Research Support, Non-U.S. Gov't]. 2004 Jan;6(1):47-54.
415. Maguire SM, Nugent AG, McGurk C, Johnston GD, Nicholls DP. Abnormal vascular responses in human chronic cardiac failure are both endothelium dependent and endothelium independent. Heart. [Clinical Trial
Comparative Study
Controlled Clinical Trial
Research Support, Non-U.S. Gov't]. 1998 Aug;80(2):141-5.
416. Wanstall JC, Homer KL, Doggrell SA. Evidence for, and importance of, cGMP-independent mechanisms with NO and NO donors on blood vessels and platelets. Curr Vasc Pharmacol. [Review]. 2005 Jan;3(1):41-53.
417. Tsai EJ, Kass DA. Cyclic GMP signaling in cardiovascular pathophysiology and therapeutics. Pharmacol Ther. [Review]. 2009 Jun;122(3):216-38.
418. Pinder AG, Pittaway E, Morris K, James PE. Nitrite directly vasodilates hypoxic vasculature via nitric oxide-dependent and -independent pathways. Br J Pharmacol. [In Vitro
Research Support, Non-U.S. Gov't]. 2009 Aug;157(8):1523-30.
419. Murrell W. Nitro-Glycerine in angina pectoris. Lancet. 1879;1:80-1.
420. Murad F, Mittal CK, Arnold WP, Katsuki S, Kimura H. Guanylate cyclase: activation by azide, nitro compounds, nitric oxide, and hydroxyl radical and inhibition by hemoglobin and myoglobin. Advances in cyclic nucleotide research. 1978;9:145-58.
421. Katsuki S, Arnold W, Mittal C, Murad F. Stimulation of guanylate cyclase by sodium nitroprusside, nitroglycerin and nitric oxide in various tissue preparations and comparison to the effects of sodium azide and hydroxylamine. J Cyclic Nucleotide Res. 1977 Feb;3(1):23-35.
422. Needleman P, Jakschik B, Johnson EM, Jr. Sulfhydryl requirement for relaxation of vascular smooth muscle. J Pharmacol Exp Ther. [In Vitro]. 1973 Nov;187(2):324-31.
423. Ignarro LJ, Gruetter CA. Requirement of thiols for activation of coronary arterial guanylate cyclase by glyceryl trinitrate and sodium nitrite: possible involvement of S-nitrosothiols. Biochim Biophys Acta. 1980 Aug 13;631(2):221-31.
424. Ludbrook PA, Byrne JD, Kurnik PB, McKnight RC. Influence of reduction of preload and afterload by nitroglycerin on left ventricular diastolic pressure-volume relations and relaxation in man. Circulation. 1977 Dec;56(6):937-43.
425. Kreye VA, Stiefel A. In vivo and in vitro studies to elucidate the hemodynamic differences of sodium nitroprusside, nitroglycerin, and two isosorbide nitrates. Zeitschrift fur Kardiologie. 1983;72 Suppl 3:52-5.
426. Rosen R, Konig E, Klaus W. Different sensitivities of arteries and veins to glyceryltrinitrate-induced relaxation and tolerance: an "in vitro" study on isolated vessels from rabbits. Arch Int Pharmacodyn Ther. 1987 Feb;285(2):226-37.
427. Mikulic E, Franciosa JA, Cohn JN. Comparative hemodynamic effects of chewable isosorbide dinitrate and nitroglycerin in patients with congestive heart failure. Circulation. 1975 Sep;52(3):477-82.
428. Miller RR, Vismara LA, Williams DO, Amsterdam EA, Mason DT. Pharmacological mechanisms for left ventricular unloading in clinical congestive heart failure. Differential effects of nitroprusside, phentolamine, and nitroglycerin on cardiac function and peripheral circulation. Circ Res. 1976 Jul;39(1):127-33.

429. Armstrong PW, Moffat JA, Marks GS. Arterial-venous nitroglycerin gradient during intravenous infusion in man. Circulation. 1982 Dec;66(6):1273-6.
430. Armstrong PW, Watts DG, Moffat JA. Steady state pharmacokinetic haemodynamic studies of intravenous nitroglycerin in congestive cardiac failure. British journal of clinical pharmacology. 1983 Oct;16(4):385-90.
431. Elkayam U, Roth A, Kumar A, Kulick D, McIntosh N, McKay CR, et al. Hemodynamic and volumetric effects of venodilation with nitroglycerin in chronic mitral regurgitation. The American journal of cardiology. 1987 Nov 1;60(13):1106-11.
432. Abrams J. Hemodynamic effects of nitroglycerin and long-acting nitrates. American heart journal. 1985 Jul;110(1 Pt 2):216-24.
433. Klemenska E, Beresewicz A. Bioactivation of organic nitrates and the mechanism of nitrate tolerance. Cardiology journal. 2009;16(1):11-9.
434. Edwards JC, Ignarro LJ, Hyman AL, Kadowitz PJ. Relaxation of intrapulmonary artery and vein by nitrogen oxide-containing vasodilators and cyclic GMP. J Pharmacol Exp Ther. 1984 Jan;228(1):33-42.
435. Collier JG, Lorge RE, Robinson BF. Comparison of effects of tolmesoxide (RX71107), diazoxide, hydrallazine, prazosin, glyceryl trinitrate and sodium nitroprusside on forearm arteries and dorsal hand veins of man. British journal of clinical pharmacology. 1978 Jan;5(1):35-44.
436. Khan SA, Higdon NR, Meisheri KD. Coronary vasorelaxation by nitroglycerin: involvement of plasmalemmal calcium-activated K+ channels and intracellular Ca++ stores. J Pharmacol Exp Ther. 1998 Mar;284(3):838-46.
437. Sutton SC, Fung HL. Effect of dosage regimen on the development of tolerance to nitroglycerin in rats. J Cardiovasc Pharmacol. 1983 Nov-Dec;5(6):1086-92.
438. Tsai SC, Adamik R, Manganiello VC, Moss J. Effects of nitroprusside and nitroglycerin on cGMP content and PGI2 formation in aorta and vena cava. Biochem Pharmacol. 1989 Jan 1;38(1):61-5.
439. MacAllister RJ, Calver AL, Riezebos J, Collier J, Vallance P. Relative potency and arteriovenous selectivity of nitrovasodilators on human blood vessels: an insight into the targeting of nitric oxide delivery. J Pharmacol Exp Ther. 1995 Apr;273(1):154-60.
440. Wakai A, McCabe A, Kidney R, Brooks SC, Seupaul RA, Diercks DB, et al. Nitrates for acute heart failure syndromes. The Cochrane database of systematic reviews. 2013;8:CD005151.
441. Fukuda S, Sakuma K, Tsukui A, Fujiwara N, Tanaka T, Fujihara H, et al. Hypoxia modifies the vasodilatory effects of nitroglycerin, prostaglandin E1, and hydralazine on isolated porcine coronary arteries. J Cardiovasc Pharmacol. 1994 May;23(5):852-8.
442. Agvald P, Adding LC, Artlich A, Persson MG, Gustafsson LE. Mechanisms of nitric oxide generation from nitroglycerin and endogenous sources during hypoxia in vivo. Br J Pharmacol. 2002 Jan;135(2):373-82.
443. Bekuzarova SA, Khromov AS, Doloman LB, Beslaneev IA, Kurdanov Kh A. [Effect of nitroglycerin on vasodilation at high altitude]. Fiziolohichnyi zhurnal. 2003;49(3):118-25.
444. Kharbanda RK, Peters M, Walton B, Kattenhorn M, Mullen M, Klein N, et al. Ischemic preconditioning prevents endothelial injury and systemic neutrophil activation during ischemia-reperfusion in humans in vivo. Circulation. 2001 Mar 27;103(12):1624-30.
445. Benjamin N, Calver A, Collier J, Robinson B, Vallance P, Webb D. Measuring forearm blood flow and interpreting the responses to drugs and mediators. Hypertension. 1995 May;25(5):918-23.
446. Markwald RR, Kirby BS, Crecelius AR, Carlson RE, Voyles WF, Dinenno FA. Combined inhibition of nitric oxide and vasodilating prostaglandins abolishes forearm

vasodilatation to systemic hypoxia in healthy humans. J Physiol. 2011 Apr 15;589(Pt 8):1979-90.
447. Dinenno FA, Joyner MJ, Halliwill JR. Failure of systemic hypoxia to blunt alpha-adrenergic vasoconstriction in the human forearm. J Physiol. 2003 Jun 15;549(Pt 3):985-94.
448. Pontin J, Blaylock MG, Walsh GM, Turner SW. Sputum eosinophil apoptotic rate is positively correlated to exhaled nitric oxide in children. Pediatr Pulmonol. 2008 Nov;43(11):1130-4.
449. Tamisier R, Norman D, Anand A, Choi Y, Weiss JW. Evidence of sustained forearm vasodilatation after brief isocapnic hypoxia. J Appl Physiol (1985). 2004 May;96(5):1782-7.
450. Shankaran S, Pappas A, Laptook AR, McDonald SA, Ehrenkranz RA, Tyson JE, et al. Outcomes of safety and effectiveness in a multicenter randomized, controlled trial of whole-body hypothermia for neonatal hypoxic-ischemic encephalopathy. Pediatrics. 2008 Oct;122(4):e791-8.
451. McGinnis WR, Audhya T, Walsh WJ, Jackson JA, McLaren-Howard J, Lewis A, et al. Discerning the Mauve Factor, Part 1. Altern Ther Health Med. 2008 Mar-Apr;14(2):40-50.
452. Miller SM, Narasimhan RA, Schmalz PF, Soffer EE, Walsh RM, Krishnamurthi V, et al. Distribution of interstitial cells of Cajal and nitrergic neurons in normal and diabetic human appendix. Neurogastroenterol Motil. 2008 Apr;20(4):349-57.
453. Feelisch M, Noack E. Nitric oxide (NO) formation from nitrovasodilators occurs independently of hemoglobin or non-heme iron. Eur J Pharmacol. 1987 Oct 27;142(3):465-9.
454. Townsend N WK, Bhatnagar P, Smolina K, Nichols M, Leal J, Luengo-Fernandez R, Rayner M. Coronary heart disease statistics 2012 edition. London: British Heart Foundation.
455. Bridgewater B GS, Hickey G, Fazal N. National Adult Cardiac Surgery Audit Annual Report 2010-11. London: National Institute for Cardiovascular Outcomes Research (NICOR).
456. Berdowski J, Berg RA, Tijssen JG, Koster RW. Global incidences of out-of-hospital cardiac arrest and survival rates: Systematic review of 67 prospective studies. Resuscitation. [Review]. 2010 Nov;81(11):1479-87.
457. Watson L VG, Donohoe R. Cardiac Arrest Annual Report: 2009/10. London: London Ambulance Service NHS Trust.
458. Perkins GD, Cooke MW. Variability in cardiac arrest survival: the NHS Ambulance Service Quality Indicators. Emerg Med J. [Comment
Research Support, Non-U.S. Gov't]. 2012 Jan;29(1):3-5.
459. Gerczuk PZ, Kloner RA. An update on cardioprotection: a review of the latest adjunctive therapies to limit myocardial infarction size in clinical trials. Journal of the American College of Cardiology. [Review]. 2012 Mar 13;59(11):969-78.
460. Hausenloy DJ, Boston-Griffiths E, Yellon DM. Cardioprotection during cardiac surgery. Cardiovascular research. [Research Support, Non-U.S. Gov't
Review]. 2012 May 1;94(2):253-65.
461. Okamoto M, Tsuchiya K, Kanematsu Y, Izawa Y, Yoshizumi M, Kagawa S, et al. Nitrite-derived nitric oxide formation following ischemia-reperfusion injury in kidney. Am J Physiol Renal Physiol. 2005 Jan;288(1):F182-7.
462. Doganci S, Yildirim V, Bolcal C, Korkusuz P, Gumusel B, Demirkilic U, et al. Sodium nitrite and cardioprotective effect in pig regional myocardial ischemia-reperfusion injury model. Adv Clin Exp Med. 2012 Nov-Dec;21(6):713-26.
463. Raat NJ, Noguchi AC, Liu VB, Raghavachari N, Liu D, Xu X, et al. Dietary nitrate and nitrite modulate blood and organ nitrite and the cellular ischemic stress response. Free Radic Biol Med. 2009 Sep 1;47(5):510-7.

464. Birnbaum Y, Ye Y, Rosanio S, Tavackoli S, Hu ZY, Schwarz ER, et al. Prostaglandins mediate the cardioprotective effects of atorvastatin against ischemia-reperfusion injury. Cardiovascular research. [Comparative Study]. 2005 Feb 1;65(2):345-55.
465. Gao F, Gao E, Yue TL, Ohlstein EH, Lopez BL, Christopher TA, et al. Nitric oxide mediates the antiapoptotic effect of insulin in myocardial ischemia-reperfusion: the roles of PI3-kinase, Akt, and endothelial nitric oxide synthase phosphorylation. Circulation. [Research Support, Non-U.S. Gov't
Research Support, U.S. Gov't, P.H.S.]. 2002 Mar 26;105(12):1497-502.
466. Tsang A, Hausenloy DJ, Mocanu MM, Yellon DM. Postconditioning: a form of "modified reperfusion" protects the myocardium by activating the phosphatidylinositol 3-kinase-Akt pathway. Circulation research. [Comparative Study
In Vitro
Research Support, Non-U.S. Gov't]. 2004 Aug 6;95(3):230-2.
467. Hafezi-Moghadam A, Simoncini T, Yang Z, Limbourg FP, Plumier JC, Rebsamen MC, et al. Acute cardiovascular protective effects of corticosteroids are mediated by non-transcriptional activation of endothelial nitric oxide synthase. Nature medicine. [In Vitro
Research Support, Non-U.S. Gov't
Research Support, U.S. Gov't, P.H.S.]. 2002 May;8(5):473-9.
468. Ferguson TB, Jr., Hammill BG, Peterson ED, DeLong ER, Grover FL. A decade of change--risk profiles and outcomes for isolated coronary artery bypass grafting procedures, 1990-1999: a report from the STS National Database Committee and the Duke Clinical Research Institute. Society of Thoracic Surgeons. The Annals of thoracic surgery. 2002 Feb;73(2):480-9; discussion 9-90.
469. Grover FL, Shroyer AL, Hammermeister K, Edwards FH, Ferguson TB, Jr., Dziuban SW, Jr., et al. A decade's experience with quality improvement in cardiac surgery using the Veterans Affairs and Society of Thoracic Surgeons national databases. Annals of surgery. [Comparative Study
Evaluation Studies]. 2001 Oct;234(4):464-72; discussion 72-4.
470. Guru V, Tu JV, Etchells E, Anderson GM, Naylor CD, Novick RJ, et al. Relationship between preventability of death after coronary artery bypass graft surgery and all-cause risk-adjusted mortality rates. Circulation. [Multicenter Study
Research Support, Non-U.S. Gov't]. 2008 Jun 10;117(23):2969-76.
471. O'Connor GT, Birkmeyer JD, Dacey LJ, Quinton HB, Marrin CA, Birkmeyer NJ, et al. Results of a regional study of modes of death associated with coronary artery bypass grafting. Northern New England Cardiovascular Disease Study Group. The Annals of thoracic surgery. [Multicenter Study
Research Support, Non-U.S. Gov't
Research Support, U.S. Gov't, P.H.S.]. 1998 Oct;66(4):1323-8.
472. Weisel RD. Myocardial stunning after coronary bypass surgery. Journal of cardiac surgery. [Research Support, Non-U.S. Gov't]. 1993 Mar;8(2 Suppl):242-4.
473. Brener SJ, Lytle BW, Schneider JP, Ellis SG, Topol EJ. Association between CK-MB elevation after percutaneous or surgical revascularization and three-year mortality. J Am Coll Cardiol. 2002 Dec 4;40(11):1961-7.
474. Domanski MJ, Mahaffey K, Hasselblad V, Brener SJ, Smith PK, Hillis G, et al. Association of myocardial enzyme elevation and survival following coronary artery bypass graft surgery. JAMA : the journal of the American Medical Association. [Meta-Analysis]. 2011 Feb 9;305(6):585-91.

475. Kathiresan S, Servoss SJ, Newell JB, Trani D, MacGillivray TE, Lewandrowski K, et al. Cardiac troponin T elevation after coronary artery bypass grafting is associated with increased one-year mortality. Am J Cardiol. 2004 Oct 1;94(7):879-81.
476. Klatte K, Chaitman BR, Theroux P, Gavard JA, Stocke K, Boyce S, et al. Increased mortality after coronary artery bypass graft surgery is associated with increased levels of postoperative creatine kinase-myocardial band isoenzyme release: results from the GUARDIAN trial. Journal of the American College of Cardiology. [Clinical Conference
Research Support, Non-U.S. Gov't]. 2001 Oct;38(4):1070-7.
477. Lehrke S, Steen H, Sievers HH, Peters H, Opitz A, Muller-Bardorff M, et al. Cardiac troponin T for prediction of short- and long-term morbidity and mortality after elective open heart surgery. Clin Chem. 2004 Sep;50(9):1560-7.
478. Braunwald E, Kloner RA. Myocardial reperfusion: a double-edged sword? The Journal of clinical investigation. 1985 Nov;76(5):1713-9.
479. Ferrari R, Curello S, Boffa GM, Condorelli E, Pasini E, Guarnieri G, et al. Oxygen free radical-mediated heart injury in animal models and during bypass surgery in humans. Effects of alpha-tocopherol. Annals of the New York Academy of Sciences. [Research Support, Non-U.S. Gov't
Review]. 1989;570:237-53.
480. Cavalca V, Sisillo E, Veglia F, Tremoli E, Cighetti G, Salvi L, et al. Isoprostanes and oxidative stress in off-pump and on-pump coronary bypass surgery. The Annals of thoracic surgery. [Comparative Study
Randomized Controlled Trial]. 2006 Feb;81(2):562-7.
481. Chambers DJ. Oxidative stress injury during cardiac surgery: how important is it? Cardiovascular research. [Comment
Editorial]. 2007 Mar 1;73(4):626-8.
482. Garcia-de-la-Asuncion J, Pastor E, Perez-Griera J, Belda FJ, Moreno T, Garcia-del-Olmo E, et al. Oxidative stress injury after on-pump cardiac surgery: effects of aortic cross clamp time and type of surgery. Redox report : communications in free radical research. [Research Support, Non-U.S. Gov't]. 2013;18(5):193-9.
483. Sivaraman V, Mudalagiri NR, Di Salvo C, Kolvekar S, Hayward M, Yap J, et al. Postconditioning protects human atrial muscle through the activation of the RISK pathway. Basic research in cardiology. [In Vitro
Research Support, Non-U.S. Gov't]. 2007 Sep;102(5):453-9.
484. Cappellano G, Uberti F, Caimmi PP, Pietronave S, Mary DA, Dianzani C, et al. Different expression and function of the endocannabinoid system in human epicardial adipose tissue in relation to heart disease. The Canadian journal of cardiology. [Comparative Study
Research Support, Non-U.S. Gov't]. 2013 Apr;29(4):499-509.
485. Roberts LJ, Morrow JD. Measurement of F(2)-isoprostanes as an index of oxidative stress in vivo. Free radical biology & medicine. [Research Support, Non-U.S. Gov't
Research Support, U.S. Gov't, P.H.S.
Review]. 2000 Feb 15;28(4):505-13.
486. Ulus AT, Aksoyek A, Ozkan M, Katircioglu SF, Basu S. Cardiopulmonary bypass as a cause of free radical-induced oxidative stress and enhanced blood-borne isoprostanes in humans. Free radical biology & medicine. [Research Support, Non-U.S. Gov't]. 2003 Apr 1;34(7):911-7.
487. Lefer AM, Lefer DJ. The role of nitric oxide and cell adhesion molecules on the microcirculation in ischaemia-reperfusion. Cardiovascular research. [Research Support, U.S. Gov't, P.H.S.

Review]. 1996 Oct;32(4):743-51.
488. Schmidt TS, Alp NJ. Mechanisms for the role of tetrahydrobiopterin in endothelial function and vascular disease. Clinical science. [Research Support, Non-U.S. Gov't
Review]. 2007 Jul;113(2):47-63.
489. Mount PF, Kemp BE, Power DA. Regulation of endothelial and myocardial NO synthesis by multi-site eNOS phosphorylation. Journal of molecular and cellular cardiology. [Review]. 2007 Feb;42(2):271-9.
490. Kamga Pride C, Mo L, Quesnelle K, Dagda RK, Murillo D, Geary L, et al. Nitrite Activates Protein Kinase A in Normoxia to mediate Mitochondrial Fusion and Tolerance to Ischemia/Reperfusion. Cardiovascular research. 2013 Sep 30.
491. Jabbari A, Banihashem N, Alijanpour E, Vafaey HR, Alereza H, Rabiee SM. Serum lactate as a prognostic factor in coronary artery bypass graft operation by on pump method. Caspian J Intern Med. 2013 Spring;4(2):662-6.
492. Maillet JM, Le Besnerais P, Cantoni M, Nataf P, Ruffenach A, Lessana A, et al. Frequency, risk factors, and outcome of hyperlactatemia after cardiac surgery. Chest. 2003 May;123(5):1361-6.
493. O'Connor E, Fraser JF. The interpretation of perioperative lactate abnormalities in patients undergoing cardiac surgery. Anaesth Intensive Care. [Review]. 2012 Jul;40(4):598-603.
494. Pagel PS. Myocardial protection by volatile anesthetics in patients undergoing cardiac surgery: a critical review of the laboratory and clinical evidence. Journal of cardiothoracic and vascular anesthesia. 2013 Oct;27(5):972-82.
495. Jones DA, Andiapen M, Van-Eijl TJ, Webb AJ, Antoniou S, Schilling RJ, et al. The safety and efficacy of intracoronary nitrite infusion during acute myocardial infarction (NITRITE-AMI): study protocol of a randomised controlled trial. BMJ Open. 2013;3(4).
496. Siddiqi N, Bruce M, Neil CJ, Jagpal B, Maclennon G, Cotton SC, et al. Protocol: does sodium nitrite administration reduce ischaemia-reperfusion injury in patients presenting with acute ST segment elevation myocardial infarction? Nitrites in acute myocardial infarction (NIAMI). J Transl Med. [Research Support, Non-U.S. Gov't]. 2013;11:116.
497. Gladwin MT. Hemoglobin as a nitrite reductase regulating red cell-dependent hypoxic vasodilation. Am J Respir Cell Mol Biol. 2005 May;32(5):363-6.
498. Crabb DW, Matsumoto M, Chang D, You M. Overview of the role of alcohol dehydrogenase and aldehyde dehydrogenase and their variants in the genesis of alcohol-related pathology. Proc Nutr Soc. [Research Support, U.S. Gov't, P.H.S.
Review]. 2004 Feb;63(1):49-63.
499. Yoshida A, Huang IY, Ikawa M. Molecular abnormality of an inactive aldehyde dehydrogenase variant commonly found in Orientals. Proc Natl Acad Sci U S A. 1984 Jan;81(1):258-61.
500. DiFabio J, Ji Y, Vasiliou V, Thatcher GR, Bennett BM. Role of mitochondrial aldehyde dehydrogenase in nitrate tolerance. Mol Pharmacol. 2003 Nov;64(5):1109-16.
501. Hink U, Daiber A, Kayhan N, Trischler J, Kraatz C, Oelze M, et al. Oxidative inhibition of the mitochondrial aldehyde dehydrogenase promotes nitroglycerin tolerance in human blood vessels. J Am Coll Cardiol. 2007 Dec 4;50(23):2226-32.
502. Philpott AC. Dissociation of Aldehyde Dehydrogenase Activity from Nitrate Effect, Bioconversion, and Tolerance in Humans. Journal of American College of Cardiology. 2007;49:420A-3A.
503. Chen CH, Budas GR, Churchill EN, Disatnik MH, Hurley TD, Mochly-Rosen D. Activation of aldehyde dehydrogenase-2 reduces ischemic damage to the heart. Science. 2008 Sep 12;321(5895):1493-5.

504. D'Souza Y, Dowlatshahi S, Bennett BM. Changes in aldehyde dehydrogenase 2 expression in rat blood vessels during glyceryl trinitrate tolerance development and reversal. Br J Pharmacol. 2011 Sep;164(2b):632-43.
505. Maher AR, Arif S, Madhani M, Abozguia K, Ahmed I, Fernandez BO, et al. Impact of chronic congestive heart failure on pharmacokinetics and vasomotor effects of infused nitrite. Br J Pharmacol. 2013 Jun;169(3):659-70.
506. Madhani M, Scotland RS, MacAllister RJ, Hobbs AJ. Vascular natriuretic peptide receptor-linked particulate guanylate cyclases are modulated by nitric oxide-cyclic GMP signalling. Br J Pharmacol. 2003 Aug;139(7):1289-96.
507. Mulvany MJ, Halpern W. Contractile properties of small arterial resistance vessels in spontaneously hypertensive and normotensive rats. Circ Res. 1977 Jul;41(1):19-26.
508. Lundberg JO, Carlstrom M, Larsen FJ, Weitzberg E. Roles of dietary inorganic nitrate in cardiovascular health and disease. Cardiovasc Res. 2011 Feb 15;89(3):525-32.
509. Huellner MW, Schrepfer S, Weyand M, Weiner H, Wimplinger I, Eschenhagen T, et al. Inhibition of aldehyde dehydrogenase type 2 attenuates vasodilatory action of nitroglycerin in human veins. Faseb J. 2008 Jul;22(7):2561-8.
510. Keith RA, Burkman AM, Sokoloski TD, Fertel RH. Vascular tolerance to nitroglycerin and cyclic GMP generation in rat aortic smooth muscle. J Pharmacol Exp Ther. 1982 Jun;221(3):525-31.
511. Rapoport RM, Waldman SA, Ginsburg R, Molina CR, Murad F. Effects of glyceryl trinitrate on endothelium-dependent and -independent relaxation and cyclic GMP levels in rat aorta and human coronary artery. J Cardiovasc Pharmacol. 1987 Jul;10(1):82-9.
512. Borgognone A, Lowe KL, Watson SP, Madhani M. Natriuretic peptides induce weak VASP phosphorylation at Serine 239 in platelets. Platelets. 2013 Mar 7.
513. Lincoln TM, Dey N, Sellak H. Invited review: cGMP-dependent protein kinase signaling mechanisms in smooth muscle: from the regulation of tone to gene expression. J Appl Physiol (1985). 2001 Sep;91(3):1421-30.
514. British Medical Association & The Royal Pharmaceutical Society. British National Formulary: Pharmaceutical Press; 2013.
515. Marti CN, Gheorghiade M, Kalogeropoulos AP, Georgiopoulou VV, Quyyumi AA, Butler J. Endothelial dysfunction, arterial stiffness, and heart failure. Journal of the American College of Cardiology. 2012 Oct 16;60(16):1455-69.
516. Greenstein AS, Khavandi K, Withers SB, Sonoyama K, Clancy O, Jeziorska M, et al. Local inflammation and hypoxia abolish the protective anticontractile properties of perivascular fat in obese patients. Circulation. 2009 Mar 31;119(12):1661-70.
517. Jasinska-Stroschein M, Orszulak-Michalak D. The current approach into signaling pathways in pulmonary arterial hypertension and their implication in novel therapeutic strategies. Pharmacol Rep. [Review]. 2014 Aug;66(4):552-64.
518. Siddiqi N, Bruce M, Neil CJ, Jagpal B, Maclennon G, Cotton SC, et al. Protocol: does sodium nitrite administration reduce ischaemia-reperfusion injury in patients presenting with acute ST segment elevation myocardial infarction? Nitrites in acute myocardial infarction (NIAMI). Journal of translational medicine. 2013;11:116.