THE CARDIOTOXICITY OF CARBON MONOXIDE

by

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A thesis submitted to the University of Plymouth
in partial fulfilment for the degree of

DOCTOR OF PHILOSOPHY

Peninsula Medical School and the
Department of Biological Sciences
Faculty of Science

November 2003

Abstract

The mechanism(s) responsible for the cardiotoxicity of CO are not clear and do not appear to arise solely from tissue hypoxia. Tissue hypoxia is produced by CO binding to haemoglobin and reducing the amount of oxygen carried by the blood *in vivo*. CO-induced hypoxia *in vivo* may be responsible for producing conditions such as those found during ischaemia/reperfusion (I/R) injury. Reactive oxygen species (ROS) produced during I/R injury is established and responsible for cellular/tissue damage from the oxidative damage. We postulate that oxidative stress is responsible for the CO-associated cardiac morbidities found in some cases following severe acute exposure to CO.

Isolated perfused rat hearts were used to investigate the physiological and biochemical changes in hearts following CO exposure. Hearts were perfused with buffer equilibrated with different mixtures of CO (0-0.05% CO for 30 minutes) in the presence of 21% oxygen during and after CO exposure (for 90 minutes), i.e. normoxic conditions were used throughout. Some hearts were perfused with water-soluble antioxidants (ascorbic acid and TroloxC) before and during the CO exposure. The reduced heart rate and perfusate flow suggest that CO may have a direct effect in heart tissue. Biochemical measurements suggest that no tissue hypoxia occurred under these conditions. The results provide evidence to suggest that oxidative stress occurred in ventricle tissue after CO exposure and was attenuated by the antioxidants. However, isolated rat liver mitochondria exposed to CO and/or hyperoxia showed no ROS production suggesting that mitochondria may not be a source of the oxidative stress.

CO exposure may also produce altered myocardial energetics by oxidatively modifying and/or binding to myoglobin. Tissue damage initiated by CO-induced oxidative stress and hypoxia may potentiate ageing within heart tissue *in vivo* and could be responsible for producing the observed CO-associated heart morbidity in an I/R-*like* injury.

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Acknowledgement

I would like to thank the following people for their time and support: John Moody ('Jam') for allowing me to occasionally pick his brains, having the odd discussion over a cup of coffee and for being himself during the time taken to compile this comic book. (Professor) Rob Sneyd for his clinical contribution, raising vital funds and being proactive when needed. Richard Handy for being himself and trying to muck in whenever possible. Special thanks to Lennie (Leonard Hawkins) for carrying out the electrolyte analysis work; Andy Fischer (PERC) for his time and advice; and Brandon Reeder (Dept. of Biochemistry, University of Essex) for HPLC analysis. Jo & Shaun for putting up with us, being yourselves and most importantly - providing cakes. Lynne for her constant support with words of encouragement. Adrian (in the workshops) for his patience and the hard work on those 'pressure vessels'. Sally (in Chemistry) for lending various reagents/boxes/method and generally being really helpful along with the two Andy's. Special 'terima kasi' to Vinod(bhai) and Licha (the 'cacing') for being great flatmates and providing the 'chilli' experience in Plymouth. Also thanks to Dips and her family for their help in Leicester towards the final leg.

Sukhi Bansal (at KCL) for his support, friendship, and occasional chemistry advice for the 'skivvy'. Bhavesh (a.k.a. 'Dom' now in the Big Apple) for providing many articles, lending me his laptop (again), and just being a mate. Roy Moate for his humour, watery tea, use of the darkroom and preparing samples for electron microscopy. Maria for her time and assistance whilst trying to isolate those bloody cells. Julie Soane (a.k.a. Jools) for her humour and providing the odd horoscope forecast. Nick Crocker and Denise for the use of various equipment and chemicals. Everyone at Triphasic Laboratories who were involved in sample analysis. Andy Demaine for providing some input/feedback. The patience of Becky Pearce, other members of the Peninsula Medical School (PMS), and Department of Biological Sciences Office who dealt with me (particularly Anne). Simon Davies for the odd drink. Awadhesh Jha for letting me borrow the occasional book and giving us a lift to Leicester. Marilyn for her cheery smile and occasional chit chats during the few 'out of office' hours.

My school-mate Dharmesh (a.k.a. Dums, Chundoo or 'Das') for his friendship and honesty, the driving lessons, and everything else (ghandubhai) over the years. Special thanks to his family for all their love, warmth, kindness and help over the years - I'm not sure where I'd be without it. Thanks to Harjit 'Gabbar' Singh for those 'cha pani' breaks in Mary Newman, watching VCDs, burning CDs, putting me on the web, and 'helping out' in your own unique way (by making me homeless during one Christmas, and missing "Pritpal's cousins wedding"! Also to Harjit's family (in the UK and Malaysia) for being themselves and all their love and support. Paul Davies for his continual words of motivation and being a mate since the days of 'Club Trop'. Matt Whiteman (currently 'exotic' in Singapore) for the odd method and taunt. Smeeta for that free radical book. Dave (in 'West London') for being an 'inspiration', odd taxi to Heathrow, and providing that HPLC. 'Slim Shady' Dan & Nicky for their hospitality, odd weekend/food shopping trips, and the television/video package - cheers 'Bro'. "God dam it" Greg for those pizza and video nights. Paul & Emma (and now the 'little one') for their company and friendship. Gav & Emma for those relaxing evenings - say no more. Senan & Ellis for being a temporary store for my personal belongings in Plymouth. Bal for keeping me updated with job ads, providing a funny story about 'work' and being the same 'Geezer' since the dungeon days. Shariza & Mumtaz for their motivating spirit and helping the "food beggar". Savvas for your support and motivation during the 'permanent head damage (Ph.D.)' years and for the exercise equipment. To Bruno and everyone else at Gurkha Village/Himalayan Spice for feeding me. I'd also like to thank both families of Bruno and Suresh (in Nepal) for their hospitality whilst making that special journey. Zaki & Adrian for your humour, sofa, and letting us watch films whilst leading stressful lives "yaar". Nilesh (Hits's brother) for the internet access and his family for providing help when needed. Leicester Bhavesh for printing documents/articles, the use of your house address, odd lunch time taster sessions and your camera man role at my Sister's 21st (in 2003).

Finally, I'd like to thank my Family for their support: My 'Mum' whose struggle (for as long as I can remember) taught me independence and patience from an early age, and to always rely and look after yourself. Both my Sisters- Usha & Gita: for their love and support, odd fights and many laughs over the years, and other happy times to come as we get on with life in our own ways. Also my 'Dad' for his indirect teachings on life. My Rainamasi & Family for dropping me off in 'Pindland', looking after me during the many weekends (in London), and for helping Mum out when it was needed. Also to the 'new' Family of Arunaben (in 2002) for feeding me now and again, and making me feel so welcome. I'm certain to have missed out many people (like Mrs. Chauhan, Mr. Cole and Dr. Anderson), but to everyone who has contributed in whatever way (small or large; here and abroad - cheers) in getting me to this point - Thank You.

'Three rules of work'

"OUT OF CLUTTER, FIND SIMPLICITY.
FROM DISCORD, FIND HARMONY.
IN THE MIDDLE OF DIFFICULTY LIES OPPORTUNITY."

-ALBERT EINSTEIN

Author's Declaration

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award.

This study was financed with the aid of a studentship from the Diving Diseases Research Centre (DDRC), the British Antarctic Survey (BAS), and the British Journal of Anaesthesia (BJA).

Relevant scientific seminars and conferences were regularly attended at which work was often presented; external institutions were visited for consultation purposes and several papers prepared for publication.

Publications (or presentation of other forms of creative work):
Patel, A. P.; Moody, A. J.; Handy, R. D. & Sneyd, J. R. (2000) Myoglobin As A Marker Of Myocardial Damage. *Toxicology Letters* **116** (Suppl. 1), 78-79.

Patel, A. P.; Moody, A. J.; Handy, R. D. & Sneyd, J. R. (2001) Carbon monoxide toxicity in the heart: evidence for ischaemia/reperfusion like injury. *Biochemical Society Transactions* **30** (1), A34-A34.

Patel, A. P.; Moody, A. J.; Handy, R. D. & Sneyd, J. R. (2003) Carbon monoxide exposure in rat heart: glutathione depletion is prevented by antioxidants. *Biochemical and Biophysical Research Communications* **302** (2), 392-396.

These publications are presented in the thesis following the References.

Presentations and Conferences attended:

Poster presented entitled "Myoglobin as a marker of myocardial damage" at EUROTOX 2000 (17th-20th September 2000); held at Imperial College, London, England, UK.

Poster presented entitled "Carbon monoxide toxicity in the heart: evidence for ischaemia/reperfusion like injury" at the 675th Biochemical Society Meeting Joint With The Physiological Society 2001 (17th-19th December 2001); held at the University Of York, York, England, UK.

Presented talk entitled "Carbon Monoxide And The Heart" at the British Hyperbaric Association Meeting (28th-30th September 2001); held at the Exeter Court Hotel, Exeter, England, UK.

Poster presented entitled "Carbon monoxide toxicity in the heart: evidence for ischaemia/reperfusion like injury" at SETAC UK 2002 (15th-18th September 2002); held at University Of Plymouth, Plymouth, England, UK.

1ST JUNE 2004.

ASHVIN PATEL.

Abbreviations

AO	Antioxidant
BP	Blood pressure
BW	Body weight
CAT	Catalase
CK	Creatine kinase
CM	Cardiac myocytes
CO	Carbon monoxide
CO_2	Carbon dioxide
COHb	Carboxyhaemoglobin
COMb	Carboxymyoglobin
DCM	Dichloromethane (or methylene chloride)
DFO	Desferrioxamine
DMTU	Dimethylthiourea
ETC	Electron transport chain
FCS	Foetal calf serum
GPX	Glutathione peroxidase
GSH	Glutathione (reduced)
GSSG	Glutathione (oxidised)
GST	Glutathione-*S*-transferase
Hb	Haemoglobin
HEPES	*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulphonic acid
H_2O_2	Hydrogen peroxide
HBO	Hyperbaric oxygen
HBOT	Hyperbaric oxygen therapy
HO	Haem oxygenase
HR	Heart rate
Hsp	Heat shock protein
HW	Heart weight
LDH	Lactate dehydrogenase
LPO	Lipid peroxidation
LV	Left ventricle
Mb	Myoglobin
N_2	Nitrogen
NBO	Normobaric oxygen
NO	Nitric oxide
O_2	Oxygen
$O_2^{\bullet-}$	Superoxide radical

$^{\cdot}OH$	Hydroxyl radical
$^{1}O_2$	Singlet oxygen
$ONOO^{\cdot-}$	Peroxynitrite radical (anion)
PCD	Programmed cell death (or apoptosis)
Q	Cardiac output
RA	Right atrium
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RV	Right ventricle
SOD	Superoxide dismutase
TnI	Troponin I
XDH	Xanthine dehydrogenase
XO	Xanthine oxidase

The present investigation was performed in accordance with the *Home Office Guidance on the Operation of the Animals (Scientific Procedures) Act 1986*, published by HMSO, London.

In memory of All the 'little uns'
that were used for this study

Chapter One:

Introduction

1.1 Background

Carbon monoxide (CO) is an odourless, tasteless, non-irritant, and colourless gas that diffuses across lung alveoli with relative ease compared to oxygen (Traber & Bradford, 1996). Natural gas contains no CO, whereas coal gas has *ca.* 8% CO by volume (de Vries *et al.*, 1977). CO intoxication is the leading cause of unintentional death in the United States (Varon & Marik, 1997) and these properties of CO have led this gas to be termed a 'silent killer' (Henry, 1999). Admissions to hospital with CO poisoning are largely from fire smoke inhalation, suicide attempts and faulty heating systems (Cobb & Etzel, 1991). CO exposure can also occur as a result of smoking cigarettes (Allred *et al.*, 1989; Zevin *et al.*, 2001).

Fire smoke comprises a mixture of gases depending on the fuel source undergoing pyrolysis (Bucci *et al.*, 1978). These include hydrogen cyanide and other irritants (Kulig, 1991; Kulling, 1992; Shusterman *et al.*, 1996). Chemical solvents affect hepatic metabolism; a classic example is the production of cyanide via the microsomal metabolism of aliphatic nitriles, which was shown to be potentiated by acetone and inhibited by CO (Freeman & Hayes, 1988; Scolnick *et al.*, 1993; Willhite & Smith, 1981). Fire victims have a significant correlation between blood cyanide and CO concentrations (Baud *et al.*, 1991). The inhalation of dichloromethane (DCM), a solvent used in many applications, produces endogenous CO through its metabolism by oxidative dechlorination (see *1.4.2.1*). CO is widespread in domestic fire smoke and is recognized as a major determinant of patient survival after fire smoke inhalation (Traber & Bradford, 1996). CO is a toxic by-product arising from the incomplete combustion of carbon containing (carbonaceous) materials (Levasseur *et al.*, 1996). The lethal effects of combustion products were first mentioned by Aristotle in 3 BC (Weaver, 1999):

"Coal fumes lead to heavy head and death."

Exposure to CO during pregnancy (from the maternal inhalation of fire smoke, environmental exposure, or smoking) can damage the developing foetus *in utero*, producing cardiac defects, and limiting the newborns' growth and development (Finette *et al.*, 1998; Ginsberg & Myers, 1976; Nelson, 2001; Sozzi & Pierotti, 1998). It has also been found to alter nerve myelination and vascular reactivity; and change dopaminergic transmission associated with impaired sexual behaviour in newborn male rats intoxicated during prenatal CO exposure (Cagiano *et al.*, 1998; Carratù *et al.*, 2000a; Montagnani *et al.*, 1996). Sphingomyelin homeostasis is impaired in the peripheral nervous system of male rats. These rat brains showed no altered metabolism of sphingomyelin precursors after prenatal exposure to CO (Carratù *et al.*, 2000b). Severe acute exposure to CO can cause foetal death or toxic effects such as anatomical deformities and dysfunction (Norman & Halton, 1990).

Blood [COHb] are elevated in smokers and urban populations due to CO inhalation. Smoking tobacco may initiate angina pectoris as a result of the inhaled CO (Oram & Sowton, 1963). Chronic smokers have blood COHb levels ranging from 4 to 6%; this can augment myocardial hypoxia due to the nicotine-induced coronary vasoconstriction (Walden & Gottlieb, 1990). Some workers have reported these levels to be higher, with blood COHb levels approaching 14% in heavy smokers compared to the 1-2% in non-smokers (Loennechen *et al.*, 1999).

CO toxicity in mammals was solely attributed to tissue hypoxia from CO binding to haemoglobin (Turner *et al.*, 1999). CO binds to the ferrous haem of haemoglobin (Hb) with a greater affinity (*ca.* 250 times) relative to oxygen. Therefore, hypoxia results due to the decreased oxygen carrying capacity of the erythrocytes (Shimosegawa *et al.*, 1992; and described in 1.2.1). Oxygen combines with Hb *ca.* ten times faster than CO, although CO dissociates very slowly from COHb due to its high affinity for Hb (Killick, 1940). CO binds to other haem-containing proteins (haemoproteins) and this binding is also considered to play a major role in the cellular toxicity of CO (Somogyi *et al.*, 1981). One such mitochondrial haemoprotein is cytochrome *c* oxidase whose function is in cellular

respiration (see *1.4.2.3*). This inhibition of cytochrome *c* oxidase leads to impaired cellular function by the limited cellular respiration (Kulling, 1992).

The literature presented (in this chapter) emphasizes the severity of CO poisoning and that CO toxicity does not result solely from tissue hypoxia, and that it may also have direct toxic effects. This chapter will conclude with the hypothesis (**1.7**), and the overall aim and objectives (**1.8**) of the research that are described in later chapters (**1.9**).

1.2 Clinical Symptoms of CO Poisoning

CO intoxication is difficult to diagnose from the clinical symptoms (**Table 1.1**). Chronic exposure to CO can produce flu-like symptoms, headaches, fatigue, visual disturbances, diarrhoea and are easily mistaken for other ailments (Meredith & Vale, 1988; Min, 1986; Raub *et al.*, 2000). It is estimated that nearly one third of all the cases of CO poisoning are undiagnosed (Hardy & Thom, 1994). The symptoms shown in **Table 1.1** have been classified into behavioural and physiological to highlight the severity and diversity of CO poisoning.

Levels of CO can accumulate rapidly in the workplace due to poor ventilation and can be responsible for poor health. Racing drivers have poor psychomotor performance due to CO and heat exposure, i.e. analogous to house fire conditions (Walker *et al.*, 2001). Muscle necrosis has been observed in several cases of CO poisoning and rhabdomyolysis has been found in a patient following the secondary exposure to CO from inhaling fire smoke (Finley *et al.*, 1977; Shapiro *et al.*, 1989) Severe acute exposure to CO can cause heart failure (Ginsberg & Myers, 1976). Autopsy has showed signs of circulatory failure in 45% of cases following acute CO poisoning (Marek & Piejko, 1972). Other uncommon symptoms can be found in the references cited in this section (**1.2**).

Physiological presentation	*Behavioural changes*
Abnormal ECG traces	Cerebral, cerebellar, and mid-brain damage
Cardiorespiratory arrest	(e.g. Parkinsonism and akinetic mutism)
Convulsions	Hallucinosis
Fever	Memory impairment
Gastroenteritis	Mood swings
Hypotension	Reduced level of consciousness
Leukocytosis	Verbal and/or physical aggression
Necrosis of the globus pallidus	
Urinary incontinence	

Table 1.1 Common symptoms observed after CO inhalation that could be misdiagnosed. These physiological and behavioural symptoms were taken from Chiodi *et al.* (1941); Gorman *et al.* (1992); Kulling (1992); Meredith & Vale (1988); and Min (1986).

1.2.1 Carboxyhaemoglobin (COHb)

Haemoglobin (Hb) is the oxygen carrying haemoprotein in blood cells that transports oxygen to the tissues (Goldbaum *et al.*, 1976). Other than carrying oxygen, Hb behaves as a buffer in erythrocytes contributing to the bicarbonate-carbonic acid buffering system. The dissociation of oxygen (O_2) from oxyhaemoglobin (HbO_2) controls (to some degree) the amount of carbon dioxide that can be carried by the blood (Dawber & Moore, 1980).

CO binding to Hb produces carboxyhaemoglobin (COHb), producing a leftward shift in the oxygen-dissociation curve with increasing blood [COHb] (see **Figure 1.1**); therefore, resulting in tissue hypoxia (Roughton & Darling, 1944). The sigmoidal characteristic of the oxygen dissociation curve (in the absence of any CO) is due to the cooperative nature of the haemoglobin molecule, with oxygen being released (at tissues) under low partial pressures of arterial oxygen (Roughton, 1970). COHb reduces the dissociation of oxygen already bound to Hb, therefore, potentiating tissue hypoxia (Broome *et al.*, 1988). The binding of CO to the widely found normal adult haemoglobin, i.e. haemoglobin A (Hb A) has been considered. It should be mentioned for completeness that Hb taken from sickle cell containing blood (Hb S) has a small significant difference in the relative affinity for CO (Rodkey *et al.*, 1974). The lower oxygen carrying capacity of Hb in these blood conditions could exacerbate CO poisoning (Chevalier *et al.*, 1963; Rodkey *et al.*, 1969).

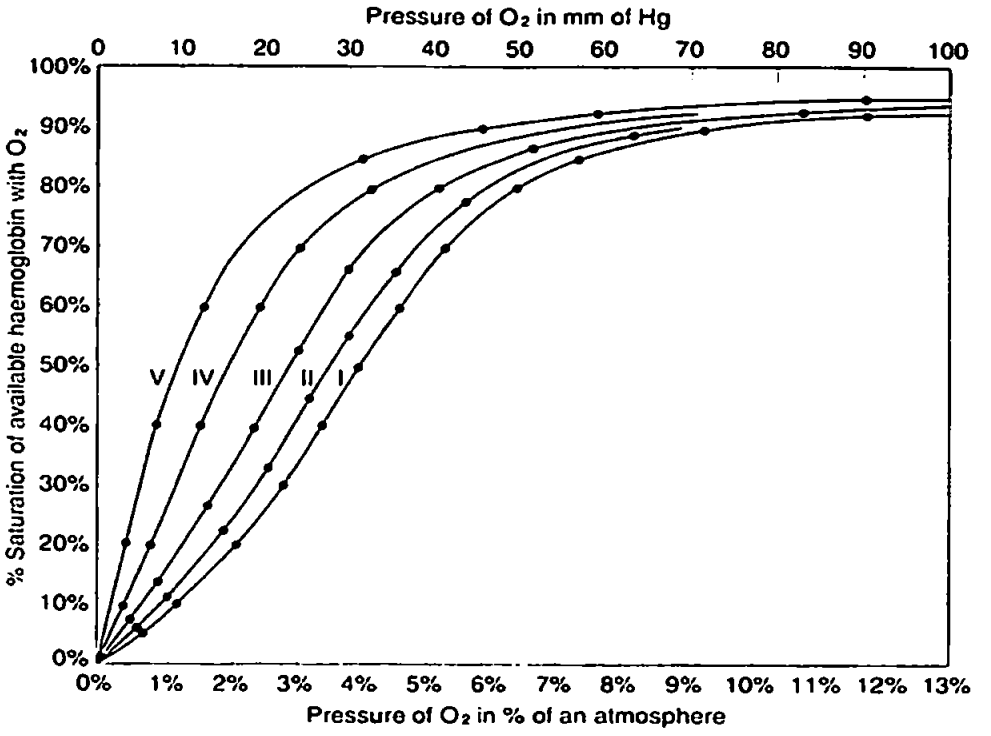

Figure 1.1 The oxygen dissociation curve for haemoglobin in the presence of carbon monoxide (CO). The plots show a loss of the sigmoidal relationship with increasing CO levels. The blood levels of COHb are 0, 10, 25, 50 and 75% are shown by I, II, III, IV and V, respectively. (The illustration is taken from: http://www.phymac.med.wayne.edu/facultyprofile/penney/COHQ/coHaldane1.htm).

The percentage of COHb and observed symptoms following exposure to CO is shown in **Table 1.2**. Blood [COHb] can indicate exposure to CO. The level cannot indicate the severity of the poisoning, predict the outcome, or suggest a suitable protocol for treatment (Brown *et al.*, 1996; Dolan, 1985; Hardy & Thom, 1994; Raub *et al.*, 2000; Scheinkestel *et al.*, 1999; Wald *et al.*, 1981).

CO level in air (ppm)	Estimated range of COHb in blood (%)	Effects
100	10 to 20	Mild headache within 4-6h; and a shift towards anaerobic metabolism.
200	25 to 35	Mild frontal headache within 2-3h; nausea and syncope; mild exertional dyspnoea; and gastroenteritis.
400	40 to 45	Severe frontal headache; nausea within 2-3h; and visual disturbances.
600	55 to 65	Severe headache; nausea in 1h; unconscious in 2-3h with death after 4h; and seizures and coma.
800	> 60	Unconscious after 1h with death in 2-3h; cardiopulmonary dysfunction and death.

Table 1.2 Steady-state concentration of blood carboxyhaemoglobin (COHb) following the inhalation of carbon monoxide (CO) produces symptoms above COHb levels of 10% (Meredith & Vale, 1988). The relationship between the CO concentration in alveolar breath and the blood COHb level is linear (with a correlation coefficient of 0.97) in smokers and non-smokers (Wald *et al.*, 1981). The above data was compiled from Raub *et al.* (2000); Scharf *et al.* (1974); and Varon & Marik (1997).

1.3 Treatment for CO Toxicity

The obvious rationale for treatment is to alleviate the CO-induced tissue hypoxia. CO toxicity and the presence of COHb in the blood has led to using artificial blood to deliver oxygen. Rats whose blood was replaced by a fluorocarbon substitute, that can carry seven percent of oxygen by volume, showed no symptoms of CO poisoning despite being exposed to 10% CO (Broome *et al.*, 1988). The primary treatment of CO intoxicated victims is to remove the CO before it causes any morbidity. Hyperbaric oxygen therapy is the chosen treatment for victims of CO poisoning (below).

1.3.1 Hyperbaric Oxygen Therapy (HBOT)

Hyperbaric oxygen therapy (HBOT) comprises exposing the patient to 100% O_2 at pressures ranging from 2-2.8 atmospheres (atm). All liquids have the ability to dissolve gases; blood exposed to pure oxygen at 38°C and 1 atm will dissolve approximately 2.3ml of oxygen in 100ml, and twice as much oxygen if the pressure is doubled in accordance to Henry's Law (End & Long, 1942). High pressures are used to increase the amount of dissolved oxygen into the blood and so displace CO from haemoglobin within erythrocytes (Smith & Sharp, 1960) and provide oxygen to hypoxic tissues.

The elimination of CO is increased when HBOT is performed compared to normoxic oxygen therapy (100% at 1 atm) or breathing air with half-lives ($t_{1/2}$) of 23, 80, and 320 minutes, respectively (Myers *et al.*, 1985; Scheinkestel *et al.*, 1999). This treatment appears beneficial with reference to the increased elimination and symptoms of CO poisoning (**Table 1.1**); therefore, this is the rationale for its clinical use. However, this treatment is restricted to the facilities that are available at the administered hospital, and guided by the protocol(s) that are based on previous cases of CO and fire smoke inhalation (Broome *et al.*, 1988).

HBOT is used as supplementary therapy in other conditions such as wound healing, arterial gas embolism, calciphylaxis (Dean & Werman; 1998), regeneration of nerves

(Haapaniemi *et al.*, 1998), reversing radiation-induced progressive obliterative endarteritis of small blood vessels (Baert *et al.*, 1998), decompression sickness and it is a primary treatment for symptomatic fire smoke victims (Hammarlund & Sundberg, 1994; Kindwall, 1993; Rogatsky *et al.*, 1999; Uhl *et al.*, 1994). The protocol(s) used for treating fire smoke victims varies between hospitals due to the differing experiences encountered by the hyperbaric teams. Oxygen inhaled at 3 atm occupies 6.4-6.6 ml of blood per 100ml; this is similar to the volume of oxygen used by tissues upon one pass through the capillary bed. This pressure is assumed to supply an adequate level of oxygen to the tissues during hypoxia resulting from the formation of COHb during intoxication (Davis & Hunt, 1977; End & Long, 1942). Haldane (1895) failed to recognise the therapeutic benefits of HBOT while studying mice. In these experiments, mice with fully saturated Hb by CO poisoning, were subjected to oxygen under pressure (2 atm). The results showed live mice after HBO was administered relative to the dead mice (in controls) that were given air (End & Long, 1942).

1.3.2. Risks of HBOT

The work carried out by Bert in 1878 (as described in End & Long, 1942) studied "excessive pressures" on various small animals. Pneumonia or convulsions were observed in these animals and attributed to oxygen toxicity. There are risks of oxygen convulsions during routine HBOT, although this associated risk is low at *ca.* 3% (Hampson *et al.*, 1996). The data (from 300 patients) studied by Hampson and workers showed that increasing the pressure (2.45-3 atm) during HBOT significantly increased the likelihood of seizures. *N*-methyl D-aspartate (NMDA) receptor antagonists have been shown to delay the incidence of these HBO-induced convulsions in male Sprague-Dawley rats (Dale *et al.*, 2000). The body tissues receive an adequate oxygen supply during HBOT. However, some cells/tissues may not be able to utilise this oxygen (due to metabolic inhibition) thereby resulting in localized tissue hyperoxia. CNS injury due to CO poisoning often requires the use of higher pressures than normally used and may produce further injury from oxygen toxicity. The work by Shilling and Adams in 1935 showed oxygen inhalation at 4 atm to be dangerous. However, it could be used at 3 atm for up to three hours and

even longer periods at lower pressures (End & Long, 1942). Morbidity following CO intoxication in man occurs commonly through a loss of higher mental functioning and depression (Gorman *et al.*, 1992).

Jiang & Tyssebotn (1997) showed that HBOT improved the "short-term" outcome assessed by mortality and neurological morbidity in rats exposed to CO (0.27% in air for 1h) compared to NBO treated rats. Lipid peroxidation in the rat brain following CO exposure was prevented by HBOT (3 atm for 45min). HBOT prevented the conversion of xanthine dehydrogenase (XDH) to xanthine oxidase (XO), which is known to be activated by leukocytes and so prevented the leukocyte-associated myeloperoxidase activity (Thom, 1993). However, treatment in this case with 1 or 2 atm HBO, or 3 atm normoxic gas (7% O_2) did not prevent the observed CO-mediated lipid peroxidation. Oxidative stress (1.5) has been reported following HBO exposure (Bearden *et al.*, 1999).

Changes have been observed in the lens nuclei within the eyes of guinea pigs treated with HBO. These observations are typical of those occurring naturally over time and were attributed to cytoskeletal proteins being oxidised due to HBO (Padgaonkar *et al.*, 1999). Acute exposure of HBO is shown to cause significant reversible changes in Sprague-Dawley rat erythrocyte rheology and morphology (Amin *et al.*, 1995). Some patients have been found to undergo middle ear trauma associated with HBOT (Blanshard *et al.*, 1996). Oxygen under high pressures can inhibit nucleic acid synthesis (Rueckert & Meuller, 1960) and shown to produce DNA damage on the first treatment followed by an adaptive response after subsequent treatments (Dennog *et al.*, 1999; Rothfuß *et al.*, 1998). HBO is also shown to be mutagenic in mouse lymphoma cells (Rothfuss *et al.*, 2000). Increased oxygen tensions can enhance Na^+/K^+-ATPase in the brain and impair the vital electrolyte balance (Schmit & Gottlieb, 1982).

1.3.3 Pregnancy and HBOT

The use of HBOT during pregnancy is still under debate, however, some pregnant women have had HBOT following fire smoke inhalation (Brown *et al.*, 1992). Early work by Telford

et al. (1969) showed foetal wastage (as assessed by foetal reabsorption) in rats subjected to HBOT (2 atm for 6h) (Van Hoesen *et al.*, 1989). There is evidence to support and contradict the use of HBOT during pregnancy. Some literature suggests that foetuses are resistant to CO poisoning (Gasche *et al.*, 1993; Mathieu *et al.*, 1996). However, a study on foetal outcome following accidental CO exposure concluded that there were short and long-term risks due to the increased accumulation of CO in the foetus relative to the mother, and a slower elimination of CO by the foetus with respect to the maternal circulation (Gabrielli *et al.* 1995; Koren *et al.*, 1991). Ethical consideration preclude human experiments in this area.

1.4 CO & The Myocardium

The brain and myocardial tissues require oxygen for their function and to sustain life. The high oxygen demand by these tissues limits their tolerance to hypoxia and so they are considered target organs for CO poisoning (Cramlet *et al.*, 1975; Silver *et al.*, 1996). Klebs first noted the involvement of the heart in CO poisoning by recording tachycardia and arrhythmias. He also observed punctiform and diffuse haemorrhages in the pericardium and endocardial tissues (Ehrich *et al.*, 1944). The following will highlight some physiological and biochemical changes that occur in the myocardium due to CO exposure.

1.4.1 Physiological Changes In The Heart

Cardiac complications after CO exposure (**Table 1.1**) arise following changes in the myocardium induced during and/or after CO exposure.

1.4.1.1 Electrocardiogram and tissue abnormalities

Contractile changes suggest immediate damage from CO as shown by changes in electrocardiography (ECG) traces. Ehrich *et al.* (1944) described an alteration in the T-waves and S-T segments of stray dogs that had inhaled CO. The T-wave changes included depression and inversions, however, permanent negative T-waves were evident when the [COHb] in blood was increased to 70-75%. Elevated R-T segment was observed at 40% COHb and heart block occurred when [COHb] exceeded 75%. These workers also found similar T-wave changes after the transfusion of CO-saturated erythrocytes. Microscopic examination of the CO-exposed hearts showed haemorrhages and necrotic regions. However, these workers also concluded degenerative changes in individual muscle fibres based on their observations. Prenatal exposure to 0.0075 and 0.015% CO altered developmental changes in ion channels responsible for the electrical properties of muscle fibres in newborn rats (Carratù *et al.*, 1993; De Luca *et al.*, 1996). CO has caused direct smooth muscle relaxation in a number of tissue preparations (Colpaert *et al.*, 2002).

Other studies conducted in man also show similar damage from CO poisoning (Kjeldsen *et al.*, 1974; Middleton *et al.*, 1961). Anderson *et al.* (1967) found inverted T-waves, depressed S-T segment and a prolonged Q-T period in several cases. Follow-up of these cases in victims who survived showed normal ECG trace recovery after periods ranging from 1 day to 2.5 months. Postmortem of the heart (in one case) showed degenerative focal lesions throughout the heart, although predominantly in the left ventricle. Swollen and necrotic muscle fibres were observed with inflammatory cells in a few regions. Other workers have reported atrial fibrillation and intraventricular block (Cosby & Bergeron, 1963; Hayes & Hall, 1964; Middleton *et al.*, 1961; Stearnes *et al.*, 1938). Focal areas of leukocyte aggregation and punctate haemmorhages are observed in heart tissue after CO exposure (Gürich, 1926; Klebs, 1865).

1.4.1.2 Heart disease and CO exposure

Patients with atherosclerotic cardiovascular disease are at increased risk of morbidity after CO intoxication. The depressed coronary blood flow (prevalent in this condition) cannot compensate for the required increase in coronary flow (CF) during elevated myocardial oxygen demand, such as in (myocardial) hypoxia caused by intense exercise, high altitude or CO exposure (DeBias *et al.*, 1976). Allred *et al.* (1989) showed that blood [COHb] of 2 and 3.9% in coronary artery disease (CAD) patients decreased the time of onset to angina by 4.2% and 7.1%, respectively. Similar findings have been reported in studies using patients with ischaemic heart disease (Anderson *et al.*, 1973; Aronow & Isbell, 1973; Aronow, 1981; Sheps *et al.*, 1987).

CO has been directly implicated by several groups in inducing myocardial infarction (MI), although these findings are rare. Ebisuno *et al.* (1986) reported the case of a 28-year old male who suffered a MI after CO poisoning. The diagnostic evidence (ECG trace, serum enzyme profile and scintigrams) reported for this case led these workers to conclude a MI attributed to CO-induced hypoxia. These workers suggested that CO may induce coronary vasospasm or increase thrombotic formation by decreasing blood flow; therefore,

producing a MI from the reduced myocardial oxygen supply. Similar findings have been reported by Anderson *et al.* (1967); Ayres *et al.* (1970); Marius-Nunez (1990); and Scharf *et al.* (1974).

Ayres *et al.* (1970) showed the development of angina pectoris in patients who showed no previous arteriographic evidence for CAD. They concluded that myocardial hypoxia arising from COHb may be responsible for producing the symptoms of angina pectoris. Scharf *et al.* (1974) estimated the maximum concentration of blood [COHb] to be 15-25% at the onset of symptoms and concluded that this level could cause cardiac alterations as assessed by ECG traces, and was responsible for the observed transmural MI. Physical exertion increases the ventilatory rate and subsequently the rate of uptake of CO. A study using firefighters found that a fixed level of atmospheric CO (0.03%) could quickly elevate the blood [COHb] to lethal levels (Griggs, 1977). An epidemiological study has shown that the cardiac mortality rate for tunnel workers was greater than bridge officers (in New York) due to the higher levels of CO found in the tunnels (Stern *et al.*, 1988).

1.4.1.3 Haemodynamic changes

CO poisoning *in vivo* produces cardiac morbidity and/or mortality. Immediate cardiovascular changes during CO exposure occur to compensate for the CO-induced hypoxia *in vivo.* Long-term change(s) are difficult to predict because of affected myocardial tissue from the initial CO insult (*1.4.1.1* and *1.4.1.2*). Haemodynamic changes in the absence of CO are regulated principally by the oxygen demand. Therefore, factors such as gaseous exchange, temperature, hormonal status and cardiac output (a reflection of the work rate) are constantly changing to maintain the oxygen demand of the tissues. Forbes *et al.* (1945) showed the relationship between COHb saturation and ventilation rate, duration of inhalation and the percentage fraction of inspired CO. Tachycardia is observed during acute exposure to CO in both anaesthetized and conscious dogs and rats. This response appears to be dependent on the [COHb] (Penney, 1988). However, this reflex change in heart rate during CO exposure is not consistent (Penney *et al.*,

1979). Dogs exposed to CO developed bradycardia, although this was later normalised to a regular rhythm by pacing the heart (Dergal *et al.*, 1976).

Other findings also support cardiovascular changes during CO-induced hypoxia. Sylvester *et al.* (1979) showed the stroke volume (SV) increased accordingly (in response to CO exposure) in anaesthetized paralysed dogs with a constant ventilation rate. Similar responses have been observed in the rat model when maintained in a narrow COHb range or increasing the blood [COHb] (Kanten *et al.*, 1983; Penney *et al.*, 1979). Extrapolation to larger mammals would be expected to give similar findings, however, discrepancies have been found. Some workers have observed no change in SV in humans with symptomatic levels of COHb at rest or during exercise (Klausen *et al.*, 1968; Vogel & Gleser, 1972). The lack of response observed in larger mammals such as humans, monkeys and dogs compared to the rat model, could be attributed to changes in peripheral resistance (or afterload) (Penney, 1988) or metabolic rate. The rate of CO uptake required to reach equilibrium with COHb (i.e. saturation) is slower in humans with respect to the rodent model (Montgomery & Rubin, 1971) and suggests that SV may be related to the rate of reaching maximal blood [COHb].

The total volume of blood pumped by each ventricle per minute is called the cardiac output and can be calculated from the product of the HR and SV (Vander, 1990). Cardiac output ($\dot{Q}$) is found to rise in animals acutely or chronically exposed to CO. However, the degree of response varies during the post exposure period and between mammal species (Penney, 1988). Stewart *et al.* (1973) showed a linear increase in $\dot{Q}$ when the percentage of blood saturation of COHb was increased from 0 to 15% in humans. Blood pressure (BP) changes due to CO exposure are less predictable and show a wide range of response with experimental conditions and animal species. A general decrease in arterial BP is observed under acute and chronic CO exposure (Penney, 1988). This CO-associated hypotension is thought to arise from the local effect of CO causing peripheral vasodilation (Coburn, 1979). It should be noted that the *normal* mechanism in response to sensing hypoxia via the carotid body receptors are not stimulated by CO-induced hypoxia,

and only from the arterial oxygen tension (Lahiri, 1980). Low blood [COHb] reduce the myocardial oxygen consumption in conscious dogs (Adams *et al.*, 1973).

Increased blood volume (BV) in cases of chronic CO exposure occurs to maintain an adequate delivery of oxygen, and lower the [COHb] by dilution (Penney *et al.*, 1979; Penney *et al.*, 1984; Theodore *et al.*, 1971; Wilks *et al.*, 1959). The changes following acute CO exposure have not been found in the literature, however, it seems likely that the response may depend upon the degree of hypoxia sustained during exposure. Penney *et al.* (1984) investigated CO-induced cardiomegaly (see *1.4.1.4*) in rat hearts and showed that carboxyhaemoglobinaemia was evident for several weeks after the CO exposure had ceased. Relevant haemodynamic parameters showed a higher working heart rate, increased blood viscosity and volume, whilst Q remained elevated and hypotension had depressed marginally (Penney & Bishop, 1978). The additional work carried out by the heart during prolonged hypoxia, either by CO exposure, high-altitude, or high intensity exercise may be responsible for the changing heart size (Zak, 1973). Stroke work was reported to remain high due to the high blood viscosity (Penney & Baylerian, 1982). The Hb content per unit volume of erythrocytes is similar for different mammalian species and any discrepancies in CO exposure outcomes may not be accounted for by the difference of CO loading in the blood (Enzmann, 1934).

1.4.1.4 Cardiomegaly

Cardiomegaly is a term defining an enlargement of the heart (Clubb Jr. *et al.*, 1986). It is established that chronic exposure to CO can induce cardiomegaly in rats (Penney *et al.*, 1974a). Cardiomegaly is produced by CO-induced hypoxia or by hypoxic hypoxia, e.g. high-altitude (Penney, 1988). This response is age-dependent and generally shows a relatively marked response in younger rats (*in utero* or neonates) relative to adult rats (Styka & Penney, 1978). Cardiac output (Q; *1.4.1.3*) is increased during hypoxia caused by CO (or high-altitude) to maintain adequate oxygen delivery to the tissues (Penney *et al.*, 1992). This larger workload is not tolerated by younger hearts and is considered to be

responsible for the greater myocardial enlargement observed in comparison to older rats (Penney *et al.*, 1979).

Chronic exposure (for 32 days) with 0.05% CO showed sex differences in the growth rates of rats based on their body weight (BW) (Penney *et al.*, 1982). Male rats had a higher growth rate relative to the female group in both air (control) and CO-treated groups. However, CO exposure decreased the growth of both sexes, with the greatest difference being observed in male rats. The ratio of both ventricles (2V) to BW was higher in the CO exposure period relative to the control group, indicating an increase in heart size due to CO exposure. These findings are similar to those reported by others (Dubeck *et al.*, 1989; Penney & Weeks, 1979; Penney *et al.*, 1988; Penney *et al.*, 1992). CO-induced cardiomegaly has been suggested to occur from the combination of rapid haemodynamic responses to COHb saturation and long-term changes that occur after CO exposure.

1.4.1.5 Hypoxia & Cardiac Physiology

Newborn rat cardiac myocytes exposed to increasing hypoxia show increased cell proliferation (Hollenberg *et al.*, 1976). Hypoxia (in foetal lambs) induced by aortic constriction produced an enlarged LV with no observed increase in the cross-sectional area of the cardiac myocytes (Fishman *et al.*, 1978). This suggests that cardiomegaly occurs by hyperplasia (increased cell proliferation) rather than hypertrophy (or increased cell mass). Furthermore, CO exposure in the neonatal period (in Sprague-Dawley rats) resulted in elevated myocyte hyperplasia of the ventricles with the greatest increase observed in the RV (Clubb Jr. *et al.*, 1986). Hyperplasia has also been observed in adult rats chronically exposed to CO. Hyperplasia is greatest in rats exposed to CO during the foetal or newborn period (Penney & Weeks, 1979).

Hypoxia (or CO-induced hypoxia) causes increases in SV and $\dot{Q}$ to satisfy tissue oxygen demand resulting from the depressed oxygen content in the blood (see *1.4.1.3)*. CO increases coronary blood flow to compensate for the myocardial hypoxia from increased

blood [COHb] (McGrath, 1984). Increased haematocrit and blood volume are also observed in response to chronic CO exposure (Penney *et al.*, 1992; Wilks *et al.*, 1959). The overall increase in workload by the heart is thought to contribute in the development of cardiomegaly by an elevated preload (Ford, 1976; Penney *et al.*, 1988). CO-induced hypoxia and hypoxic hypoxia are *not* equivalent; CO-induced hypoxia does alter the coronary perfusion, whereas hypoxia (either by CO or hypoxic conditions) produces a varying degree of cardiomegaly depending on the degree and duration of hypoxia. Hypoxic hypoxia due to high-altitude exposure (or long-term intense exercise) results in an elevated pressure loading in the right hand side of the heart inducing RV cardiomegaly of the concentric type, where wall thickness is increased (Penney *et al.*, 1984). Chronic CO exposure induces cardiomegaly in an eccentric type, where an increase in lumen volume occurs (Penney *et al.*, 1992). To summarise, chronic LV pressure overload leads to wall thickening and hypertrophy, whereas LV volume overload is characterised by an enlarged chamber (Grossman *et al.*, 1975).

1.4.2 Cardiac Biochemistry

This section will introduce some of the general changes that occur in heart tissue following CO intoxication.

1.4.2.1 Endogenous Production of CO

CO is produced endogenously via the catabolism of haem products such as haemoglobin (Coburn *et al.*, 1964) and haemin (White, 1970). The CO produced in this two-step pathway is responsible for the low levels of COHb normally found in the blood (Brouard *et al.*, 2000). **Figure 1.2** shows the mechanism of CO production via haem metabolism. Although CO is produced by this route, this may not actually occur in heart tissue as Vreman *et al.* (1998) found no production of CO in heart homogenates. The role of haem oxygenase (HO) is crucial as its inducible form is expressed under conditions of stress such as hypoxia and hyperoxia (Dennog *et al.*, 1999; Marilena, 1997; Padgaonkar *et al.*,

1997). Cytokines can also induce haem oxygenase (inducible isoform; HO-1) and contribute in the production of CO *in situ* (Marilena, 1997).

CO is also produced through the metabolism of dichloromethane (DCM). The metabolism of DCM proceeds through the cytochrome P450-dependent monoxygenase system and requires NADPH and molecular oxygen (Jung, 2002). This detoxification of DCM results in CO, carbon dioxide and chloride being produced (Wirkner *et al.*, 1997). The detoxifying enzyme is identified as the ethanol-inducible form of cytochrome P450, i.e. cytochrome P450 2E1 (CYP2E1; **Figure 1.3**). DCM is also detoxified by a glutathione dependent pathway that also produces CO (Oh *et al.*, 2002). Cytochrome P450 is suggested to be more inhibited by CO than cytochrome *c* oxidase, therefore, this may play a role in the toxicity of some drugs (Jaffe, 1997).

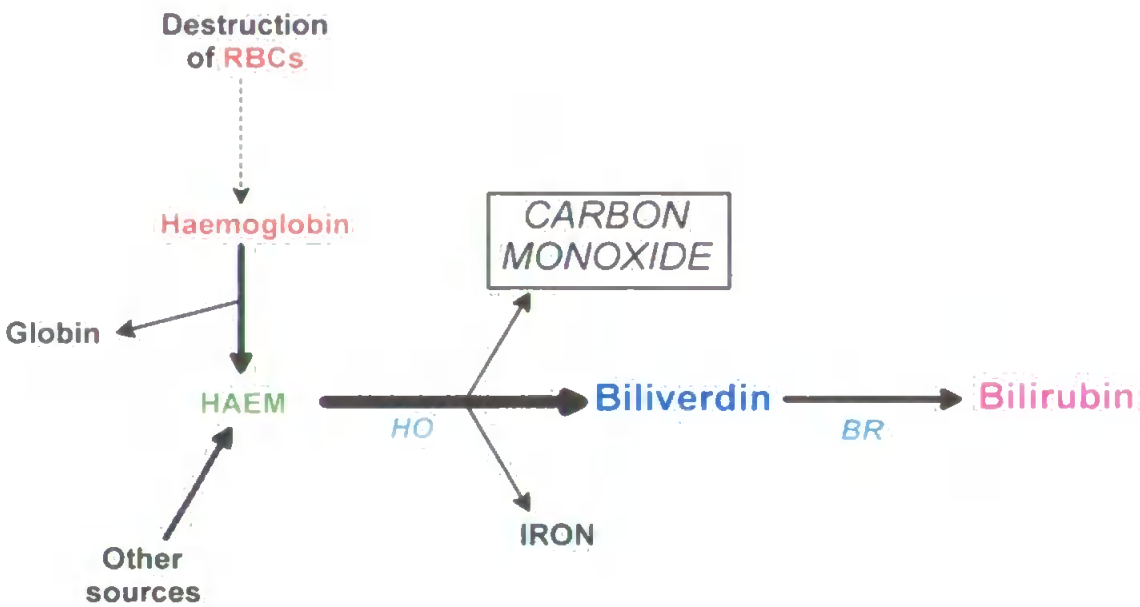

Figure 1.2 The endogenous production of carbon monoxide (CO) through the catabolism of haem. The destruction of red blood cells (RBCs) eventually produces haem. This is enzymatically converted to biliverdin and bilirubin by haem oxygenase (*HO*) and biliverdin reductase (*BR*), respectively. The action of HO produces iron and CO.

CO is also suggested to be metabolized to CO_2 (Tobias *et al.*, 1945) by cytochrome *c* oxidase (Breckenridge, 1953). This oxidative pathway is thought to be a minor detoxification route in clearing CO from the body, however, this pathway may be upregulated under chronic exposure to CO. Mixtures of CO and oxygen have been reported to increase the oxygen uptake of rat skeletal and cardiac muscle via its oxidation to CO_2 (Clark Jr., 1950).

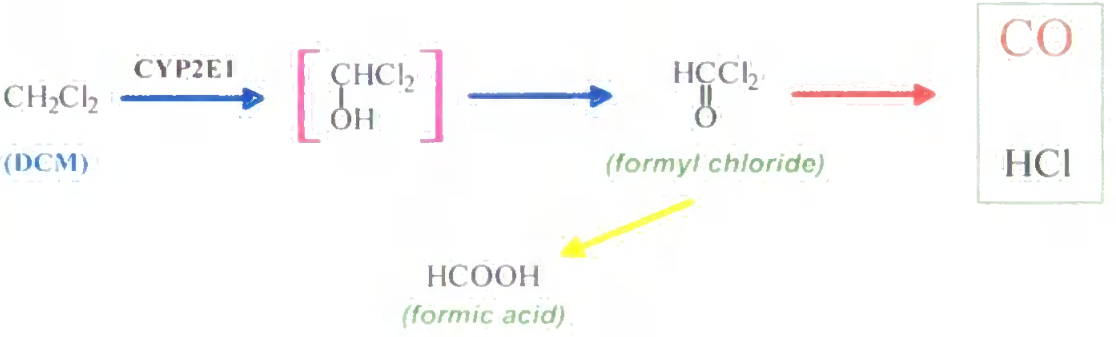

Figure 1.3 The metabolism of dichloromethane (DCM) by cytochrome P450 2E1 (**CYP2E1**) produces CO.

1.4.2.2 Hypoxia & Hyperoxia

CO-induced and hypoxic hypoxia produce biochemical changes that underlie the physiological changes (described in 1.4.1). The underlying biochemical mechanism(s) of these changes are unclear. CO-induced hypoxia may produce metabolic acidosis, changes in plasma levels of glucose, a decrease in the myocardial ATP levels and an increase in the plasma uric acid levels (Hattori *et al.*, 1990). These particular changes are typical of ischaemia/reperfusion and involve oxidative stress (see **1.5** and **1.6**) that is established to be the likely cause of tissue dysfunction (Korthius *et al.*, 1992). Nitric oxide (NO) has also been implicated in various hypoxic conditions. This role of reactive oxygen species (ROS; **1.5**) and reactive nitrogen species (RNS) such as NO are established in the underlying pathogenesis of many tissues. NO has been shown to lower the myocardial oxygen consumption *in vivo* (Sherman *et al.*, 1997).

1.4.2.2 Cellular respiration and mitochondria

CO binds avidly to some haems and its targets include haemoproteins such as Hb, myoglobin and cytochrome P450s (Piantadosi, 1987). One such haemoprotein is cytochrome *c* oxidase (or Complex IV) which has an important role in the generation of ATP via oxidative phosphorylation. The electron transport chain (ETC) has a 'leakage' of ROS and so mitochondria are considered a major site of free radical production (Staniek & Nohl, 2000). The inhibition of cytochrome *c* oxidase by CO (binding to the reduced haem centre) is suggested to increase ROS production in a similar manner to that observed when the ETC is blocked using antimycin (Staniek & Nohl, 1999). This may potentiate tissue dysfunction in accordance to the free radical theory of aging. In brief, the free radical theory of aging proposes that ageing is a result of deleterious tissue damage caused by free radicals. (Cadenas & Davies, 2000; Harman, 1993). These cellular conditions suggest that impaired mitochondrial component(s) may contribute to the underlying cardiomyopathies and other morbidities associated with CO intoxication (Coburn, 1979; Zhang & Piantadosi, 1992).

1.4.2.4 Cell Signalling

The possible involvement of oxidative stress suggests that other diverse mediators may be involved as oxidative stress is responsible for stimulating other signalling pathways in heart tissue (Omura *et al.*, 1999; Shin & Murray, 2001; Tanaka *et al.*, 2001). These include stress kinases, ion homeostasis by disruption of ion-channel regulatory proteins and the recruitment of inflammatory mediators. ROS/RNS have been found to initiate apoptosis (or programmed cell death; PCD) and may contribute to cardiac dysfunction following CO exposure (Piantadosi *et al.*, 1997).

CO is responsible for neuronal signalling and modulating vascular tone (Maines, 1993; Marilena, 1997; Seki *et al.*, 1997). This precedes through the production of guanosine-3',5'-cyclic monophosphate (cGMP) by activating guanylate cyclase (GC; Downard *et al.*, 1997). cGMP is involved in the recruitment of neutrophils and is enhanced by CO (VanUffelen *et al.*, 1996). In bacteria, CO can regulate gene transcription by binding to haemoproteins that serve as sensors (Aono *et al.*, 1996; Fox *et al.*, 1996). The M-subunit of lactate dehydrogenase (LDH) is increased in the hearts of rats exposed to 0.05% CO in air over 42 days (Penney *et al.*, 1974b). CO reduces the number of lysosomes in cultured neonatal rat cardiomyocytes (Brenner & Wenzel, 1972). Levels of endothelin-1 (Et-1) are increased in heart tissue subjected to chronic CO- or hypoxic hypoxia (Loennechen *et al.*, 1999). Et-1 is expressed in endothelial cells, cardiomyocytes and fibroblasts of the myocardium and this may contribute to endothelial dysfunction, which is an early event in atherogenesis, and other vascular diseases such as heart failure, stroke, and transplant vasculopathy. Upregulation of Et-1 is thought to involve the hypoxia-inducible factor-1 (Hif-1; a haemoprotein).

1.5 Oxidative Stress

In general, any condition that generates free radicals and/or other oxidising species (such as reactive oxygen species) or reduces the antioxidant capacity constitutes oxidative stress. Reactive oxygen species (ROS) comprise the hydroxyl radical ($^{\bullet}OH$) (Vlessis *et al.*, 1991), the superoxide radical ($O_2^{\bullet-}$) (Lebovitz *et al.*, 1996) and singlet oxygen ($^1O_2$) (Lee *et al.*, 1999). Hydrogen peroxide (H_2O_2) is also a ROS, however, it has no unpaired electrons and is not classed as a radical (Halliwell & Gutteridge, 1985). These ROS are involved in many pathological states and can also regulate cell/tissue function by modulating numerous signalling pathways (Russell *et al.*, 1989).

Antioxidant capacity is comprised of enzymatic and non-enzymatic components. Enzymatic antioxidants comprise glutathione reductase (GR) and glutathione peroxidase (GPX; Reed, 1990), superoxide dismutase (SOD; Fridovich, 1974), and catalase (CAT; Das *et al.*, 1986). Catalase itself has a high affinity for CO (Coburn, 1979) and this may cause oxidative stress by lowering the activity of CAT. Some non-enzymatic components include ascorbate (Maranzana & Mehlhorn, 1998), reduced glutathione (Kleinman & Richie, 2000), vitamin E (a mixture of the tocopherols; Milchak & Bricker, 2002), albumin (Watts & Maiorano, 1999), histidine (Lee *et al.*, 1999) melatonin (Kondoh *et al.*, 2002) and bilirubin (Asad *et al.*, 2000; and mentioned briefly in *1.4.2.1*). Other cellular/tissue non-enzymatic components are emerging which show protective roles in oxidative stress such as adenosine (Maggirwar *et al.*, 1994). Some of the non-enzymatic antioxidants also exhibit pro-oxidant activity.

The cumulative cascade of oxidant-initiated reactions produces damage to an array of biomolecules including structural, contractile, transport proteins, enzymes, receptors, lipids and nucleic acids (Halliwell & Gutteridge, 1999). These oxidant-induced changes produce a loss of cellular activity or function. For example, the peroxidation of linolenic acid in the chains of cardiolipin, can lead to a loss of cytochrome *c* oxidase activity (described briefly in *1.4.2.3*) which is critical for cellular function (Soussi *et al.*, 1990). The widespread occurrence of lipids and ease of peroxidation can yield devastating cell

dysfunction via changes in membrane fluidity and cellular compartmentalization. These changes can lead to altered membrane receptor mobility, impaired second messenger functioning and cause leakage of intracellular enzymes (such as lactate dehydrogenase and creatine kinase) into the plasma (Jaffe, 1965; Shapiro *et al.*, 1989).

Heart tissue is abundant in haemoproteins (typically myoglobin; and perfused by erythrocytes containing haemoglobin) that may lose their iron and participate in the Fenton reaction (Gutteridge, 1986). The Fenton reaction produces hydroxyl radicals in the presence of peroxides and reactive iron (under appropriate conditions). Oxidant-mediated damage to structural, transport and contractile proteins in the post-ischaemic myocardium and skeletal muscle may explain the contractile dysfunction observed in reperfused muscle tissue (Korthius *et al.*, 1992; and **1.6**). Recent work suggests the role of nitric oxide (NO) in causing the negative inotropic responses observed in heart muscle tissue (Igarashi *et al.*, 1998; Ohashi *et al.*, 1997). CO-induced hypoxia is shown to produce oxidative stress in the mitochondria of rat brains (Zhang & Piantadosi, 1992). Exposure to CO produced a 9-fold production of NO in the brains of rats exposed to CO (Ischiropoulos *et al.*, 1996). Cigarette smoke is also shown to significantly increase oxidative stress in several organs by elevating the level of oxidative adduct 8-hydroxydeoxyguansoine (8-OHdG; Howard *et al.*, 1998). Oxidative stress could induce DNA damage in the organogenesis-stage conceptus that may produce organ dysfunction *in utero* (Vinson & Hales, 2001).

1.6 Ischaemia/Reperfusion (I/R) Injury

In addition to the hypoxic/anoxic injuries caused by CO poisoning *in vivo*, reoxygenation may initiate its own toxic insult and/or potentiate the damage from CO hypoxia, and simulate an ischaemia/reperfusion (I/R) *like* injury. The ischaemia described in this injury refers to the partial or complete cessation of blood supply to a tissue/organ. This injury has been discussed briefly, in light of the likely hypoxia/reoxygenation subjected to victims of fire-smoke inhalation and subsequently treated with hyperbaric oxygen therapy.

The I/R concept was first described for post-ischaemic intestinal tissue and has since been extended to other organs such as the heart, pancreas, liver, kidneys and brain (Korthius *et al.*, 1992). The importance of re-establishing an adequate blood supply to primarily provide oxygen and nutrients to tissues is without question. Much evidence has established that reperfusion results in a number of events that cause tissue injury. Cardiac reperfusion injury is associated with the generation of reactive oxygen species (Das *et al.*, 1986; **1.5**) and can also indirectly produce local ischaemia (or hypoxia) through the 'no-reflow' phenomenon. This event is characterised by capillaries failing to reperfuse once blood flow is restored. Minor morphologic changes are seen in the heart during ischaemia (Hearse, 1977). Reperfusion injury in the heart is also characterised by major ultrastructural changes including cell swelling, release of cytosolic enzymes and depressed contractile function. Other findings include a number of electrophysiologic abnormalities that can lead to potentially lethal arrhythmias (Hearse, 1977).

Reperfusion extends the damage associated with ischaemia and may have a likely role during the oxygenation (by HBOT or NBO) in victims of CO intoxication. The initial hypoxia caused by COHb formation following CO exposure *in vivo* may mimic ischaemia at the molecular level, the subsequent reoxygenation via HBOT (or NBO) could simulate reperfusion and may be responsible for some cardiac dysfunction found in cases following severe CO exposure.

Reperfusion of ischaemic tissues is associated with massive leukocyte infiltration (Carden & Korthius, 1989). Studies in the small bowel and skeletal muscle have shown that neutrophil accumulation after reperfusion is decreased by pretreatment with various oxidant scavengers, for example, SOD, CAT, dimethylthiourea (DMTU; Mohazzab-H *et al.*, 1997) and desferrioxamine (Link *et al.*, 1999). These findings implicate reactive species in the recruitment of neutrophils to post-ischaemic tissues (Granger, 1988) and highlight oxidative damage as a contributing factor in tissue morbidity. The participation of many other signalling pathways in heart tissue is also expected to contribute to reoxygenation injury (Kuzuya *et al.*, 1993).

1.7 Hypothesis

Inhalation of carbon monoxide (CO) via the lungs introduces CO into the body. The inspired CO binds to haemoglobin (Hb) in erythrocytes producing elevated blood [COHb]. This process occurs very rapidly owing to its rapid diffusion during gas exchange and its high binding affinity for Hb relative to oxygen. The COHb has a lowered oxygen carrying capacity as CO occupies the binding sites for oxygen (described in **1.2**). COHb also decreases the oxygen dissociation from Hb by altering the functional cooperativity of Hb. Therefore, CO poisoning *in vivo* produces tissue hypoxia that results in a range of morbidity depending on the degree (or dose) and duration of CO exposure.

The high affinity of haemoproteins for CO is thought to contribute to the toxicity of CO. The binding of CO to haem groups influences its biochemical toxicity and affects cell/tissue function. CO can bind to the reduced cytochrome *c* oxidase (Complex IV of the mitochondrial ETC). Inhibition of mitochondrial respiration is thought to produce an accumulation of reducing equivalents upstream of Complex IV. Therefore, this results in an increased production of ROS, in particular superoxide ($O_2^{\bullet-}$). Myoglobin (Mb) is thought to facilitate the diffusion of O_2 in cells (Adachi & Morishima, 1989; Coburn, 1979; Turek *et al.*, 1973; Wyman, 1966). CO binding to Mb may limit the intracellular diffusion of oxygen to mitochondria for respiration The accumulated $O_2^{\bullet-}$ is likely to cause oxidative stress and lower the antioxidant capacity in affected cells/tissues.

Tissue hypoxia resulting from the formation of COHb can be considered to produce chemical ischaemia (or hypoxia), i.e. similar to ischaemia caused by limiting blood supply. Upon cessation of the CO exposure, either by removing the subject to a CO-free environment (normoxia), or treating the patient with hyperbaric oxygen (HBO) reoxygenation will occur. The mechanism of cardiotoxicity by CO is thought to occur similarly to tissue dysfunction observed in I/R injury (described in **1.6**). The hypothesis postulates that the cardiotoxicity of CO occurs in an I/R-*like* injury and is responsible for the observed CO-associated tissue morbidity. The hypothesis is illustrated in **Figure 1.4**.

Figure 1.4 *(overleaf)* The mechanism(s) of the cardiotoxicity of carbon monoxide (CO) is hypothesised to occur in an ischaemia/reperfusion (I/R)-*like* injury. Carboxyhaemoglobin (COHb) is produced from inhaled CO binding to haemoglobin (Hb) in blood cells. Tissue hypoxia results from the limited binding and dissociation of oxygen (O_2) from Hb. CO also binds to myoglobin (Mb) and produces carboxymyoglobin (COMb) that may limit the availability of O_2 for respiration by the mitochondrial electron transport chain (ETC). These events are considered to produce *chemical ischaemia* (or hypoxia) at the molecular level within cells. In subjects removed to a CO-free environment (or treated with hyperbaric oxygen) this scenario is thought to mimic reoxygenation (or reperfusion). The hypothesis is that this I/R-*like* injury may produce oxidative stress that is responsible for the observed CO-associated heart morbidity. *Key*: HbO_2 and MbO_2 are the oxygenated forms of Hb and Mb, respectively. C*c*O is cytochrome *c* oxidase (or Complex IV) of the ETC. Reactive oxygen species and nitric oxide are denoted by ROS and NO, respectively.

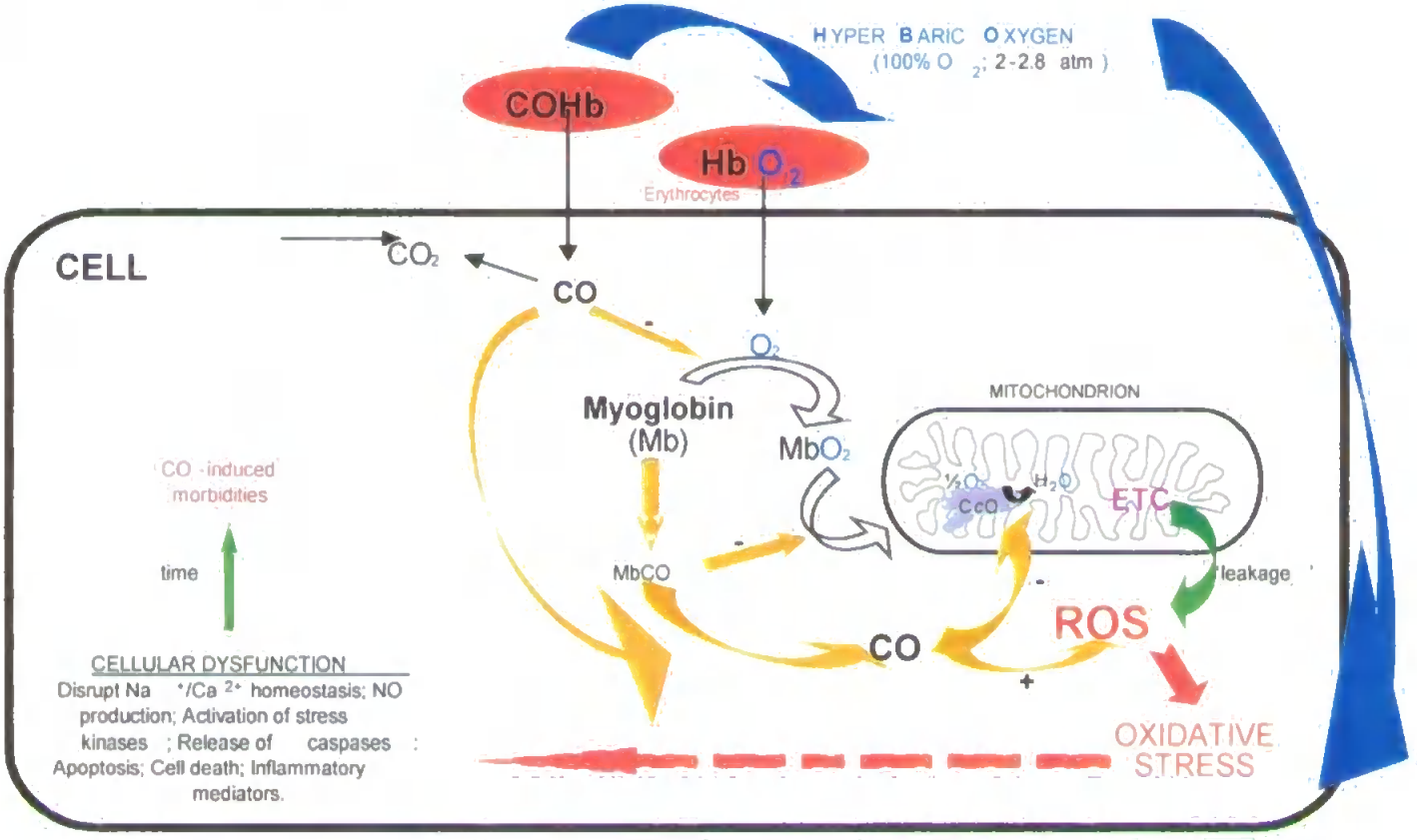
HYPER BARIC OXYGEN
(100% O 2; 2-2.8 atm)
COHb
Hb O2
Erythrocytes
CELL
CO2
CO
O2
Myoglobin
(Mb)
MbO2
MITOCHONDRION
½O2
H2O
CcO
ETC
leakage
MbCO
CO
ROS
OXIDATIVE
STRESS
CO -induced
morbidities
time
CELLULAR DYSFUNCTION
Disrupt Na +/Ca 2+ homeostasis; NO
production; Activation of stress
kinases ; Release of caspases :
Apoptosis; Cell death; Inflammatory
mediators.

Figure 1.4

1.8 Aims & Objectives

Tissue hypoxia occurs during exposure to CO *in vivo*, although the cause(s) of tissue dysfunction in the heart associated with CO-induced hypoxia are not clear at present. The overall aim is to elucidate the mechanism(s) of cardiotoxicity following CO exposure using a suitable model. The chosen cellular model comprised using adult rat ventricular myocytes. This model was chosen firstly, due to its established isolation procedure; and secondly, to allow the cellular changes in cardiac physiology and biochemistry to be determined. This study aims to test that:

(a) CO has direct toxicity in exposed heart cells under normoxic conditions;

(b) cellular/tissue changes in heart tissue occur after acute CO exposure;

(c) intracellular hypoxia produced from carboxymyoglobin (COMb) formation may be a key component in the cardiotoxicity of CO;

(d) test the hypothesis that an I/R-*like* injury may be responsible in producing ROS that may be responsible for heart tissue morbidity following CO exposure; and

(e) administration of antioxidants prior to or during CO exposure may limit the degree of heart tissue damage following treatment with CO exposure.

Specific objectives were:

(i) to establish a suitable model to carry out the desired aims of the research;

(ii) to establish cell markers to measure cell/tissue viability over time under the experimental conditions;

(iii) to identify specific CO-induced morphological cellular changes as well as identifying likely organelle structural aberrations;

(iv) to associate changes in the identified biochemical markers with physiological alterations to examine the underlying occurring events and elucidate the mechanism of CO toxicity;

(v) to show ROS production during CO exposure by looking at appropriate markers of oxidative stress, the role of antioxidants in CO toxicity and identifying the source(s) of ROS production resulting from CO exposure; and

(vi) to evaluate oxidative stress in causing CO-associated heart tissue morbidity by investigating the modification(s) of protein targets such as myoglobin.

1.9 Overview of Thesis

Chapter 1 has highlighted the present findings of CO toxicity with specific emphasis on heart tissue, stated the hypothesis that this study wishes to address, and highlighted the aims and objectives of the research.

The initial *in vitro* model to investigate CO toxicity was to be conducted using isolated adult rat heart muscle cells (in **Chapter 2**)). This was later abandoned due to time constraint, therefore, this work is presented as a developmental chapter for possible reference. The isolated perfused rat heart was chosen as an alternative *in vitro* model to the isolated cardiac myocytes (**Chapter 3**) to examine the cardiotoxicity of CO. **Chapter 3** describes the suitability of this model and presents the preliminary work performed to validate its use in addressing the aims and objectives. The chosen isolated rat heart model was suitable for the devised experiments in assessing the physiological and biochemical changes in heart tissue exposed to CO. **Chapters 4 and 5** show some novel physiological and biochemical changes, respectively.

Chapter 6 addresses the role of oxidative stress in CO poisoning. This chapter features a novel application of experiments that investigate the possible role of mitochondria in producing reactive oxygen species (ROS) during or after CO exposure. **Chapter 7** examines covalently modified myoglobin (via oxidative stress) as a potentially novel marker to determine oxidative stress in rat hearts. Other novel work is included that examined the role(s) of antioxidants in preserving myoglobin function under oxidative conditions. The overall findings from the work described (in the above chapters) are drawn together and addressed as a general discussion with conclusions in **Chapter 8**.

Chapter Two:

Isolated Cardiac Myocyte Model

2.1 Introduction

2.1.1 Background in Using Isolated Heart Muscle Cells

Isolated heart muscle cells (or cardiac myocytes) have been used to examine various changes in cardiac function. Viable cardiac myocytes (CM) show a phenotype found in the intact organ (**Figure 2.1**), therefore, CM are used to assess morphological changes during experimental treatment(s). Isolated CM retain some of their metabolic and biochemical activity (Altschuld *et al.*, 1987). CM have been used previously to evaluate signalling cascades in the heart (Farmer *et al.*, 1977; Laderoute & Webster, 1997; Mizukami *et al.*, 2001); investigate atherosclerosis (Bourne *et al.*, 1997); examine hormonal responses (Eckel *et al.*, 1983; Eckel *et al.*, 1985; Liu *et al.*, 1999; Onorato & Rudolph, 1981); bioenergetic changes (Altschuld *et al.*, 1987; Spanier & Weglicki, 1982), assess the temperature dependency of contractile activity (Vahouny *et al.*, 1970); and examined gene expression by reactive oxygen species (ROS; Cheng *et al.*, 1999; Sabri *et al.*, 1998).

CM have also been used to assess myocardial ion homeostasis (by using fluorescent dyes and voltage-clamp techniques) as these cells retain their electrophysiology (Dow *et al.*, 1981; Shattock & Matsuura, 1993). The role of intracellular calcium in damaging heart tissue during hypoxia/reoxygenation has been shown using this model (Allshire *et al.*, 1987). Relevant articles are cited for interested readers to highlight the many uses of this *in vitro* model in myocardial research: Balligand *et al.* (1994); Boivin & Allen (2003); Brunner *et al.* (2003); Daleau (1999); Hansen & Stawski (1994); Hattori *et al.* (1997); Ikeda *et al.* (1997); Kan *et al.* (1999); Kasai *et al.* (1997); Kukielka *et al.* (1995); Kumar *et al.* (1999); Kuzuya *et al.* (1993); Li *et al.* (1998); Liu & Schreur (1995); Martin *et al.* (2000); Massey *et al.* (1995); Nakajima *et al.* (2000); Nyui *et al.* (1998); Oddis *et al.* (1995, and 1996); Oddis & Finkel (1996); Oh *et al.* (1998); Ohashi *et al.* (1997); Rumsey *et al.* (1990); Sandirasegrane & Diamond (1999); Sarti *et al.* (1994); Shindo *et al.* (1994); Simm *et al.* (1998); Singh *et al.* (1996); Tanaka *et al.* (1998); Turner *et al.* (1991).

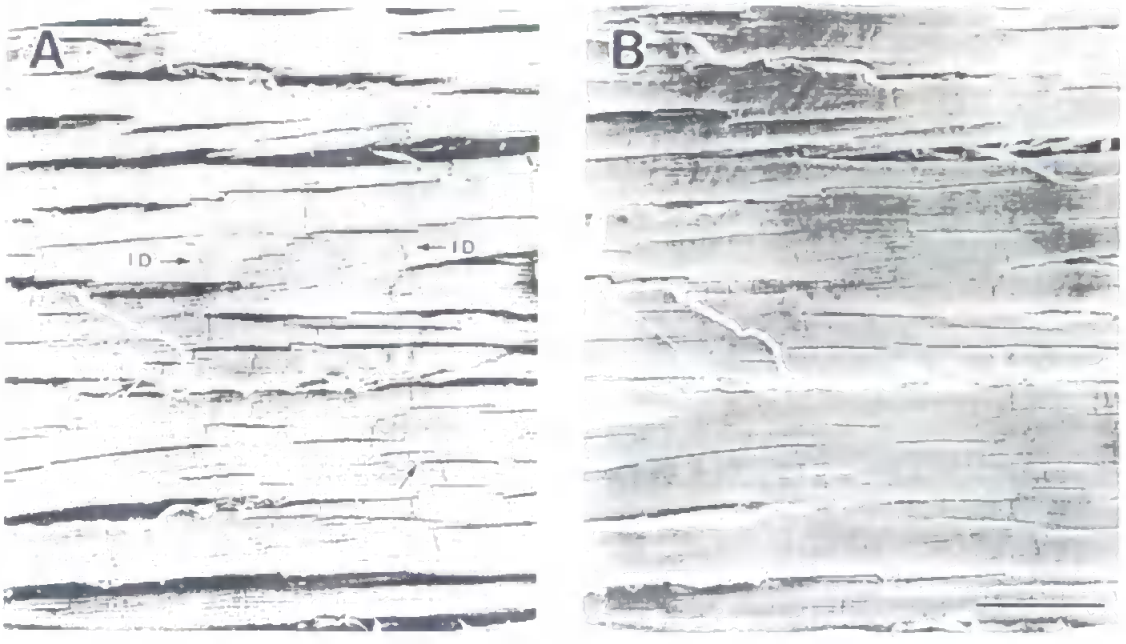

Figure 2.1 The architecture and the arrangement of cardiac myocytes in myocardial fibres with their intercalated discs (marked as ID). Scanning electron micrographs in backscattered electron (**A**) and secondary electron (**B**) images of the same area of healthy ventricle. The scale indicates 20μm (*in panel B*). The electron micrographs were taken from Okabe *et al.* (2000).

This model permits *in vitro* analysis of the properties of CM without interference from other systemic variables such as different cell types, the exogenous influences of hormones, and altered blood flow. Many studies have used this isolated cell model, therefore, this model could be used to examine the myocardial toxicity of CO. The isolated cell model has shown significant uses in many *in vitro* studies. However, further studies are required before any results derived from a model can be extrapolated to the *in vivo* setting or to other species. The following excerpt elegantly summarises this:

"Cells are isolated from the extracellular matrix and from association with other cell types with which they may be interdependent." (Rojkind *et al.*, 1980)

Carbon monoxide (CO) poisoning is lethal owing to its chemical properties (**Chapter 1**). The use of hyperbaric oxygen treatment (HBOT) is established in increasing the rate of elimination of CO from the blood (**1.3**). HBOT has side-effects in some patients undergoing this therapy such as convulsions induced by oxygen under pressure (Hampson *et al.*, 1996). HBOT has been suggested to produce oxidative stress as shown by an elevation in markers of oxidative stress (Bearden *et al.*, 1999; Dennog *et al.*, 1999). This model would also allow the effects of HBOT (following CO exposure) to be studied.

Very few studies investigating CO toxicity using isolated CM are found. Earlier studies examined the effects of CO on several cultured cell types from neonatal hearts (such as endotheloid and muscle cells). The findings can be summarized to the extent that CO inhibited the cell growth, depressed the contractile rate, and decreased specific enzyme activities *in vitro* (Brenner & Wenzel, 1972; Wenzel & Brenner, 1973). Another study has examined the role of myoglobin in CM exposed to CO (Wittenberg & Wittenberg, 1993). No other studies have been performed using CM to specifically evaluate the cardiotoxicity of CO.

2.1.2 Rationale for Isolating Heart Cells & Their Culturing

2.1.2.1 Isolation of cardiac myocytes (CM)

The first goal is to achieve a primary tissue digest of viable cells that are fully differentiated and morphologically similar to the intact heart (De Young *et al.*, 1989). In heart tissue, the muscle cells are firmly connected to each other via intercalated discs and the extracellular matrix (Piper *et al.*, 1990). These connections must be disrupted enzymatically to isolate cells, although the conditions used for cellular dissociation can damage the cells. Cell dissociation requires the absence of calcium to separate the basement and plasma membranes prior to reintroducing calcium to the cells for normal function (De Young *et al.* 1989). Loss of the glycocalyx is not thought to play a role in the biochemistry/physiology of these tissues (Isenberg & Klöckner, 1980). Isolated myocytes

have an external membrane that is selectively permeable to electrolytes. CM have a negative resting potential (-60 to -70mV depending on experimental conditions) and retain the ion conductances and voltage-sensitivities that are essential for action potentials (Powell *et al.*, 1980).

The heart is highly dependent on aerobic metabolism and requires oxygen (**Chapter 1**), therefore, heart tissue must be adequately reoxygenated after being removed from the animal. To prevent tissue hypoxia during cell isolation, the isolated heart is placed on an artificial circulation system, the Langendorff perfusion apparatus (described in **Chapter 3**). This ensures adequate perfusion of the heart to maintain tissue homeostasis and integrity. The perfusion apparatus allows the appropriate balance of salts, nutrients, glucose, and dissolved oxygen at 37°C to be delivered to the organ *ex vivo* for several hours (De Young *et al.*, 1989).

Most methods used to isolate CM involve perfusing isolated hearts with proteolytic enzymes (Kono, 1969) in the absence (or nominally free) of calcium. The gradual reintroduction of external calcium to isolated cells is a major factor in the successful preparation of heart cells. The duration of calcium-free perfusion influences the viability of the isolated cell preparation; it must allow adequate cell dissociation to occur without damaging cells, yet balanced to minimise membrane damage during the reintroduction of calcium (Zimmerman & Hülsmann, 1966). This 'calcium paradox' renders difficult the preparation of viable calcium tolerant heart cells (De Young *et al.*, 1989; Haworth *et al.*, 1989; Montini *et al.*, 1981). However, successful high quality cell isolations are possible, and the calcium paradox has been extensively investigated (Allshire *et al.*, 1987; Alto & Dhalla, 1979; Frangakis *et al.*, 1980; and Lambert *et al.*, 1986).

Ischaemia and anoxia play a vital role in the calcium paradox. Therefore, a rapid excision of the heart and short period prior to the Langendorff perfusion are essential to maximize the yield of viable CM (Cheung *et al.*, 1984; Spanier & Weglicki, 1982). Isolated viable cells may be further purified with gentle centrifugation, with or without density

centrifugation using Percoll or Ficoll (Glick *et al.*, 1974; Powell, 1984; and *2.5.1.6*). However, Percoll is not used as it inhibits ion transporters in the cell membrane (personal communication with Dr. Richard Handy, University Of Plymouth). The dimensions of viable rod-shaped CM vary from widths of 10-35μm and lengths of 80-150μm (Powell & Twist, 1976). The viability of isolated cells is determined from their morphology using light microscopy or by using marker dyes such as trypan blue (Black & Berenbaum, 1964). The cardiac myocyte is a rigid polygonal cell that is easily damaged by mechanical shock compared to other cell types. CM from adult heart ventricle tissue are considered terminally differentiated and non-proliferating. Therefore, they are used at the viability and yield that they were isolated at (Stemmer *et al.*, 1992; Yasui *et al.*, 2000). Determining the viability of intact cells using trypan blue has been shown to depend on other factors such as pH, concentration of dye and the protein content of the medium (Black & Berenbaum, 1964; Piper *et al.*, 1982). Therefore, the viability of the isolated cells were determined from their morphological appearance.

Atrial tissue from adult hearts contain cells that can differentiate, although age is another factor that can influence the isolated yield and cell types (Barnes, 1988; Cantin *et al.*, 1981; Farmer *et al.*, 1977). By using adult rat heart ventricle tissue, CM can be obtained lacking contamination from other cells such as endothelial cells, fibroblasts, and the extracellular matrix (ECM). It has been found that using rats older than four days yield CM that do not develop rhythmic spontaneous contractions in culture, and the yield of viable cells declines with age from 5 day-old animals (Kasten, 1973; Stemmer *et al.*, 1992). Therefore, this gives an impression that using rats older than 5d does not yield good isolated cell preparations, although optimal cell preparations can be isolated from 180-220 grams post-weaned adult Sprague-Dawley rats (personal communication with Dr. Richard Handy). Numerous biochemical and physiological differences exist between embryonic, neonatal, and adult cells. The number of nuclei in CM vary with species and age. Human myocytes are predominantly mononucleated, whereas rat myocytes have one or two nuclei depending on the age of the animals. Binucleation develops during the early postnatal period of rats, whereas adult values are reached at 12-14 days of age (Korecky

et al., 1979). Adult mammaliam CM do not undergo DNA synthesis and show no mitotic activity (Cantin *et al.*, 1981).

2.1.2.2 Primary cell culture of isolated cardiac myocytes (CM)

Cell isolation renders the freshly isolated cell preparation to lose some of its intracellular content; therefore, studies using this model have to ensure some degree of similarity to the *in vivo* state. The isolation procedure produces a large number of damaged cells (Piper *et al.*, 1982). Isolated CM are cultured to mirror or approach the *in vivo* intracellular content. In brief, a period of recovery aids the repair of this superficial damage and allows the physiological state of the cells to stabilize. Freshly isolated calcium tolerant CM lose some of their intracellular ATP and creatine phosphate content (Farmer *et al.*, 1983) and show a gradual replenishment over the first day in culture (Piper *et al.*, 1982). The loss of ATP may be attributed to a compromised sarcolemma (Stemmer *et al.*, 1992).

Adult ventricular CM do not divide in culture and so these cultures have to be prepared using freshly isolated cells. These cells have to be used at the density at which they are plated, although CM from atrial tissue show some potential to divide (Stemmer *et al.*, 1992). The short-term culturing of CM can enrich the viability of the cells. Long-term culturing is not applicable for the proposed experiments as cell morphology is shown to change significantly (Piper *et al.*, 1990). The viable rod-shaped cells do not form confluent cultures as they do not attach to the dish without spreading. Long-term cultures can spread and form a few contacts, however, these contacts are not over the whole culture region.

2.2 Aims

The *in vitro* cellular model uses adult rat ventricular myocytes to allow cellular changes in cardiac morphology and biochemistry to be studied following CO exposure. Cell biochemistry would be performed to associate any structural changes with biochemical measurements to evaluate the order of events. Biochemical parameters for examination include the cellular antioxidant status, mitochondrial function, monitoring the release of intracellular enzymes to determine cell viability, assess for any production of reactive oxygen species (ROS), and examine ion fluxes based on the cellular electrolyte content. The aims were to:

(a) to investigate the immediate effects of CO exposure and subsequent HBO on cellular oxidative metabolism by monitoring mitochondrial (respiratory) enzyme activities;

(b) to investigate ROS production by looking for appropriate markers of oxidative stress; and

(c) to look at cellular changes after acute CO exposure (and/or HBO) with particular emphasis on cytochrome *c* oxidase inhibition and mitochondrial function.

2.3 Materials & Methods

Animals were supplied from A. Tuck & Son Ltd. (Essex). Chemicals and reagents were provided from Sigma Chemicals (Dorset) unless otherwise stated.

2.3.1 Animal Handling and Removing the Heart

Hearts from adult male Sprague-Dawley rats (230-250 grams) were rapidly excised after an intraperitoneal (*i.p.*) injection of 60mg sodium pentobarbitone (Sagatal, Rhone Merieux) and 10mg heparin (sodium salt from porcine intestinal mucosa; 1820 USP units) and immediately transferred to ice-cold cardioplegic (in mM: NaCl 130; KCl 20, $MgCl_2$ 1, $CaCl_2$ 1, NaH_2PO_4 1, Na_2HPO_4 4, procaine 1; HEPES 10, and D-(+)-glucose 11; adjusted to pH 7.4 with 4M NaOH). Unwanted tissues such as the lungs and thymus were removed to allow access to the aorta whilst maintaining the heart in ice-cold cardioplegia (to reduce its metabolism). The heart was then weighed in a tared Petri dish containing cardioplegia. The aorta was cannulated to achieve retrograde perfusion. A secure fit was tested by passing ice-cold cardioplegic (*ca.* 10ml) and observing blood leave the heart via the micro-circulation indicating good perfusion, i.e. no damage to the aortic valve (personal communication with Dr. Maria Ödblum). The cannulated heart was then fitted to the Langendorff perfusion apparatus to commence cell isolation (2.3.2). The heart was perfused at $6ml.min^{-1}.g^{-1}$ of heart tissue. Care must be taken to ensure that no air bubbles are introduced to limit the tissue perfusion. Note, at this point the heart beats itself and aids in ejecting the remaining blood.

2.3.2 Isolation of Calcium Tolerant Cardiac Myocytes (CM)

This was performed using the modified method of Montini *et al.* (1981). Medium A is Joklik's Modification of Minimum Essential Medium (ICN) supplemented with 20mM HEPES. Medium B is 0.1% BSA (fatty acid free) and 0.05% collagenase (Type II; Batch number S7B951, Worthington Biochemical) in medium A. Medium C contained 0.25mM $CaCl_2$ in Joklik's medium (as above) with 1% BSA. Calcium-free Joklik's solution (medium A) was perfused for 2min (at 36.9°C and pH 7.0), with a flow rate of two $drops.s^{-1}$ (of

perfusate) from the apex of the heart. This was collected and discarded to remove the blood and prevent unwanted material being recirculated (below). Collagenase (medium B; pH 7.2) was then perfused for 2min to washout medium A, at a flow rate equivalent to one drop.s^{-1}. At this stage the heart swells with no severe colour change. Medium B is recirculated for 16min.g^{-1} of tissue (wet weight).

The heart was removed and the ventricular tissue sliced away from the unwanted atria and blood vessels. The ventricle tissue was homogenised rapidly with two scalpels in a gentle manner to yield soft tissue. This tissue was transferred to a 50ml sterilin pot containing *ca.* 8ml of collagenase (medium B) at 37°C and gently pipetted to aid cell dissociation and placed on a metabolic shaker at 100 strokes.min^{-1} with gentle bubbling of medical oxygen (100% O_2). This incubation produces a heavy suspension of cell debris that was filtered through nylon gauze (200μm pores supplied from Lockertex Ltd., Warrington, Cheshire) after 5-10 min; carefully ensuring not to pour off the large tissue pieces. The remaining tissue was incubated in fresh collagenase (medium B) and was repeated another 5-7 times. An equivalent volume of medium C (pH 7.3) was added slowly to the filtrate to reduce the digestion by collagenase; also to introduce calcium to the cells to heal gaps in their glycocalyx, and to minimise the calcium paradox occurring later. The filtrate fractions were stored at ambient temperature to minimise Na^+-loading; with constant gentle bubbling of oxygen to prevent hypoxia and maintain the cell viability. These fractions were then centrifuged at 30*g* for 50s in 13ml centrifuge tubes (Sarstedt). The pellet was *gently* resuspended using a Pasteur pipette, as mechanical force can damage these sensitive cells (personal communication with Dr. Richard Handy), and assessed for viability by counting the elongated rod-shaped cells and expressing this as a percentage relative to the total number of cells (using a haemocytometer and cell tally counter). The trypan blue assessment for viability was not performed due to time and the good association of elongated morphology with viability (Piper *et al.*, 1982; Powell & Twist, 1976).

The fractions with good viability's, i.e. greater than 60%, were pooled and washed in medium C by centrifugation (as described above). The pellet was resuspended in a *ca.* 7ml Joklik's medium containing 0.5mM $CaCl_2$, and left at room temperature for 10-15min with gentle inversion of the centrifuge tube and regular oxygenation. These cells were then washed and resuspended in Joklik's medium containing 1mM $CaCl_2$ for 10min before washing. The cells were washed in 1mM calcium to remove unwanted cells (i.e. those cells with a round morphology due to hypercontracture via Ca^{2+}-overload) by two centrifugations at 30*g* for 70 and 60s, respectively. The cell suspension was resuspended in 1ml culture medium (below) at room temperature, and its viability determined followed by a cell count to dilute the cells appropriately for culturing (2.3.3).

2.3.3 Primary Cell Culture

Isolated CM were plated at a density of 2 x 10^4 cells per well in 6-well culture plates (Nunc) using glutamine- and serum-free medium M199 containing Earle's salts with $NaHCO_3$ and 1.2mM calcium (Piper *et al.*, 1982). Six-well culture plates were pretreated with 1ml 4% foetal calf serum (FCS; for 2-3h at 37°C) in a humidified air atmosphere with added CO_2 (5%) to the container. The serum was removed by washing each well twice with 2ml of culture medium (glutamine-free medium M199 containing Earle's salts with $NaHCO_3$) prior to plating. The isolated CM were diluted to a density of 2 x 10^4 cells.ml^{-1} in culture media M199 supplemented with (in mM): creatine 5, L-carnitine 2, taurine 5; and essentially fatty acid free BSA (0.2% w/v), insulin (0.1μM), penicillin (100 U/ml) and streptomycin (100μg/ml). Cells were plated by adding 1ml of cell suspension to each well containing 2ml of culture media. Cells were plated by aspirating the cells evenly over each well to obtain a primary culture of CM. The cells were incubated under similar conditions used for pretreating the culture plates. Cell media was changed at 3h, 24h and then every 24h by slowly removing 2ml of media from the centre of each well, and replaced with 2ml of oxygenated (95% O_2/5% CO_2) culture media at 37°C. The elongated rod-shaped cells adhere at a faster rate compared to the dead (rounded) cells, and an increase in the viability is observed relative to the viability determined after cell isolation. Cultures were

maintained for 2-3d and the cells were removed for experimental use by gently agitating the plates in a circular manner.

2.3.4 CO/HBO Experiments

To evaluate the cardiotoxicity of CO, we proposed to expose the cultured CM to different duration and doses of CO, whilst performing biochemical assays and monitoring the morphological cellular changes. The viability of the CM was assessed by scraping off the attached cells, quickly spinning down the cells, and assaying the cell culture media (supernatant) for intracellular enzymes. The intracellular enzymes chosen for assessing cell viability were lactate dehydrogenase (LDH) and creatine kinase (CK) (Cheung *et al.*, 1984; Penney & Maziarka, 1976). Viability would be expressed as a percentage from experimental and detergent (0.1% Triton-X 100 at final concentration) lysed cells using specific enzyme activities. Some of the CM pellet would be fixed with buffered glutaraldehyde to assess ultrastructural changes. The cell pellets were to be stored for isolating mitochondria to evaluate mitochondrial function following CO exposure.

The duration of CO exposure would be: 0, 2, 5, 10, 20, 30 and 60min. The use of 6-well plates would allow three wells to be treated with CO prior to biochemical/histological analysis. The remaining three wells would undergo HBOT with suitable air-matched (normoxic) controls from another plate. The 3 wells would serve as replicates; with a total of 6 plates to be used at each dose and duration of CO exposure to allow satisfactory data analysis. The HBO exposure would be performed at 1-3 atm to evaluate the effect on cellular function and assess for any recovery following CO exposure (below).

The doses of CO to be used were: 0, 0.05, 0.1 and 0.2%. The certified gas mixtures were purchased from BOC Gases (Dorset) and all contained 21% oxygen (O_2), 5% carbon dioxide (CO_2) balanced with nitrogen. The gases contained 5% CO_2 to be used in conjunction with the bicarbonate buffer present in the cell culture media. The HBO treatment was to be performed in pressure vessels that can hold up to 4 cell culture plates. These pressure vessels were tested for air tightness and withstand pressures up

to 3 bars. These vessels were constructed from stainless steel to prevent CO adsorption, therefore, ensuring adequate CO exposure to the cells. Some of the treated cells would be fixed for histological preparation to assess for ultrastructural change(s) to comment on tissue physiology and associate any biochemical changes.

2.4 General Findings

This section shows and discusses the work performed to isolate cells for culturing purposes. The format of this chapter is not consistent with following chapters due to the developmental nature of this work. The data shown is expressed as the **mean ± SEM** (unless otherwise stated) with the number of hearts used (*n*). No statistical analysis was performed to determine the significance of each variable changed; instead, the effectiveness of each change was evaluated qualitatively using the determined viability.

2.4.1 Cell Isolation

Adult ventricular cardiac myocytes (CM) were isolated from male Sprague-Dawley rats (230-260 grams) using aortic retrograde digestive perfusion with collagenase (Montini *et al.*, 1981). In brief, the method produced intercellular dissociation by perfusing the isolated heart with an essentially calcium-free media. This is followed by cell dissociation from the extracellular connective tissue using collagenase with a low level of calcium. The aim is to isolate calcium tolerant CM (Frangakis *et al.*, 1980; Rodrigues & Severson, 1997). The rapid introduction of calcium to physiological levels can damage the cells and this event is termed the calcium paradox. Calcium tolerant CM were required for our purposes and obtained by gradually reintroducing calcium. Initially, the cell isolation resulted in low yields of viable cells. Cell viability was determined by counting the rod-shaped (viable) cells using a light microscope and expressing it as a percentage of the total cells. Dead cells appeared as round hypercontracted 'blebs' as illustrated in **Figure 2.2**.

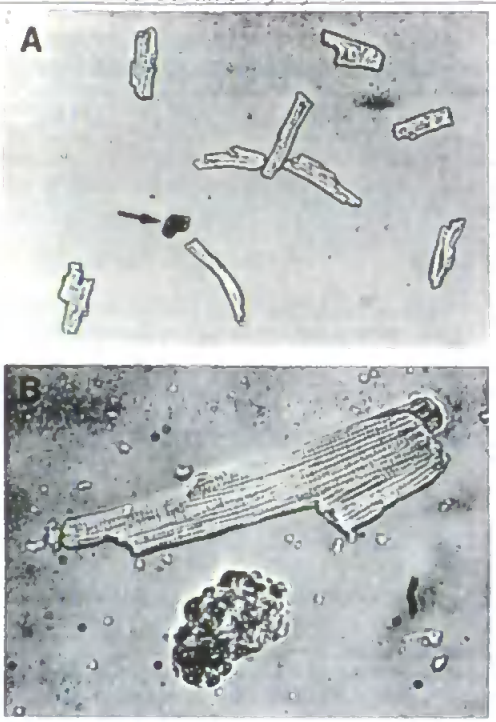

Figure 2.2 Isolated cardiac myocytes. Viable myocytes exhibit a long rod-like shape (*ca.* 20 x 100μm; Nash *et al.*, 1979), whereas, dead or dying cells appear highly rounded (*arrow*) or in transition (**A**). Higher magnification light micrograph illustrates the intercalated disc region and crossed striations in a viable cell (**B**). The intercalated disc regions have the presence of protein and polysaccharide (Yokoyama *et al.*, 1961). The figure is taken from Rodrigues & Severson (1997).

Firstly, the *in house* method was employed (as used previously by Dr. Maria Ödblom; described in 2.3.1 and 2.3.2). This method isolated calcium tolerant CM of 18 ± 24% viability (mean ± SEM; range: 0 to 50%; n = 6 hearts). The greatest viable cell preparation was obtained from the first heart (50% viability of $4x10^4$ cells), whereas the other hearts showed decreasing viability and cell numbers. The total numbers of cells obtained from the other 5 hearts were not determined following their poor viability.

Many changes were performed to improve the viability and yield (see below). In brief, a minimum of 3 hearts was used (where stated otherwise) to evaluate each variable of the protocol. The reason(s) for the change are discussed with the results of the modified step.

The number of animals used to optimise this model was 48 and highlights the amount of work performed (over 8 months). Although 48 animals were used for isolating and culturing the cardiomyocyte cells, the number of hearts used was 39 and 14 for the isolation and culturing of cells, respectively. The loss of 9 hearts were attributed to technical faults and are described below (*2.4.1.1* to *2.4.1.8*). The primary culture of isolated CM was performed when there were sufficient numbers of cells to use; otherwise it would be costly and labour intensive.

2.4.1.1 Contamination of the Langendorff apparatus

The administration of anaesthesia, removal of the heart from the animal, and the preparation of reagents (for washing and perfusions) were conducted using sterile utensils and working conditions. The presence of organic and inorganic impurities in the water for preparing the perfusing and incubation buffers was of analytical grade (purified by using the Milli-Q filtration system) as myocardial cells are sensitive to impurities (Piper *et al.*, 1990). The initial low viability (above) was overcome by flushing the Langendorff perfusion apparatus (with 70% ethanol) followed by water. A white emulsion was observed when rinsing with water and was considered to be cellular material, as the apparatus had not been used for several months (prior to commencing work). These isolated cells had 41 ± 29% viability (range: 8-62%; n = 3 hearts) and the total cell count ranged from 7.21×10^5 to 2.28×10^6 cells ($1.65 \times 10^6 \pm 8.22 \times 10^5$ cells).

2.4.1.2 Osmolarity and pH of perfusing media

The pH and osmolarity of each reagent used was measured prior to their use to ensure that no shock treatment was subjected to the hearts. The osmolarity of all the solutions were in the ideal range for cardiac tissues of 290-320 $mmol.kg^{-1}$ (or $mOsm.l^{-1}$; Starr *et al.*, 1999). No marked deviations were found to account for the poor yield and viability of cells.

2.4.1.3 Perfusion of heart tissue

The heart weight (HW) was estimated from the body weight (BW) for the first 11 hearts; based on the assumption that HW is 0.3% of the BW (as used previously by Dr. Maria Ödblom and Dr. Richard Handy). This was found to be incorrect with the HW being greater than 0.3% of the BW (*ca.* 0.6%). This may have produced hearts to be underperfused when perfused using a flow rate of $6ml.min^{-1}.g^{-1}$ (of wet heart tissue). Therefore, to reduce the risk of error hearts were weighed prior to cannulating the organ. Underperfusion of the tissues may account for the low cell viability and/or cell yield observed during the cell isolation. Any underperfusion may also exacerbate any tissue ischaemia incurred following the removal of the organ (from the anaesthetized animal).

Following this the hearts were perfused at the same flow rate (FR) of $6ml.min^{-1}.g^{-1}$ based on the actual HW, rather than the estimated HW (above). However, this FR appeared to show no marked difference as observed by the similar FR of perfusate falling from the apex of the heart (*ca.* 2 $drops.s^{-1}$). The viability also showed no improvement to that obtained previously. The mean viability was 49 ± 10% with a total cell count ranging from $8.3x10^5$ to $1.8x10^6$ cells (mean total cell count of $1.27x10^6 \pm 4.16x10^5$) from 4 hearts. Five animals were used to evaluate the correct FR; although only 4 hearts were used. One heart was rejected as a thrombus was observed inside a ventricle when removing the atria following its perfusion. An anticipated low viability due to a poor tissue perfusion prevented any further use of this heart.

2.4.1.4 Collagenase

Calcium is a cofactor for collagenase and tissues tend to soften faster when calcium is restored (Haworth *et al.*, 1989). The incubation of collagenase (in nominally calcium-free medium) has *ca.* 50μM trace calcium that is sufficient to activate the enzyme. Collagenase disrupts the cellular connections with the extracellular matrix (ECM) for a suitable period prior to producing cellular/tissue damage. Therefore, it has to be used in a controlled manner to avoid damaging isolated cells. The proteolytic enzymes in collagenase are

poorly defined, however, the enzyme and batch used in our methods were tested and shown to work prior to their use (by Dr. Maria Ödblom).

Collagenase perfused the hearts for 16.5min.g^{-1} (wet weight of tissue) following the calcium-free perfusion (medium A for 2min). The period of calcium-free perfusion was increased to 3min to allow greater loss of calcium from the intercalated discs to assist the action of the collagenase. Although the period is *ca.* 2min to completely switch over (equilibrate) with the previous perfusing media (by personal communication with Dr. Richard Handy). All the reported viability's are from cells in the final suspension, i.e. in the presence of 1mM $CaCl_2$. Perfusion with collagenase for 2min ensured that the perfusion buffer was equilibrated with collagenase prior to recirculating the heart (for 16.5min.g^{-1} tissue). However, the extended calcium-free perfusion (of medium A by 1min) may have resulted in a loss of essential cofactors (for collagenase) from the heart. Some components of the ECM are necessary for cell dissociation by providing cofactors for collagenase (personal communication with Dr. Maria Ödblom). This may appear to be the case as Piper *et al.* (1982) also recirculated collagenase to aid digestion of the ventricle tissue.

Collagenase was also added to the stock bottle prior to its use during the calcium-free perfusion (rather than standing *ca.* 10min prior to use). This was performed routinely after the 9th heart. This change may have reduced some loss of collagenase activity from its incubation at 45°C prior to perfusing the heart (at 37°C). The cell numbers did not increase compared to the previous levels and the cell viability decreased. The cell viability and yields were 30 ± 20% (n = 3) and $1.43 \times 10^6 \pm 1.06 \times 10^6$ (n = 2; as the information to determine the yield was not recorded for 1 heart), respectively.

To maintain a stable preparation of active collagenase to perfuse the heart and/or compensate for loss of the necessary ECM cofactors (above), we increased the level of collagenase from 0.05 to 0.06% (w/v). This change was considered to be satisfactory and lay within the recommended range, as too much collagenase can dissociate CM quickly

and render them susceptible to later damage by the calcium paradox. The increase in [collagenase] produced no improvement in cell viability or yield. The mean cell viability and yield of isolated cells was 46 ± 3% and $1.16x10^6$ ± $7.26x10^5$ cells, respectively (from *n* = 2 hearts). Three animals were used to determine the viability when using 0.06% collagenase, however, 1 heart could have been poorly perfused due to the absence of tissue swelling after its perfusion with collagenase. This may be accounted for by damage to the aortic valve, as the tissue was stiff relative to the soft state normally found (following its perfusion with collagenase). This heart was cannulated in the normal manner prior to its perfusion. However, the heart had slipped off its cannula during the perfusion (with collagenase) and may have been damaged when being recannulated.

2.4.1.5 Introduction of calcium to the cell suspensions

The level of calcium in the perfusate during cell isolation is critical and a sensitive window exists between 20-25μm Ca^{2+} (Haworth *et al.*, 1989). Calcium was added back slower than before to ensure we were not rapidly introducing calcium to the cells. This was performed by adding calcium solutions made at a lower concentration. This change did not improve the cell viability or yield. Cell viability was 44 ± 12% (*n* = 6 hearts) with cell numbers ranging from $2x10^5$ to $1.68x10^6$. All the reported viability's are from cells in the final suspension, i.e. in the presence of 1mM $CaCl_2$ (i.e. prior to culture) The mean cell yield was $8.60x10^6$ ± $6.32x10^5$ (*n* = 5 hearts, as the information from 1 heart was not recorded to determine its yield). The stepwise addition of calcium chloride ($CaCl_2$) lowered the cell viability at each addition. However, the viability remained for at least one hour when gently oxygenated similar to the findings of Montini *et al.* (1981).

Eight animals were used to evaluate this variable; although 6 hearts were used to compile this information as 2 hearts were rejected. One heart was discarded after air had entered the organ and led to a poor perfusion. Therefore, this was expected to produce a cell preparation of low viability as the poor perfusion produced patches of yellow/white regions

indicating the calcium paradox. The other heart failed to swell after the collagenase perfusion and could suggest a poor perfusion and/or inactive collagenase.

2.4.1.6 Introduction of calcium to the perfused heart

We next adopted the method of De Young *et al.* (1989), whose rationale introduced calcium back to the perfusing heart during the collagenase perfusion rather than isolated cells (*2.4.1.5*). Unfortunately this method did not improve the viability or yield of cells. The mean cell viability was 33 ± 9% (n = 3) and the mean yield was $9.15 \times 10^5 \pm 7.78 \times 10^4$ (n = 2; as the information to determine the yield for 1 heart was not recorded). Five animals were planned to evaluate the method of De Young *et al.* (1989), although only 3 animals (hearts) were used successfully. One heart was rejected after air entered the organ and this was observed too late to salvage the tissues. The other heart appeared dead before being perfused as observed by the lack of contractile activity when placed in ice-cold cardioplegic. This heart was anticipated to yield no viable cells, although it was perfused before being discarded when it showed no signs of contraction during the perfusion. De Young *et al.* (1989) obtained *ca.* 70% viability with low yields, however, they also used Percoll to purify out dead cells by centrifugation. Using Percoll (or Ficoll) sedimentation step(s) were not used as these are difficult to remove, and their presence could interfere with the proposed biochemical assays (from personal communication with Dr. John Moody, University Of Plymouth).

2.4.1.7 Pore size of nylon gauze

A range of nylon gauze with different pore sizes were used to rule out that some viable cells may be retained from the filtrate resulting in low viability's. The final cell suspensions (in Joklik's-medium containing 1mM $CaCl_2$) were divided into 3 batches before passed through nylon gauze with pore sizes of 150, 200 and 250μm. The ideal pore size was found to be 250μm as this had higher viability compared to those cells passed through the smaller pore sized gauzes. These findings are consistent with the gauze used by De

Young *et al.* (1989). This is reported using 2 hearts, although 4 animals were used to evaluate this variable. Two hearts were expected to yield cells of poor quality as one perfusion was inevitably prolonged (due to the fire alarm); and the other may have become ischaemic during the removal of blood (from the anaesthetized animal). The latter heart showed contractile activity, although it displayed signs of a poorly perfused heart, i.e. not swollen (in this case).

2.4.1.8 Increasing the cell yield

The low cell yields obtained did not allow the required number of culture plates to be made to perform the any experiments. Therefore, in order to increase the yield of cells the perfusion apparatus was modified to perfuse two hearts. This modification comprised a double luer fitting to allow two hearts to be perfused simultaneously with an appropriate set-up to allow the recirculation (of collagenase).

The cell isolation protocol perfused hearts at a flow rate (FR) of 6ml.min^{-1}.g^{-1}, therefore, a higher flow rate was used to ensure satisfactory perfusion of both hearts. The FR used was 10ml.min^{-1}.g^{-1} as described in the method of Piper *et al.* (1990) for perfusing two hearts simultaneously. The FR was not uniform as shown by a different flow of perfusate from each heart (in drops.s^{-1}). This could be due to the difference in heart weight producing an uneven perfusion by gravimetric flow. Using two hearts resulted in increased cell numbers (not determined), however, the observed cell viability was very poor (15 ± 3%; n = 3, i.e. 6 hearts in this case). This was anticipated from the poor tissue perfusion (suggested above) occurring in at least one heart. A poor perfusion may have led to a greater loss of calcium, and the subsequent loss in viability may be potentiated by a poor oxygen supply.

2.4.2 Primary Cell Culture

Primary cell culture was incorporated to allow time to perform the proposed CO experiments (described in 2.3.5). The cell isolation procedure may be responsible for producing oxidative damage similar to an ischaemia/reperfusion-like injury during organ excision and prior to its perfusion (**1.6**). Oxidative damage to freshly isolated cells is shown to occur in the liver, however, no references were found to support this for heart tissue. The work performed to increase (or at least maintain) the viability of CM following their isolation is shown below (in *2.4.2.1* to *2.4.2.4*).

2.4.2.1 Pretreating of culture plates

The presence of foetal calf serum (FCS) in the culture medium following the pretreatment of culture plates produces CM that retain their elongated shape during the first 12-24h and most become spherical by the end of day two. The absence of FCS in the culture media (after pretreating plates) has been shown to preserve the elongated morphology of CM for up to 2 weeks (Piper *et al.*, 1990). The role of FCS in the primary culture of CM is for cellular attachment. However, the precise nature of the constituents of FCS and its mode of action during attachment is not clear (Piper *et al.*, 1990). Serum-free culture conditions were used for the culturing of adult CM, although prior to plating cells the culture plate(s) were pretreated with FCS (in media). Neonatal CM require different culturing conditions and contain a diverse range of cell types due to the presence of fibroblasts (Polinger, 1970; Van Der Laarse *et al.*, 1979). Cultured neonatal CM exhibit different phenotypes compared to when isolated and do not accurately represent the *in vivo* picture. Neonatal cells were not used because they are not fully differentiated and may introduce other variables into the study.

The duration of pretreatment determined the degree of attachment. It was found that a pretreatment period of 6h required careful handling during media change. However, a longer pretreatment (28h) allowed harsher conditions to be used due to the greater attachment (of both dead and viable cells). It was also observed that viable cells that had

attached loosely prior to the media change would not survive (in most cases). Longer pretreatment conditions (>8h) showed aggregates as cells adhered rapidly between attached viable and unattached viable/dead cells. Cells also attached at a faster rate relative to a shorter pretreatment period (such as 2-4h). The adhesion of viable and dead cells is unaccountable. The shorter period appeared to favour the attachment of viable cells. Although it may occur due to a higher level of calcium (1.2mM) in the culture medium relative to 1mM calcium (in Joklik's media). Excluding the pretreatment step resulted in the attachment of both dead and viable CM. This absence also produced a greater loss of cell viability (in the same batch of isolated cells) compared to cells cultured in pretreated plates. These findings support the use of pretreated culture plates. The pretreatment period must be used to compromise between attaching predominantly viable cells from freshly isolated cells and maintaining the attachment (of viable cells) during media changes.

Tissue culture plates used from different manufacturers are shown to produce different outcomes when culturing CM. Tissue culture grade polystyrene by Falcon and Costar (used here) were shown to be successfully pretreated by serum (Piper *et al.*, 1990). The pretreating of plates (with FCS) prior to culturing adult CM was shown to be necessary. The duration of FCS incubated in culture plates (for pretreatment) was found to vary between methods (shown in **Table 2.1**). The difference in pretreatment time may arise from the initial cell viability prior to culturing. However, most of these workers (in **Table 2.1**) did not cite their isolated cell yield and viability prior to culturing.

Table 2.1 (*overleaf*) Cell culturing conditions used for cardiac myocytes isolated from rat hearts.

* personal communication with Dr. Richard Handy (Department of Biological Sciences; University of Plymouth). [a] M199 is Medium 199 culture media; [b] BSA is bovine serum albumin; [c] FCS is foetal calf serum; [d] PBS is phosphate buffered saline; [e] HEPES (buffer) is *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid); [f] MEM is Minimum Essential Medium; [g] NEAA is non-essential amino acid.

Culturing conditions	Culture media	Supplements used	Reference
Plated 10^5 cells per well (in pretreated 6-well culture plates). Used 4% FCS[c] or laminin (10μg/ml) to pretreat culture plates.	Gln-free M199[a] with Earle's salts. 37°C; 95% O_2: 5% CO_2	Creatinine (5mM), L-carnitine (2mM), taurine (5mM), 0.2% essentially fatty acid-free BSA[b], insulin (100nM), penicillin (100 U/ml) and streptomycin (100μg/ml).	Handy*
Plated 1.5 x 10^5 elongated cells per 60mm culture dish. Attached cells for 30min after pretreatment (24h) with 4% FCS in M199[a]. Used PBS[d] for cell washes.	Modified Gln-free M199[a] with Earle's salts	0.2% BSA[b], insulin (100nM), penicillin (100 U/ml) and streptomycin (100μg/ml).	Volz *et al.* (1991)
Pretreated tissue culture plastic or glass with 4-8% FCS[c] in 3ml M199 medium per 60mm dish (5-15h). Change media 1-4h after plating cells if cells are required for ≥12h. Media changed normally every third day after the first media change.	M199 with Earle's salts buffered with bicarbonate-CO_2 (or HEPES[e] with no CO_2)	Creatinine (5mM), L-carnitine (2mM), taurine (5mM), 0.2% BSA[b], insulin (100nM), penicillin (100 U/ml) and streptomycin (100μg/ml).	Piper *et al.* (1990)
Plated 7 x 10^4 cells in 35 plastic dishes coated with 0.1% gelatin.	Gln-free MEM[f] with Earle's salts; 10% FCS[c]; vitamins (x2) and 1 x NEAA[g]. 37°C; 5% CO_2 in air	For first 7 days media had 10mM araC to prevent fibroblast growth. Always supplemented with 1% penicillin and streptomycin.	Eppenberger *et al.* (1988)
3h attachment period after ≥5h pretreatment period. Used 35mm petri dishes.	BM 86-Wissler media with 4% FCS[c]. 37°C; 16% O_2: 5% CO_2: 79% N_2	Streptomycin (100μg/ml) and Penicillin G (100 U/ml).	Eckel *et al.* (1985)
3h attachment period after pretreated overnight with 4% FCS[c] in M199 (37°C and 5% CO_2). Used 60mm petri dishes.	M199 media	0.2% BSA[b], insulin (10nM), penicillin G (250μM) and streptomycin (250 μM).	Piper *et al.* (1982)

2.4.2.2 Culturing conditions

Glutamine and serum-free media supplemented with creatine, carnitine and taurine prolong the survival of elongated CM in culture (Volz *et al.*, 1991). Glutamine was omitted from the culturing conditions as this amino acid serves as a signal for proliferating fibroblasts (Clark Jr., 1976) and shown to develop the spherical shape of CM in serum-free conditions (Piper *et al.*, 1990). Taurine (2-aminoethanesulphonic acid) was supplemented in the culture media as it is found in mammalian tissues. In heart tissue, levels range 20-26μmoles.g^{-1} (wet weight), and this has been suggested to be equivalent to a concentration of at ≥20mM (Militante *et al.*, 2000). Taurine is thought to modulate the movement of sodium ions across the sarcolemma (osmoregulation), modulate protein phosphorylation and calcium fluxes in heart tissue. Carnitine and taurine have a similar effect to creatine and together prevent cell rounding and contribute in replenishing intracellular ATP levels (Piper *et al.*, 1990).

Culture medium should be changed between 1-4h after plating the isolated cells (Piper *et al.*, 1990). This ensures the removal of non-attached cells and other cell debris (**Table 2.1**). The pH of the culture media was assessed after observing the red colour of the supplemented media turn pink (under the culturing conditions used). The colour dye indicating pH qualitatively showed alkalosis in the culture media. The bicarbonate buffer system used was dependent upon 5% CO_2. However, this buffer system did not appear to be effective and the addition of HEPES to the culture media was thought to maintain a stable pH. This was performed by supplementing the culture media and adjusting to pH 7.4 at 37°C. This resulted in more cells surviving (after being plated) as observed prior to the first media change. The media change resulted in cell viability's of *ca.* ≥70%, although with a marked loss of cell numbers. Supplementing culture media with HEPES led us to use commercially available Medium 199 with HEPES. The culture media was oxygenated prior to its use. However, this oxygenation was difficult to perform as it caused frothing of the culture media due to the presence of BSA. This frothy media is not recommended during cell culture (personal communication with Dr. Christine King, University of

Plymouth); therefore, the container holding the culture plates was oxygenated prior to incubating the plates.

The findings of Eppenberger *et al.* (1988) showed that cultured adult CM undergo extensive morphological transitions after 4d in culture to a less differentiated state. Small myofibrils were identified in the cells and these cells showed altered shapes and distribution of the myofibrils. The inclusion of serum increases the rate of differentiation in the cellular myosin profile (Nag & Cheng, 1986). This suggests that the cultured cells may have to be used within 4d (after being cultured) to maintain a consistent cell type. These morphological changes question the validity of published studies using these culturing conditions.

2.4.2.3 Calcium in cell culture

A gradual introduction of calcium is necessary to avoid the calcium paradox and to allow the intercalated disc region to recover from the cell dissociation process (Isenberg & Klöckner, 1980). The calcium (in the culture media) could contribute in restoring the extracellular structures found in the intercalated disk regions and may account for the aggregation of cells (observed here).

2.4.2.4 Distribution of cultured cardiac myocytes

One common observation was the absence of an uniform distribution of cells after plating isolated CM. Unattached cells tended to congregate towards the centre of each well. This could not be resolved either by gentle agitation of the culture plates, or by spreading the cells over the well area during plating. The layer of unattached cells may starve the attached viable cells by consuming oxygen and limiting oxygen for the attached viable cells, as CM are highly dependent upon oxygen (De Young *et al.*, 1989). Furthermore, CM do not like to be in contact with each other in culture, as either one or all the cells in contact die (personal communication with Dr. Richard Handy). Therefore, this led us to plate cells at lower densities, i. e. 2×10^4 cells compared to 1×10^5 cells. The cells plated

at a higher density showed a greater viability relative to those plated at a lower density after the 3h wash. These findings agree with Yasui *et al.* (2000) who showed cell-cell interaction to be important for cell function and survival (**Figure 2.1**).

2.5 Discussion

The low cell viability may have been caused by hypoxic injury resulting in the hypercontracture of isolated cells (Hohl *et al.*, 1982). These hypercontracted cells are shown to lack the mechanisms for normal calcium homeostasis (Allshire *et al.*, 1987). Oxygen utilization is increased in myocytes following the addition of calcium (Frangakis *et al.*, 1980). The increasing presence of calcium may have raised the oxygen consumption of these cells. Therefore, it may be worthwhile to increase the oxygen levels gradually in parallel with the calcium levels. However, this may compromise cell viability by the 'oxygen paradox'.

Dead (rounded) CM may release pro-apoptotic factors during their hypercontracture that may be responsible for a low stability of the isolated viable cells. This suggests a threshold effect, where the loss of viability may trigger cell death by (necrosis or apoptosis) in the remaining viable cells. This could explain the requirement of high viability preparations of isolated cells, typically to exceed ≥70% based on the numerous studies that have used this model. The preparation of high viable fractions may not release a lethal level of mediators responsible for producing death in neighbouring cells.

2.6 Conclusions

Cell culture could not be used to maintain the viability of the isolated cardiac myocytes, possibly due to the poor viability obtained after their isolation. The discussed findings may serve as a reference for other workers who propose to use this model. The expenditure of resources and time led us to consider using another model.

Chapter Three:

Isolated Perfused Heart Model

3.1 Introduction

The isolated rat heart preparation were evaluated as an alternative *in vitro* model to investigate the cardiotoxicity of CO. In brief, this model should allow a rapid approach (at a lower cost) to examine cardiac changes compared to using isolated cardiac myocytes (**Chapter 2**). This chapter examined the possible use of this model to study the cardiotoxicity of CO. We have considered the appropriateness of the model; shown the technical feasibility in preparing this model; examined its validity; found suitable markers to determine tissue viability; and demonstrated a range of CO levels to investigate the toxicity of CO.

3.1.1 Isolated Perfused Rat Heart Model

Isolated perfused hearts allow biochemical, physiological function (i.e. contractile and electrophysiology), pharmacological, vascular biology, and tissue histology to be studied. These experiments are performed in the absence of other influencing factors such as organs/tissues, hormones, and neural control. Therefore, allowing changes to be examined that may otherwise be unobserved. Such cardiac changes include those that occur to maintain adequate oxygenation of tissues during chronic exposure to CO (1.4.1 and 1.4.2). Isolated hearts are not usually perfused with blood. Crystalloid solutions are usually used for delivering oxygen and substrates, for example, the Krebs Henseleit buffer (KHB; Neely *et al.*, 1967). An absence of blood allows the direct effect(s) of CO to be studied in heart tissue under conditions lacking systemic hypoxia (due to the absence of carboxyhaemoglobin from blood). The loss of peripheral innervation may be rectified by using perfusion buffers with added chemicals such as neurotransmitters to approach *in vivo* conditions.

Biochemical analysis can be performed on heart tissues by preparing tissue homogenates. This allows time course studies to be carried out (Yue *et al.*, 2002). Isolated rat hearts have been used to examine the effect of free radical producing systems

in situ (Chahine *et al.*, 1997); to study ischaemia (Mousa *et al.*, 1987); and protein turnover (Morgan *et al.*, 1971; Rannels *et al.*, 1975). Hearts from numerous species can also be used by this model to evaluate species differences. However, the issues of cost, animal ethics and appropriateness have to be considered (Galinanes & Hearse, 1990a). Previous related work (using this model) mostly studied the effects of CO- and hypoxic-hypoxia on the vasculature (McGrath & Chen, 1978; McGrath & Smith, 1984). Other work has shown histological changes to occur in the heart following CO poisoning (Kjeldsen *et al.*, 1974). The established use of isolated hearts to examine ischaemia/reperfusion (I/R) injury (**1.6**), and the postulated role of I/R-like injury in the cardiotoxicity of CO (**1.7**) favour its use for this study.

Most models have limitation(s) that vary in degree with the nature of the conducted experiments. The advantage of removing other factors that influence tissue function *in vivo* yields preparations that dissociate from the *in vivo* setting. The lack of sympathetic and vagal tones can be compensated (to some extent) by artificially pacing the heart preparation. This requires the necessary hardware and could mask ventricular arrythmias that may arise from the treatment(s). This *ex vivo* preparation gradually deteriorates, however, it does allow studies for several hours. Rat hearts have a very short action potential duration and this can limit its value (in terms of extrapolating any findings to the human heart) in arrhythmogenic studies (Galinanes & Hearse, 1990b). The species used is important in any study considering extrapolating the findings from one species to another.

3.1.2 Heart Preparations: Langendorff versus Working Heart

The isolated hearts can be prepared in two ways.

3.1.2.1 Langendorff preparation

The Langendorff preparation comprises cannulating the aorta of the isolated heart prior to its perfusion with oxygenated buffer. The perfusion buffer is delivered in a retrograde manner to the organ, by either constant pressure (60-100 mmHg) or flow (delivered by infusion or a peristaltic/roller pump). The perfusing buffer forces the aortic valves to close and the buffer perfuses into the coronary arteries. This perfuses the heart tissues via the coronary arteries before draining into the coronary sinus (Wexler & Walsh, 1996). The coronary drainage occurs in the right side of the heart from the pulmonary artery. Most of the present methods are adapted from the original design by Langendorff (1895). The Langendorff preparation was chosen due to its simplicity in setting up (relative to the working heart preparation; *3.1.2.2*), and it is established in evaluating cardiac performance. This model does not require pacing as the working heart preparation.

3.1.2.2 Working heart preparation

This is more complex relative to the Langendorff preparation (described above). This preparation comprises ventricle filling prior to ejection through the aorta, i.e. the heart behaves as *in vivo*, hence its name. It is essentially a left heart preparation that conducts its own work whilst perfusing its own coronary system, and it is paced to maintain oxygenation close to the physiological range (Mousa *et al.*, 1987). This preparation requires the Langendorff preparation to be modified after the heart is stabilised following its isolation. This preparation has a greater expenditure of energy and subsequently a higher consumption of oxygen. These particular attributes are likely to render the working heart more susceptible to ischaemic injury (Galinanes & Hearse, 1990a). However, this preparation allows other indices of cardiac function to be determined, i.e. different filling pressures and afterloads (Neely *et al.*, 1967) relative to the Langendorff preparation (Galinanes & Hearse, 1990b; Grupp *et al.*, 1999).

3.1.3 Aims & Objectives

The aim to investigate the toxicity of CO in the heart appears feasible using the Langendorff heart preparation. It is envisaged that the isolated heart model will allow the effects of CO and hyperbaric oxygen treatment (HBOT) to be investigated to address the hypothesis (**1.7**).

Specific objectives include:

(a) to establish a protocol for using the Langendorff heart preparation;

(b) to evaluate lactate dehydrogenase and creatine kinase as markers of tissue viability of the isolated heart preparation; and

(c) to determine a suitable range of CO exposure to produce results that may be extrapolated for clinical use.

3.2. Material & Methods

3.2.1. Isolation of Rat Hearts

The heart was isolated following the *i.p.* administration of anaesthesia and heparin as described previously (in 2.3.1).

3.2.2 Langendorff Perfusion

The aorta was cannulated using surgical silk to attach the heart to the Langendorff apparatus for retrograde perfusion (Powell, 1984). Prior to perfusing the heart with the Langendorff apparatus, the cannulated fitting was tested by passing ice-cold cardioplegia (*ca.* 10ml), to ensure that the suture was secure to prevent the heart from detaching off the perfusion apparatus (as described in 2.3.1). This also ensured that no damage was caused to the aortic valve and removed some blood from the tissues. Observing blood perfuse out from the heart surface in the coronary ostia region during the slow infusion of cardioplegic indicates no damage to the aortic valve. The heart was attached to the Langendorff apparatus with the perfusion buffer flowing at $0.1ml.min^{-1}$ to prevent air bubbles entering the heart. Perfusion buffer (118mM NaCl, 4.7mM KCl, 1.2mM KH_2PO_4, 1.2mM $MgSO_4$, 1.8mM $CaCl_2$, 20mM $NaHCO_3$ and 11mM glucose) was constantly gassed with 21% oxygen, 5% carbon dioxide (balanced with nitrogen) to pH 7.4. The flow rate (FR) was increased gradually so as not to cause any damage to the aortic valve and coronary vasculature to $6ml.min^{-1}.g^{-1}$ (wet weight). At this point the heart beats itself and this aids in removing unwanted blood from the tissues. The heart was maintained in a humid environment by placing cling-film over the chamber housing the isolated heart. Hearts that failed to beat within 30s were removed and discarded from the study.

3.2.3 Experimental Design

In brief, the hearts were exposed to CO and examined for any changes with the CO-treatment. Hearts were isolated and perfused for *ca.* 10min to remove blood and allow the

organ to stabilise following the excision procedure (pretreatment period). Hearts were perfused with buffer saturated with 95% oxygen and 5% carbon dioxide during this pretreatment period. CO exposure was started by perfusing hearts with buffer saturated with 0, 0.05, 0.1 or 0.2% CO (with 21% oxygen, 5% carbon dioxide, and balanced with nitrogen) for 30min, followed by a 90min CO-free period (by perfusing the hearts with petreatment perfusion buffer). Three hearts were used for each treatment with 0-0.2% CO. The heart rate and perfusate flow was determined in some hearts (3.2.4). The CO gas mixtures used were certified mixtures supplied from BOC Gases (Dorset).

3.2.4 Heart Rate & Perfusate Flow

The basal or pretreatment (i.e. pre-CO) heart rate (HR) was determined 5 minutes prior to treating the heart by perfusing equilibrated CO solution (3.2.3) and recording the HR at various timepoints during and after the CO exposure. The HR of the isolated organ was determined by visually counting the number of heart beats during a 30s period and expressing the HR in beats.min^{-1} (bpm) at that timepoint. Perfusate flow (PF) out of the organ was determined (in some hearts) by measuring the collected volume of perfusate at various time points to assess the myocardial coronary function. Perfusate samples were collected from the heart at various time points and kept on ice (in 1.5ml eppendorf tubes) prior to assaying (3.2.5).

3.2.5 Biochemical Assays

The spectrophotometric assays (below) were conducted using a HeλIOSβ UV-visible spectrophotometer (UNICAM) that was fitted with a temperature controlled cuvette holder. The activity of the markers (and other analytes) in the perfusate were determined from the assays (below) and expressed per gram (wet weight of heart tissue) to normalise the data.

3.2.5.1 Lactate dehydrogenase (LDH)

The LDH activity was measured after each perfusion in fresh perfusate samples that were placed on ice prior to determining the LDH activity (≤1h after completing each perfusion). The perfusate sample (0.1ml) was added to 2.9ml of assay reagent (in a 3ml cuvette), rapidly mixed using a paddle and the initial decrease in absorbance at 340nm was recorded over 30s at 25°C. The assay reagent used comprised 50mM sodium phosphate buffer (pH 7.5) containing 0.6mM pyruvate (sodium salt) and 0.2mM NADH (final assay concentrations) that were added in powder form prior to use. Enzyme activity was determined by running known standard activities (0-5 $U.ml^{-1}$) prepared from L-lactic dehydrogenase (Type XXXV) from porcine heart (Sigma).

3.2.5.2 Creatine kinase (CK)

The activity of creatine kinase was determined in stored perfusate samples using a commercial assay kit (catalogue number: 47-20, Sigma). The assay was performed as described in the manufacturers instructions.

3.2.5.3 Nitric oxide (NO)

Nitric oxide was measured by determining its immediate breakdown product, nitrite (NO_2^-), using the Griess assay. This was performed after each perfusion using the method in Yamamoto *et al.* (1998).

3.2.5.4 Ferric reducing ability of plasma (FRAP) assay

The FRAP assay was performed as described by Benzie & Strain (1996).

3.2.6 Data analysis

The heart rate and assay measurements were performed in triplicate and showed good agreement. The data shown is the mean ± SEM of 3 hearts at each CO dose tested. No statistical analysis was performed in this preliminary study.

3.3 Results

The Langendorff model was tested using a range of dissolved CO gas mixtures containing 0, 0.05, 0.1 and 0.2% CO (in 21% O_2 with 5% CO_2 and balanced with N_2). The heart is very sensitive to acid so an effective buffer was employed. The established bicarbonate buffering system was used in conjunction with 5% CO_2 as performed by other studies using isolated hearts (Galinanes & Hearse, 1990; Humphrey *et al.*, 1987).

Please note that no statistical analysis of the data was performed (in this chapter). The study was performed to examine whether the isolated heart model is a valid model to test our hypothesis (**1.7**).

3.3.1 Heart Rate & Perfusate Flow

Control hearts (with no added CO) appeared to beat constantly at a similar level and showed no visible colour change during the entire experimental period. The heart rate (HR) was not recorded to allow quantification of the control data for comparison to the other CO-treated groups. The 0.05% CO perfused hearts showed a decrease in HR after 2min of CO perfusion as observed qualitatively. A negative inotropic response was observed in parallel with the chronotropic response. A decrease in perfusate (coronary) flow was observed by an increase in back pressure (seen by an increased volume of perfusing buffer in the bubble trap of the Langendorff apparatus). After the 30 minute CO perfusion period (with 0.05% CO), the hearts displayed an increase in HR within 2min, and contractile recovery was further observed by 15min. However, the inotropic response did not recover to that observed (qualitatively) prior to CO exposure. No colour change was observed in hearts perfused with 0-0.1% CO.

Hearts perfused with 0.1% CO showed similar changes in their inotropic and chronotropic responses as the 0.05% CO perfused hearts. The pre-0.1% CO HR was 168 ± 12 beats.min^{-1} (expressed as the mean ± SEM; from n = 3 hearts) at 5min prior to CO

exposure. The HR decreased during CO exposure to 146 ± 8 bpm at 27min of CO exposure (or 87 ± 5% of the pre-CO value). The HR increased during the CO-free period (after 30min of CO exposure) to 132 ± 20, 144 ± 28 and 160 ± 11 bpm at 31, 92 and 122min, respectively. The pre-CO HR values were taken as 100% for each heart and other values of HR determined during and after the CO-treatment were expressed as a percentage of the pre-CO level for each heart (prior to averaging for the group). These HR values were equivalent to 79 ± 15%, 86 ± 19% and 95 ± 7% of the pre-CO level for 31, 92 and 122min of the perfusion, respectively. Arrthymias were clearly observed when determining the HR following the CO exposure. These arrhythmias were seen as phases of irregular spasmic-like beating.

The 0.2% CO perfused hearts also showed a similar decrease in HR (as the 0.1% CO-treated group). The pre-CO HR was 198 ± 7 bpm and fell to 112 ± 34 bpm at 29min of CO exposure, then recovered over the 90min CO-free period to 190±3 bpm at 120min (**Figure 3.1**). The HR for the 0.2% CO-treated group was 56 ± 15%, 87 ± 8% and 96 ± 2% (of the pre-CO level) at 29, 90 and 120min, respectively. However, no arrhythmias or vasoconstriction (indicative of perfusion back pressure) were observed in these hearts. The perfusate flow (PF) was measured and decreased during the CO exposure period and increased gradually over the post-treatment period (**Figure 3.1**). The pre-CO PF (determined 5min prior to CO-treatment) was 5.62 ± 0.08ml.min^{-1}. The PF did not recover to the observed pre-CO levels. The PF was not determined for the 0.1% CO-treated group to compare between these CO-treated groups.

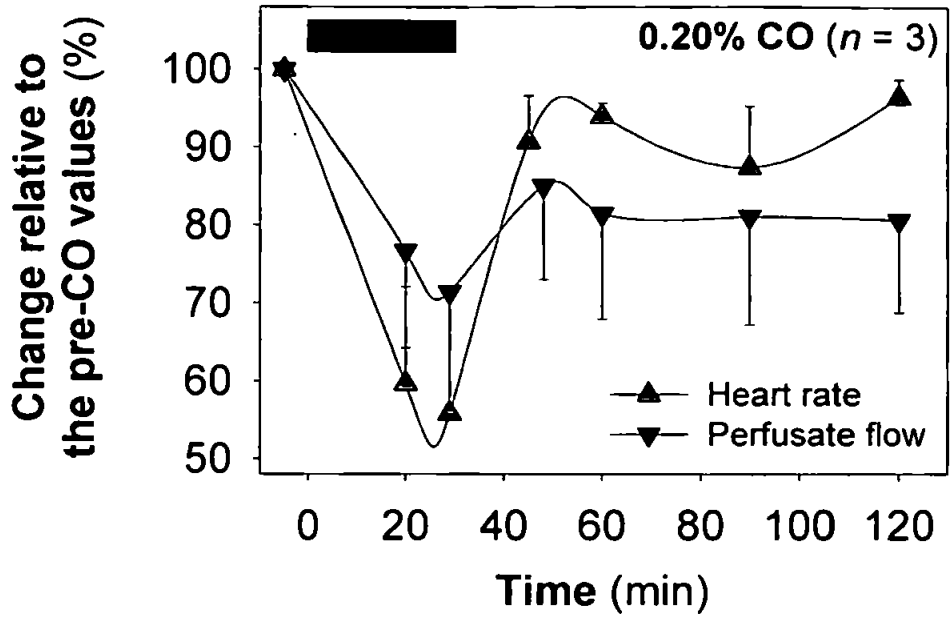

Figure 3.1 Heart rate and perfusate flow of isolated rat hearts exposed to 0.2% carbon monoxide (CO). Hearts were perfused with buffer saturated with 95% O_2 and 5% CO_2 during the pretreatment period (*ca.* 10min). CO exposure was started by perfusing hearts with buffer saturated with 0, 0.05, 0.1 or 0.2% CO (with 21% oxygen, 5% carbon dioxide, and balanced with nitrogen) for 30min, followed by a 90min CO-free period (by perfusing hearts with petreatment perfusion buffer). Data shown is the mean ± SEM (*n* = 3 hearts). The black bar shows the period of CO exposure in perfused hearts.

3.3.2 Tissue Viability

Lactate dehydrogenase (LDH) and creatine kinase (CK) are both intracellular enzymes. The release of LDH and CK into blood serum indicates cellular, tissue, or organ damage (Penney & Maziarka, 1976; Swaanenburg *et al.*, 1998). Their activities were determined in perfusate samples from hearts perfused with CO. Control hearts (0% CO) had a constant release of LDH ($\leq$0.04 units.ml^{-1}.g^{-1}) during the experimental period. Hearts perfused with 0.05% CO showed maximum LDH activity in the 30min perfusate samples followed by a decline (**Figure 3.2**). However, it remained elevated above the LDH activity of control hearts. Perfusions with 0.1% and 0.2% CO had similar LDH release profiles and showed no difference relative to the control hearts (0% CO). The CK activity had a similar profile as the LDH release for the 0.1 and 0.2% CO groups, however, no elevation in CK activity was found for the 0.05% CO-treated group (**Figure 3.3**). The CK activity was not determined for the control hearts.

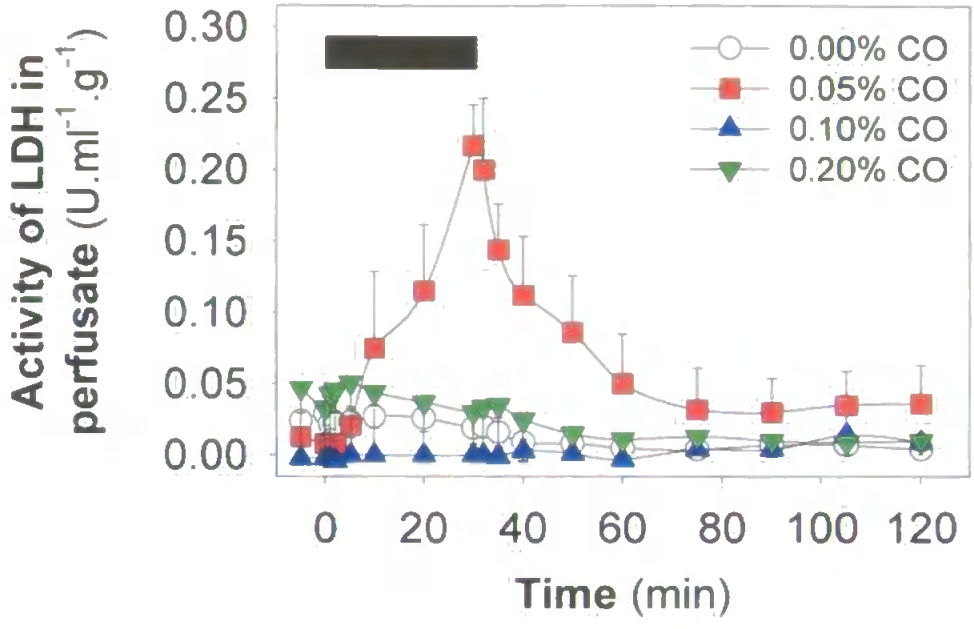

Figure 3.2 Release of lactate dehydrogenase (LDH) into the perfusate of CO-treated rat hearts (0-30min; black bar) followed by a 90min CO-free period. The LDH activity was determined (as described in *3.2.5.1*; Materials & Methods) immediately after each perfusion was performed. Same experimental conditions as described in Figure 3.1. The white circles, red squares, blue triangles and green upside-down triangles show the mean ± SEM of three hearts for the 0, 0.05, 0.1 and 0.2% CO-treated groups, respectively.

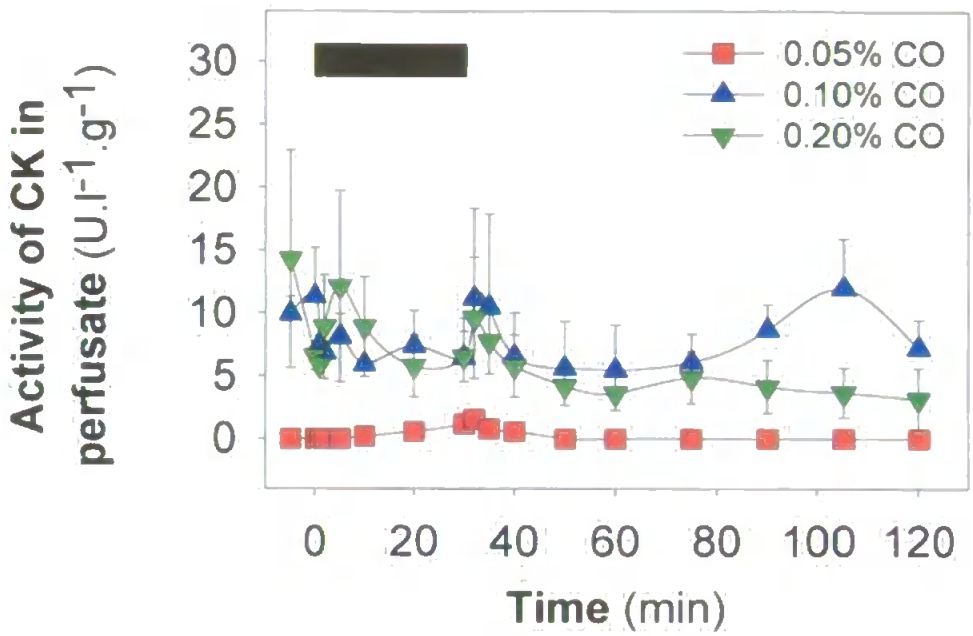

Figure 3.3 Activity of creatine kinase (CK) in perfusate samples from CO-treated hearts. The assays were performed using a commercial assay kit on perfusate samples that had been stored frozen (at -20°C for at least two days) prior to their analysis. Same conditions as described in Figure 3.2, although no control data was determined.

3.3.3 Nitrite

Nitric oxide (NO) is produced after CO exposure in the brain tissue of rats (Ischiropoulos *et al.*, 1996). To address this issue for heart tissue, we examined whether CO could produce its toxicity/morbidity from a production of reactive oxygen species (ROS) and/or reactive nitrogen species (RNS). Therefore, the possible production of NO was investigated by measuring its immediate breakdown product, nitrite (using the Griess assay; *3.2.5.3*). The Griess assay was performed on perfusate samples taken on the same day as the perfusion. No differences were observed between the control and CO-treated hearts at the three doses used (**Figure 3.4**).

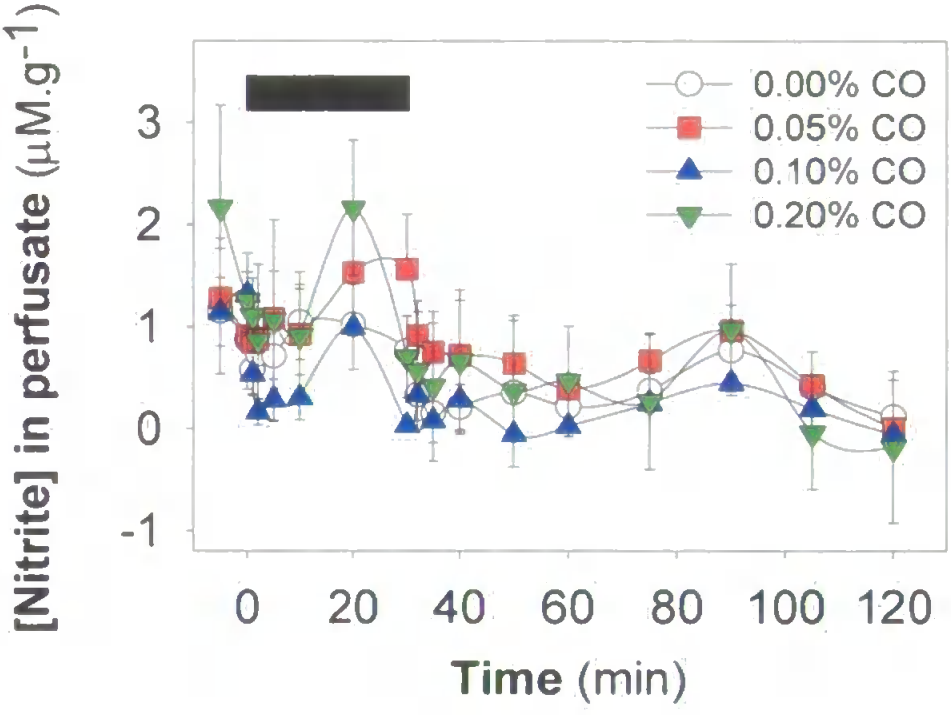

Figure 3.4 Nitric oxide levels were determined from the nitrite concentration (normalised per gram of heart tissue) in the perfusates of CO-treated hearts. Same key and experimental conditions as described in Figure 3.2.

3.3.4 Ferric Reducing Ability of Plasma (FRAP)

To address our hypothesis (**1.7**), we need to examine for the production of reactive oxygen species (ROS) during and/or following CO exposure. The FRAP assay determined the antioxidant potential (in perfusate samples) from its ability to reduce ferric (Fe^{III}) to ferrous iron (Fe^{II}). Ferric iron (at low pH) will form a complex with tripyridyltriazine (Fe^{III}-TPTZ) that can be reduced (to ferrous iron) producing a stable coloured complex that is measured (λ_{max} at 593nm). If the production of ROS is elevated in the heart it may reduce the antioxidant capacity of the tissue(s). No qualitative differences were observed between the control and CO-treated hearts at the three doses used (**Figure 3.5**). The perfusate from the 0.05% CO-treated group showed an elevated level of antioxidant potential similar to the release of LDH, although it does not appear to be significant.

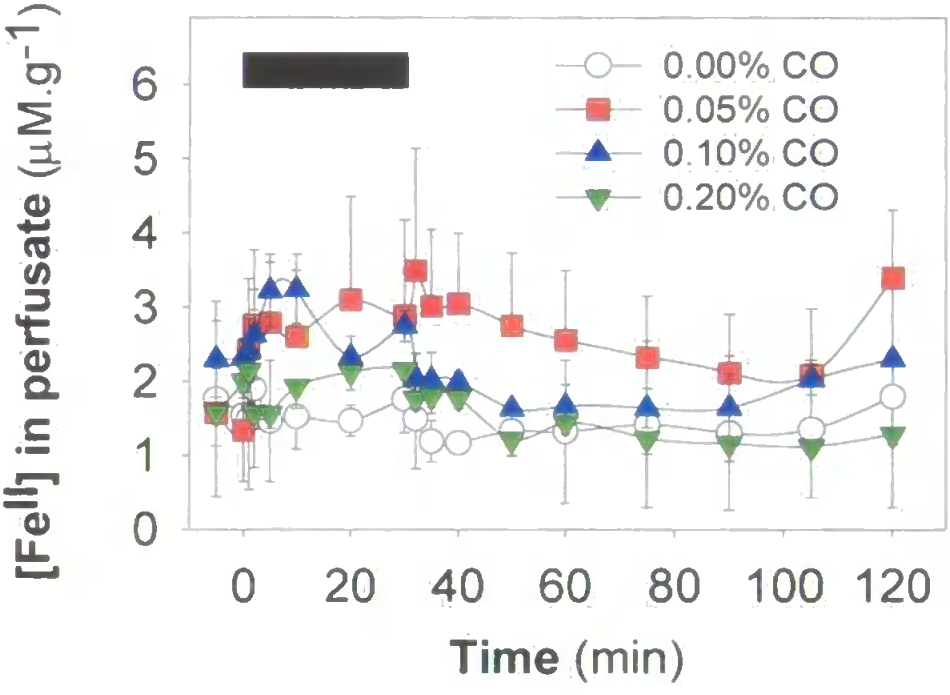

Figure 3.5 Antioxidant potential of the perfusate of isolated rat hearts exposed to CO. The ferric reducing ability of plasma (FRAP) assay was used to assess the antioxidant potential (as described in *3.2.5.4*).

3.4 Discussion

Pentobarbitone is a widely used anaesthetic that is a cardiovascular and respiratory depressant that can reduce the intracellular level of high energy phosphates (Bretschneider, 1975). Tissue hypoxia could potentiate this loss of high energy phosphate when the heart was removed from the animal (Grupp *et al.*, 1999). The maintenance of a high ATP to ADP ratio is critical in determining tissue viability. The removal of the heart (after administering pentobarbitone) was performed quickly and followed by rapid perfusion to minimise global ischaemia in the heart. Stressed (frightened) animals have an altered hormonal blood chemistry (particularly catecholamines) that may influence the perfused heart at the biochemical and physiological level (Bolli *et al.*, 1985; Bristow *et al.*, 1993; Vleeming *et al.*, 1989). Stressing the animals was avoided at all costs by keeping the animals in a quiet environment prior to anaesthesia and by minimising their handling. The animals were handled confidently and administered anaesthesia without causing them any visible distress following the experience gained (in **Chapter 2**).

The levels of CO used (0-0.2%) are similar to those producing a broad range of blood [COHb] found during CO intoxication. Raub *et al.* (2000) showed equilibrium levels in blood COHb ranging from 2-80% following the steady-state exposure to air containing 0.001-0.195% (10-1950ppm). These blood [COHb] produce a range of effects following CO exposure *in vivo* from subtle flu-like symptoms to death (**Table 1.2**), i.e. non-lethal to lethal. Therefore, using [CO] that reflect the spectrum of symptoms produced following CO poisoning ensured a clinically relevant dimension to the study. The level of CO may be lower than those cited due to the decreasing solubility of CO in solution with increasing temperature. The solubility of CO in water is 3.54, 2.14, and 1.83ml.100ml^{-1} at 0, 25, and 37°C, respectively (World Health Organization, 1979). The use of plastic tubing throughout the perfusion apparatus may also reduce the [CO] through adsorption. Most of this loss was thought to arise from the lowered solubility with temperature (above) and the presence of other solutes in the perfusion buffer. The degree of loss of CO through solubility and adsorption was not determined. To minimise any loss of CO through

adsorption between the stock gas cylinder to the glass bottle (holding the CO-containing perfusion buffer); therefore, tubing that could withstand high pressure and not adsorb CO was found and ordered for later work (from Altec). Therefore, the [CO] used here reflect nominal values and not the actual level.

The changes in mechanical function were assessed by measuring the heart rate (HR). The bradycardia (observed from the determined HR in 3.3.1) suggest a dose-dependent effect of CO at similar time-points for the 0.1 and 0.2% CO-treated groups. The absence of a blood compartment (as found *in vivo*) rules out the formation of COHb. Therefore, these changes in HR do not result from systemic hypoxia, or the decreased perfusing O_2 level during CO exposure relative to the pre-CO PO_2 (discussed below). The vasoconstriction, arrythmias and bradycardia observed in hearts perfused with 0.1 and 0.2% CO may suggest a direct effect of CO. The perfusate flow (PF) may also have a dose-dependent relationship (similar to the HR), however, this remains to be determined. These changes in HR and PF are not discussed here as the primary purpose (of this chapter) is to evaluate the feasibility of using isolated hearts. However, this work does suggest a direct effect of CO on heart function (under the conditions used).

The markers used (lactate dehydrogenase and creatine kinase; LDH and CK, respectively) are established in cardiovascular research (Van Der Laarse *et al.*, 1979). These markers were used for two purposes: to dissect out the deterioration of the perfused organ with time and to examine the experimental effects of CO. Some release of LDH was found from control hearts and this leakage may indicate washout of LDH arising from tissue damage during heart isolation and/or deterioration of the isolated preparation over time. The release of LDH during the 0.05% CO-treatment indicates some tissue damage produced by CO. The mechanism(s) responsible for producing the tissue damage (with 0.05% CO) are not discussed as this preliminary study was performed to evaluate the isolated heart model (**1.8**). However, the higher doses of CO (0.1 and 0.2%) showed no elevation in LDH activity (**Figure 3.2**). These findings suggest that sub-acute doses of CO ≤0.05% CO may warrant further examination prior to making any conclusions

(**Chapter 4**). Furthermore the reduced PF observed in the 0.2% CO-treatment (above) may lower the activity of LDH (and also CK) by reducing the rate of release of these markers from the heart tissue(s). The increase in PF after the 0.2% CO-treatment could have occurred in the 0.05% CO-treated group (not determined here) and may account for the decrease in LDH activity following the CO-treatment (**Figure 3.1**). The absence of LDH activity (in the perfusate) from hearts exposed to ≥0.05% CO suggests that the changes in PF may have masked any LDH release and could be determined more accurately by measuring the activity in perfusate fractions.

The activity of CK in the perfusate samples did not allow any conclusions to be made as no control data was determined. The CK assays were performed at a later date after the experiments and this storage could have resulted in a loss of activity from the samples (**Figure 3.3**). The duration of storage at -20°C (prior to assaying for CK) in the samples was 2-3, 6, and 68-75 days for 0.10%, 0.20% and 0.05% CO, respectively. Any likely loss of CK activity may mirror the duration that the samples were stored at. Assuming there was a loss of activity with time, it suggests that there may have been a release of CK (as observed for LDH). Whether a dose-dependent release occured remains to be determined. Damage to the heart following CO exposure could be examined using sensitive markers of viability to indicate more specific tissue damage. Such markers include the troponins (**Chapter 4**) and myoglobin (**Chapter 7**) due to its specificity in showing early myocardial damage and its abundance in myocardial tissue, respectively (Ooi & Le May, 2000; Stokke *et al.*, 1998).

Inducible nitric oxide synthase (iNOS) is shown to be stimulated following ischaemia/reperfusion (I/R) and also after CO exposure (**1.5**). The role of nitric oxide (NO) during and/or after CO exposure may be ruled out in heart tissue at the levels of CO used here. The nitrite may have oxidised to nitrate (prior to measuring nitrite) and this may explain the absence of any difference in NO levels (as reflected by nitrite). However, this does not rule out iNOS, as NO may be found elevated in heart tissues following a period of induction post-CO exposure (**Chapter 1**). Therefore, this measurement will be excluded

in further studies, although its role in cardiac mortality following CO exposure cannot be eliminated.

The antioxidant potential of perfusate samples showed no differences using the FRAP assay (*3.2.5.4*). Several flaws were identified in the method that could render it useless for this study. The use of acidic solutions (to maintain the solubility of iron) may prevent some antioxidants in reducing ferric iron. This change in pH may alter the redox potential and interfere with the antioxidant chemistry. Whether antioxidants were released from heart tissue (into the perfusate) cannot be concluded as used (or oxidised) antioxidants may not participate in the FRAP assay, unless they undergo redox recycling to their antioxidant state, i.e. become reduced. The FRAP assay is shown to determine the antioxidant activity in samples of plasma (Benzie & Strain, 1996). This could restrict the assay to using samples with a high content of antioxidants such as the plasma or heart tissue homogenates (relative to using perfusate samples). Conversely, other measurements such as the glutathione content may be determined to evaluate oxidative stress (**Chapter 5**).

The gases used here comprised 95% O_2 (and 5% CO_2) during the pre-CO stage that was changed to a CO-containing mixture (of 21% O_2 and 5% CO_2 balanced with N_2) during CO-treatment, followed by the pre-CO (CO-free) gas mixture. This use of gas mixtures (during CO-treatment) may have introduced a reduction in PO_2 during the CO exposure in comparison to the basal (pre-CO) and the post-CO period (following CO-treatment); therefore, masking the effect(s) of CO by possibly introducing hypoxia. Using gases that remove this variable would have to be employed. Use of gas mixtures containing 21% O_2, 5% CO_2, 0-0.1% CO (balanced with nitrogen), i.e. maintain 21% O_2 throughout the entire perfusion would eliminate this error. The 0.05% CO-treated hearts showed the release of LDH into the perfusate (above) suggesting that CO may have a direct cardiotoxic effect irrespective of hypoxia produced following the changes in O_2 levels during CO exposure. The absence of an elevated LDH activity in the perfusate of control hearts (0% CO) suggests that no hypoxia(-reoxygenation) injury may have occurred, therefore, ruling out

this introduction of hypoxia under the experimental conditions used. Conversely, no hypoxia may have been introduced as oxygen (at ≥21%) was used throughout the experiments.

Hyperbaric oxygen therapy (HBOT) following CO exposure may be studied by perfusing the heart preparations with buffer equilibrated with a greater content of dissolved oxygen, i.e. hyperoxic relative to the buffer equilibrated with 21% O_2. This appears to be a valid experimental model as hyperoxia of the blood is essentially what occurs during HBOT. Blood can carry 0.3ml of dissolved oxygen per 100ml under physiological conditions, i.e. inhaling air (21% O_2) at sea level (one atmosphere). The inhalation of 100% O_2 at 1 atmosphere (atm) results in 1.5 ml O_2 per 100ml blood, an increase by a factor of five (according to Henry's Law); at 3 atm (i.e. HBOT) increases the amount to 4.5ml O_2. By using 95% O_2 to perfuse the heart (and maintain the bicarbonate buffering with 5% CO_2) may simulate these conditions, although the dissolved O_2 content of the perfusing buffer may have to be determined to quantify the level of hyperoxia. Using different levels of O_2 may allow other levels of hyperoxia to be studied. The use of a high O_2 level after CO exposure may indicate some positive effects of HBOT as shown by the increase in HR, PF and the decreased loss of LDH from the heart following CO-treatment. However, this is not conclusive as hyperoxia was used here prior to any CO exposure.

Experimental hearts should be perfused with gas mixtures containing 21% oxygen throughout the entire period to rule out hypoxia (above). This is not difficult to conduct (above), however, whether the heart preparation is able to use 21% O_2 remains to be determined as other organ studies use 95-100% O_2 with 0-5% CO_2 depending on the buffer used (McGrath & Martin, 1978). Examination of the oxygen consumption *in vivo* would appear to support the use of 21% O_2 in Langendorff perfusing hearts. Dissolved O_2 in the plasma is used by the cells/tissues following its diffusion through the capillary walls. The content of dissolved O_2 in the blood is 0.3ml per 100ml of arterial blood following the inhalation of atmospheric air, i.e. 21% O_2 (**1.3** and above). Considering that this model comprises an isolated heart, it should be able to sustain itself using 21% O_2 (as other

compartments such as the tissues and blood are not present). Taking the above into consideration, it appears likely that the heart preparations using 21% O_2 throughout are likely to work and has been confirmed (in **Chapter 4**). Using 21% is also more clinically relevant and strengthens the use of this model for our purposes.

3.5 Conclusions

The Langendorff isolated heart preparation was used to evaluate and validate its use for investigating the cardiotoxicity of CO. This preliminary study identified some improvements (**3.4**) that support its use to address the hypothesis (**1.7**). The levels of CO used were chosen by examining the literature values and the symptoms produced (given in **Chapter 1**). The 0.05% CO dose produced tissue damage, however, the higher concentrations of CO may have had their toxicity masked due to an inaccurate sampling method. As the isolated heart was exposed to CO, the chosen concentrations of CO gas mixtures may exceed (theoretical) blood levels that could surpass death. Therefore, using a lower concentration of CO such as 0.01% CO may prove to be suitable for this study.

3.6 Further Work

3.6.1 Nitric oxide

A fluorometric assay with increased sensitivity and including nitrate reductase may be more suitable to detect any production of NO (Misko *et al.*, 1993). Alternatively, tissue homogenates could be prepared from heart tissue to eliminate the above issue of sensitivity. A recent method that continually monitors NO released from perfused hearts may be used (Tsukada *et al.*, 2003).

Chapter Four:

Cardiotoxicity of CO During Normoxia - Physiological Changes

4.1 Background

Previous work has shown ultrastructural changes in rabbit hearts after exposing live animals to CO (Kjeldsen *et al.*, 1974). There are also studies that have reported changes in tissue viability following CO exposure (Penney & Maziarka, 1976) and some studies have assessed cardiac function using isolated hearts from rats exposed to CO. However, at present no studies have been found examining tissue biochemistry with physiological changes (in structure and function) following the CO exposure. This study will incorporate the necessary work to provide a detailed insight to elucidate the mechanism(s) of CO toxicity in the heart.

Other studies used chronic exposure to CO and showed that significant changes occurred following CO exposure (1.4.1). Such changes include an increased haematocrit content, cardiomegaly, and other changes in the levels of various blood enzymes. Many of these changes were attributed to CO-induced hypoxia, therefore, the direct effect of CO in myocardial tissue is masked by other influencing variables (**Chapter 1**). For example, a cardiotoxic chemical species (or modified biomolecules) produced by other organs (such as the liver) following chronic exposure to CO may be responsible for the cardiotoxicity of CO. These early studies that examined chronic CO exposure failed to account for the associated heart failure that at present appears to be a direct effect of CO. Previous chronic studies also used non-lethal doses that were lower than the doses found in severe cases of acute CO poisoning.

Studies are routinely performed solely examining the physiological changes without considering any biochemical changes. Our model used isolated rat hearts to assess the cardiotoxicity of acute CO poisoning, however, the blood component is missing and the organ is perfused using a modified Krebs-Heinshelt buffer (KHB; 3.2.2). Therefore, blood (or systemic) hypoxia cannot occur as CO cannot bind to haemoglobin (in erythrocytes). This model allowed the examination of CO directly in the heart and was performed by dissolving the CO-containing gas mixture into the buffer that perfused the isolated organ.

The novel aspect is that normoxic conditions were used throughout, i.e. ***all perfusions with CO were conducted in the presence of 21% oxygen***. Biochemical measurements were also undertaken to look for evidence of a reperfusion-like injury to investigate the hypothesis (**1.7**). This chapter presents the physiological findings from this study (and **Chapter 5** shows the biochemical aspects).

4.2 Materials & Methods

4.2.1 Isolation & Langendorff Preparation

Isolation of rat hearts and the Langendorff preparation were carried out as described previously in 3.2.1 and 3.2.2, respectively. No heparin was administered to the unconscious animal. (The reasons for not administering heparin in this study are stated in 5.2.3). Isolated hearts that showed arrhythmia's immediately or other visible signs of the calcium paradox after being perfused were discarded.

4.2.2 Experimental Protocol

This was performed exactly as described by Patel *et al.* (2003). In brief, hearts were perfused for 15 minutes to wash out contaminating blood. The basal physiological parameters (4.2.3; heart rate and coronary flow) were determined over the next 15 minutes. Exposure to CO was started by perfusing with buffer that was saturated with 0, 0.01 and 0.05% CO (0, 100 and 500ppm, respectively) for 30 minutes, followed by a 90 minute CO-free period. Note, the perfusing buffer was constantly saturated with 21% oxygen and 5% carbon dioxide (balanced with nitrogen). The heart ventricle was then blotted and weighed prior to snap-freezing for biochemical analysis (**Chapter 5**).

Some hearts were perfused with perfusion buffer supplemented with antioxidants. The antioxidants were perfused during the pretreatment period (i.e. whilst establishing basal levels of heart rate and coronary flow over 15min; 4.2.3) and during the 30min exposure to CO. The antioxidants used were TroloxC and ascorbic acid, at 0.2mM and 1mM, respectively. The stock solution of ascorbate was prepared and added to the perfusion buffer before the start of the antioxidant perfusion. The inclusion of ascorbate required greater buffering in the perfusion buffer and was supplemented with 10mM HEPES. Other heart perfusions were performed that involved taking hearts immediately off the Langendorff apparatus after the basal parameters had been determined or after being treated with 0 and 0.01% CO (for 30 min).

4.2.3 Physiological Parameters

Heart function was evaluated by monitoring the heart rate (HR; *4.2.3.1*) at various fixed time points. This was performed to examine any change(s) in cardiac performance and investigate any direct effects of CO on the heart. The coronary flow (CF; *4.2.3.2*) was measured to assess changes in coronary vasculature produced as a result of CO exposure. The time points used were -15 to -10, -10 to -5, -5 to 0, 0-5, 5-10, 10-15, 15-20, 20-25, 25-30, 30-35, 35-45, 45-60, 60-80, and 80-120 minutes.

4.2.3.1 Heart rate (HR)

The heart rate (in beats.min^{-1}) was determined by counting the number of heart beats in a 30 second period three times around the midpoint of each time fraction (above). The mean HR was determined in each timepoint for each heart.

4.2.3.2 Coronary Flow (CF)

The coronary flow (CF) was determined from the volume collected (ml) over the time period for each fraction (in minutes) and normalised per gram of heart tissue (wet weight). The collected perfusate was immediately placed on ice for biochemical analysis (5.2.3).

4.2.4 Changes in Heart Weight (HW)

Prior to cannulating the heart (for Langendorff preparation; 4.2.1) the heart was blotted and quickly weighed to determine the pre-perfusion HW. Following the perfusion the heart was quickly removed from the Langendorff apparatus, taken off the cannula and weighed to determine the post-perfusion HW.

4.2.5 Tissue Water Content

The water content was determined in heart tissue from the difference in wet and dry tissue weights. Hearts were immediately taken off the perfusion apparatus following the experiment (4.2.2 and 4.2.4). The ventricle tissue was obtained (firstly, by removing the atrial and vascular tissue) using a scalpel, then some tissue (*ca.* 30-100mg wet weight) was rapidly removed from the top portion of the ventricle mass opposite the apex. The remaining ventricle mass was gently blotted and weighed prior to being snap frozen and stored at -80°C (5.2.4). The removed portion of ventricle tissue was weighed (after gentle blotting) to give the wet weight (WW). The pieces of tissue were dried by incubating in an oven and drying to constant weight (at 80°C over 11 days). The tissue water content was expressed as a percentage (determined using (WW-DW/WW)x100%, where DW is the dry weight).

4.2.6 Metal Ion Analysis

Dried ventricle tissue (from above; 4.2.5) was reconstituted in 2ml nitric acid (70%, 1.42 SG; Fischer Chemicals Ltd) in a fume hood and heated at 55°C (for 10min) to dissolve the dry tissue. The following electrolytes: sodium (Na^+), potassium (K^+), calcium (Ca^{2+}), magnesium (Mg^{2+}), iron (Fe), copper (Cu^{2+}), and manganese (Mn) were quantified using inductively coupled plasma mass spectrometry (ICP-MS). The analysis was performed using a PlasmaQuad PQ2+ Turbo ICP-MS with the following default lens setting: extraction 1.00-2.00; collector 7.70; lens 1 7.70; lens 2 5.40; lens 3 5.00; lens 4 3.85; and pole bias 4.60-5.50. The instrument was calibrated using appropriate metal solutions, and indium ($100ng.ml^{-1}$) as the blank. The sample preparation and electrolyte analysis was performed by Mr. Leonard Hawkins (as part of a final year undergraduate project).

4.2.7 Osmolarity

The osmolarity of the perfusate were measured to evaluate any changes in the electrolyte content of heart tissue (4.2.6) following CO-treatment. The frozen perfusate samples were thawed on ice prior to determining its osmolarity.

4.2.8 Histology

Two to four tiny pieces (*ca.* 2-4mm length) of ventricle tissue from each heart were removed and immediately placed in 3% glutaraldehyde in 0.1M sodium phosphate buffer for primary fixation (1h at 4°C). In brief, the pieces were then washed twice with buffer (5min each). Secondary fixation was performed using 1% osmium tetraoxide (OsO_4) in buffer (for 1h at 4°C) prior to washing three times with buffer. Tissue pieces were dehydrated using successive incubations (10min each) in ethanolic solutions (30, 50, 70, 90% and absolute alcohol). The samples were then infiltrated with Spurr's resin in a graded manner prior to polymerisation in embedded blocks of resin. Ultra-thin sections (*ca.* 100nm) were prepared using a Reichert ultramicrotome and collected onto copper support grids for routine staining for contrast by Dr. Roy Moat (Electron Microscopy Unit, University Of Plymouth, UK).

4.2.9 Data Analysis

All data shown is expressed as the mean ± SEM or SD with the number of hearts (*n*) cited for each group. Unless otherwise stated the Student's *t* test was used for statistical comparisons of the means using StatGraphics 5.0 software.

4.3 Results

4.3.1 General Observations

The rat hearts were excised, cannulated and mounted on the Langendorff perfusion apparatus as quickly as possible in order to minimise tissue ischaemia (as discussed in **Chapter 2 and 3**). Isolated perfused hearts that beat regularly and showed no visible signs of the calcium paradox (**Chapter 2**) were used. The total number of hearts (or animals) used solely for this work including unsuccessful hearts were 40. The number of successful perfusions (used here and **Chapter 5**) were 36. The weights of the hearts used in the perfusions are presented in **Table 4.1**. Also shown are the body weights (BW) of the animals used from which the hearts were taken, and these were determined in unconscious animals prior to excising the heart. The start-stop perfusions were conducted using nine hearts (three in each group) and had a mean body and heart weight of 292 ± 38 and 1.53 ± 0.19 (mean ± SD), respectively.

The region of the heart surrounding the coronary arteries was observed to swell up during the CO-free period in hearts exposed to CO. However, control hearts showed no sign of swelling. Some CO-treated hearts showed an increase in the volume of perfusion buffer in the bubble trap (placed above the heart to prevent air bubbles entering the heart). From the 0.01% and 0.05% CO-treatment, respectively, four and two hearts showed an increase in the volume of after CO exposure. This increase in buffer volume occurred at different periods after the CO-treatment. In general, these identified hearts appeared to have an increase in back pressure before their decline in heart rate (HR). Some hearts from the preliminary study (**Chapter 3**) also showed this increase in volume, however, those CO-treated hearts showed this trend either during and/or after the CO exposure.

Control hearts had regular heart beat and showed no altered HR. Most of the CO-treated hearts displayed signs of arrhythmias indicated by fibrillation and irregular heart beat. Eight hearts (of ten) showed arrhythmias at some periods with 0.01% CO-treatment; 2, 3 and 3 hearts showed these signs during CO-treatment, during and after CO-treatment,

and only after CO exposure, respectively. All hearts treated with 0.05% CO showed signs of arrythmia at some point: 3, 1 and 2 hearts showed these signs during CO-treatment, during and after CO-treatment, and after CO exposure, respectively.

The coronary ostias region of the heart was close (in proximity) to the atria in control hearts indicating no swelling. A swollen appearance in the vasculature was observed in all hearts after the period of CO-treatment. However, this could not be presented here as this observation was not quantified or recorded. Two of the hearts (out of six) exposed to 0.05% CO had two to three small red areas resembling haemmorhagic zones. These focal regions were found predominantly around the apex of the heart (after the perfusion). No tissue specimens were taken from these areas for histological examination.

Group (*n*)	***Body weight*** *(grams)*	***Heart weight*** *(grams)*
0% CO (*3*)	261.33 ± 5.35	1.37 ± 0.10
0% CO + **AO** (*3*)	258.23 ± 3.91	1.30 ± 0.07
0.01% CO (*10*)	246.21 ± 11.90	1.51 ± 0.11
0.05% CO (*6*)	229.73 ± 11.09	1.42 ± 0.10
0.05% CO + **AO** (*5*)	278.08 ± 7.82	1.61 ± 0.15
0% CO; 0min	284.80 ± 39.07	1.43 ± 0.05
0% CO; 30min	308.67 ± 44.94	1.69 ± 0.26
0.01%; 30min	283.77 ± 38.97	1.46 ± 0.10
All (*36*)	261.70 ± 30.21	1.48 ± 0.15

Table 4.1 The body and heart weights of adult male Sprague-Dawley rats in each treatment group. This includes the CO-treated groups, antioxidant (**AO**) perfusions, and start-stop perfusion. The data shown is expressed as the mean ± SD for each group with the number of hearts in each group (*n*).

4.3.2 Heart rate (HR)

The basal heart rates of the perfused hearts ranged from 78-274 beats.min^{-1} for the hearts perfused with or without antioxidants (AO). The mean heart rate (HR) for the pretreatment period are shown in **Table 4.2**. To allow comparisons to be made between the CO-treated groups; the three pretreatment HR levels were expressed as a mean pretreatment HR and the other HR were expressed as a percentage of the mean pretreatment level (taken as 100%) for each heart. The data were expressed as the mean ± SD for each group (**Figure 4.1**).

Control hearts beat regularly and had no significant deviation to the pretreatment (basal) HR. The CO-treatment had a negative chronotropic effect. Hearts treated with 0.01% CO showed a decline and a recovery in HR during the 30min period. These hearts had a decline in the CO-free perfusion period. Those hearts exposed to 0.05% CO showed a similar decrease, however, there were no signs of recovery during the CO-treatment and the mean HR were similar to the 0.01% exposed levels at the end of the perfusion. The decline in HR showed no significant differences to the control hearts (0% CO) throughout the perfusions. The control antioxidant group had no difference to the control group without AO, however, the CO-treatment had a negative chronotropic effect that was augmented with the antioxidants.

Time	*Heart rate* (beats.min^{-1})				
(min)	0% CO (n = 3)	0% CO with AO (n = 3)	0.01% CO (n = 10)	0.05% CO (n = 6)	0.05% CO with AO (n = 5)
-12.5	191 ± 69	189 ± 14	131 ± 19	142 ± 32	151 ± 42
-7.5	185 ± 62	183 ± 11	132 ± 18	142 ± 29	160 ± 53
-2.5	186 ± 75	184 ± 15	135 ± 20	144 ± 30	163 ± 55

Table 4.2 The pretreatment heart rate (HR, in beats.min^{-1}) of isolated perfused rat hearts. Hearts were perfused in a retrograde manner through the aorta using a Langendorff apparatus at 6ml.min^{-1}.g^{-1} (wet weight). The hearts were perfused with a modified Krebs-Henseleit buffer at 36.9°C (see Materials & Methods for the composition of the perfusion buffer). The buffer was equilibrated with a certified gas mixture containing 21% oxygen, 5% carbon dioxide, balanced with nitrogen and gassed constantly with this mixture for the entire perfusion period to establish pH 7.4. Hearts were perfused for 15min to wash out blood and allow the organ to stabilise from the excision procedure. The basal HR (or pretreatment HR) were determined over another 15min period prior to commencing the carbon monoxide (CO) treatment. Some hearts were treated with antioxidants (**AO**): TroloxC and sodium ascorbate were included in the perfusion buffer (at 0.2 and 1mM, respectively) during the 15min equilibration (basal) period and during the exposure to CO. The CO exposure (for 30min) used *normoxic* conditions with 0% CO (n = 3 hearts; controls), 0% CO with AO (n = 3 hearts; AO controls), 0.01 and 0.05% CO (n = 10 and 6 hearts, respectively) and 0.05% CO with AO (n = 5). Normoxic conditions were used throughout the entire perfusion using gas mixtures with varying nitrogen levels to compensate for the different CO level (0 to 0.05%). Hearts were treated by switching the buffer with a pre-equilibrated buffer containing the desired CO concentration. The time to attain this new equilibrium was *ca.* 2min. Following the 30min CO exposure, hearts were perfused for 90min to assess the post-CO HR; not shown. The data shown is the mean ± SD for n hearts in each group.

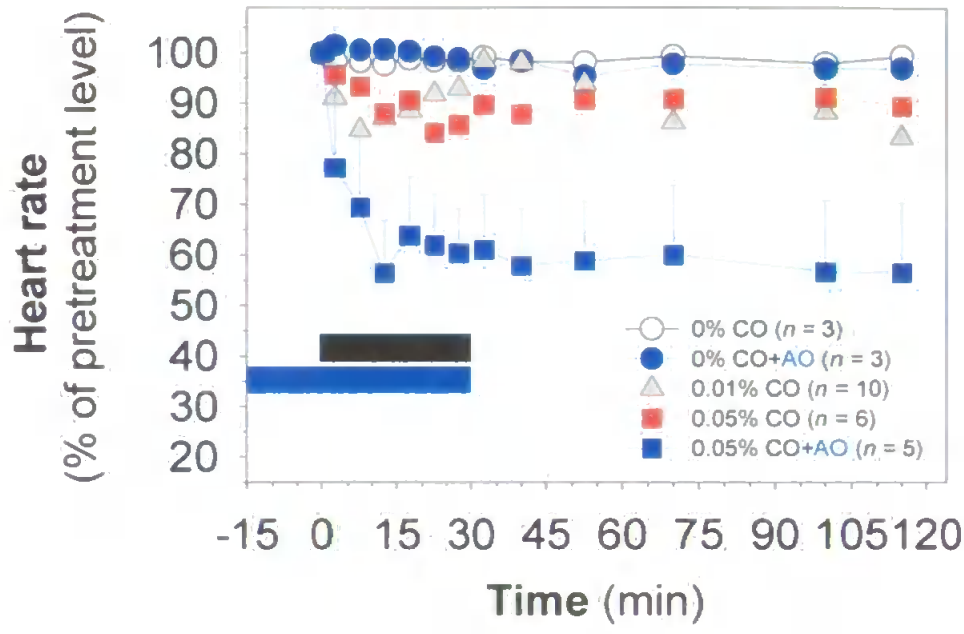

Figure 4.1 The heart rate of isolated rat hearts exposed to carbon monoxide (CO) by perfusion using a Langendorff perfusion apparatus. Hearts were perfused in a retrograde manner through the aorta using a Langendorff apparatus. The hearts were exposed to 0, 0.01 and 0.05% CO and 21% O_2 (balanced with N_2) for 30 minutes followed by a CO-free gas mixture (90 min) by perfusion. Some hearts were perfused with antioxidants (AO; 0.2mM TroloxC and 1mM sodium ascorbate) during the pretreatment period (15min) and during the CO exposure (30min). The black bar denotes the period that hearts were exposed to CO, whereas, the blue bar shows when AO were perfusing the hearts. Same conditions as described in the legend for Table 4.1. The mean basal (or pretreatment) HR were taken as 100% and the HR expressed as a percentage of the determined basal HR. The data shown is the mean ± SEM with the number of hearts (*n*) used in each group depicted in the figure. No significant differences were found at the 95% confidence level between the controls (with or without AO) and the CO-treated groups.

4.3.3 Coronary Effluent

The coronary flow (CF) was determined to assess any change in the perfusion of hearts exposed to CO. The terms coronary flow (CF) and perfusate flow (PF) is used interchangeably as exemplified in other literature, however, some readers may disagree with this. The isolated hearts were perfused using a calibrated pump that was validated before and after completing the study (not shown). All the hearts were perfused at $6ml.min^{-1}.g^{-1}$ (see 4.2.1) and the mean pretreatment CF are shown in **Table 4.3**. These values were transformed as performed for the heart rates (described in 4.3.2) and shown in **Figure 4.2**. The CF declined significantly in a dose-dependent manner after the CO-treatment relative to the control group (**Figure 4.2**). The 0.05% CO-treated group had a significant decline in cumulative CF over the CO-free perfusion period relative to the control group (not shown) and is expected from the observed decline in CF. The inclusion of AO prevented the decline in CF produced after the 0.05% CO-treatment (**Figure 4.2**).

A

Time	***Coronary flow*** (ml.min^{-1}.g^{-1} wet weight of heart)				
(min)	**0% CO** (*n* = 3)	**0% CO with AO** (*n* = 3)	**0.01% CO** (*n* = 10)	**0.05% CO** (*n* = 6)	**0.05% CO with AO** (*n* = 5)
-12.5	5.61 ± 0.22	6.87 ± 0.11	5.73 ± 0.30	5.61 ± 0.23	7.12 ± 0.41
-7.5	5.56 ± 0.19	6.87 ± 0. 11	5.75 ± 0.33	5.60 ± 0.24	7.15 ± 0.38
-2.5	5.56 ± 0.14	6.87 ± 0. 11	5.74 ± 0.33	5.59 ± 0.23	7.14 ± 0.40

B

Time	***Cumulative volume of perfusate*** (ml.g^{-1} wet weight of heart)				
(min)	**0% CO** (*n* = 3)	**0% CO with AO** (*n* = 3)	**0.01% CO** (*n* = 10)	**0.05% CO** (*n* = 6)	**0.05% CO with AO** (*n* = 5)
0	84 ± 2	103 ± 1	86 ± 1	84 ± 0	107 ± 3
30	250 ± 3	308 ± 1	256 ± 4	250 ± 1	322 ± 7
120	745 ± 10	919 ± 4	739 ± 13	708 ± 13	953 ± 26

Table 4.3 The coronary flow (CF) of isolated rat hearts perfused with carbon monoxide (CO) with or without antioxidants (**AO**). Hearts were treated as described earlier (in Table 4.1 and Figure 4.1) and perfused at a flow rate of 6ml.min^{-1}.g^{-1} (wet weight). The CF was determined by measuring the collected volume of perfusate (or coronary effluent) in the perfusion fractions and normalising this volume per gram (wet weight) of heart tissue (***A***). The times stated above (in min) represent the midpoint of the time fractions used in the experiments: -15 to -10, -10 to -5, and -5 to 0 minutes. The data shown is the mean ± SD for *n* hearts in each group. (***B***) The cumulative volume of perfusate following CO-treatment with or without antioxidants (**AO**). The perfusate were collected from hearts exposed to CO in the time fractions (under the same conditions as described previously) and the volume of perfusate was measured prior to any biochemical analysis. The cumulative volume were normalised (as described above) and the mean ± SEM expressed for each group of treated hearts.

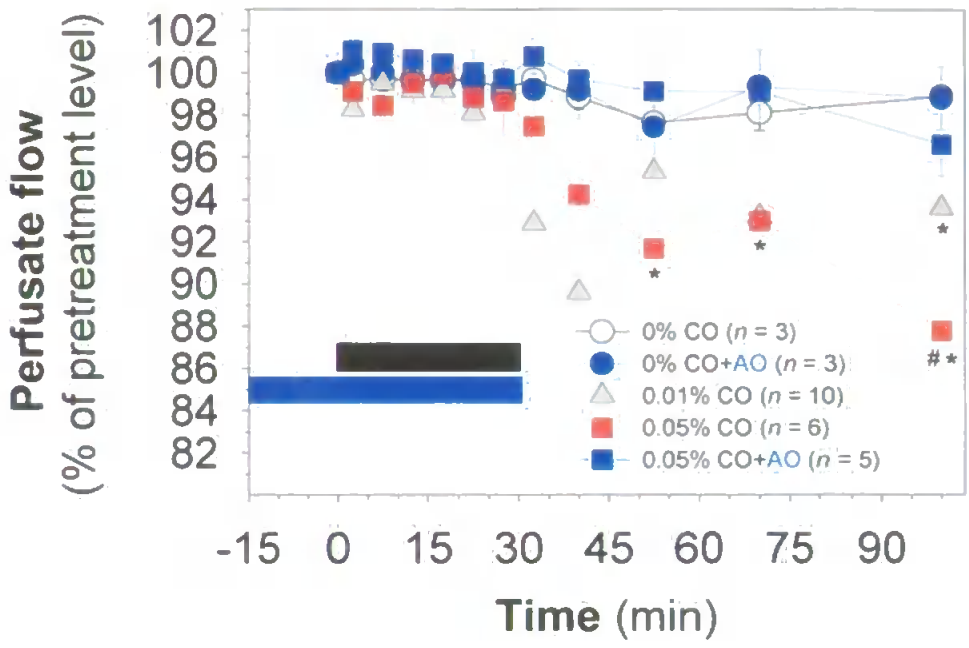

Figure 4.2 The coronary flow (CF) of isolated rat hearts exposed to CO with or without antioxidants (AO). The same experimental conditions were used as described in Figure 4.1. The black and blue bar denotes the period that hearts were perfused with CO (30min) and AO (45min), respectively. The pretreatment CF values were taken as 100% with the other measurements expressed as a percentage of the basal levels. The number of hearts used in each group is the *n* number shown in the figure (above). The data shown is the mean ± SEM for the hearts in each group. The asterisk (*) and hatched symbol (#) indicate a significant difference using ANOVA multivariate analysis at the 95% confidence level between the controls (with or without AO) and 0.01% CO groups, respectively.

4.3.4 Heart Weights & Tissue Water Content

The hearts were weighed prior to and after the perfusion to estimate changes in wet weight following CO exposure. All the groups had an increase in mean heart weight (HW) after being perfused compared to their pre-perfusion HW (**Table 4.4**). The increases were not significantly different to the control group (with or without AO). The water content of the ventricle tissue from perfused hearts (without AO) were determined by drying the tissues to constant weight (**Table 4.4**). No significant difference were found in these groups relative to the controls.

Measurement	**0% CO** (*n* = 3)	**0% CO with AO** (*n* = 3)	**0.01% CO** (*n* = 10)	**0.05% CO** (*n* = 6)	**0.05% CO with AO** (*n* = 5)
Pre-perfusion HW	1.37 ± 0.10	1.30 ± 0.07	1.51 ± 0.11	1.42 ± 0.10	1.61 ± 0.15
Post-perfusion HW	1.57 ± 0.11	1.44 ± 0.10	1.68 ± 0.09	1.50 ± 0.08	1.77 ± 0.08
Increase in HW (%)	15.00 ± 7.50	10.72 ± 12.56	11.58 ± 3.47	5.97 ± 5.26	10.62 ± 5.88
Tissue water content (%)	82.5 ± 0.9	*nd*	83.0 ± 1.4[†]	82.3 ± 2.7	*nd*

Table 4.4 The wet heart weights (HW) before their perfusion and after the 30min CO exposure and CO-free (90min) perfusion period. The post-perfusion water content (%) were determined by drying preweighed ventricle tissue (taken from perfused hearts) to constant weight. The data shown is the mean ± SD for the hearts in each group; and *nd* is not determined. [†]The *n* number of hearts was 8 (and not 10) through no collection and loss of the tissue sample.

4.3.5 Tissue Electrolyte Content

The electrolyte content was determined in the dried ventricle tissue of perfused hearts (described in 4.2.6). The electrolyte content were measured using ICP-MS for the following metals: sodium, potassium, calcium, magnesium, iron, copper, and manganese. The data shown below (in *4.3.5.1-4.3.5.8*) is the mean ± SD from 3, 3, 8, 6 and 5 hearts for the 0% (controls), 0% with AO (AO control), 0.01 and 0.05% CO, and 0.05% CO with AO (0.05% CO+AO) groups, respectively.

4.3.5.1 Sodium

The sodium content in the dried ventricle tissue of each treatment group were 8497 ± 1116, 9014 ± 2366, 6217 ± 1757, 9537 ± 2211, and 5891 ± 1178 $\mu g.g^{-1}$ (dry weight) for the control (n = 3 hearts), AO control (3), 0.01 (n = 8) and 0.05% CO (n = 6), and 0.05% CO+AO groups, respectively. There were no significant differences in the levels of sodium between the control and CO-treated groups ± AO. Although the sodium content in the 0.01% CO and 0.05% CO+AO groups were lower relative to the other groups. The ratio of sodium to potassium content was also determined (see *4.3.5.8*).

4.3.5.2 Potassium

The potassium content in the dried ventricle tissue for each group were 12672 ± 1483, 12629 ± 2562, 7827 ± 1492, 13680 ± 2839, and 6516 ± 2328 $\mu g.g^{-1}$ (dry weight) for the control, AO control, 0.01 and 0.05% CO, and 0.05% CO+AO groups, respectively (with the same n numbers as cited above for sodium). There were no significant differences between the CO-treated (± AO) groups relative to the control, however, the 0.01% CO and 0.05% CO+AO groups were lower as the sodium content (above). The ratio of sodium to potassium content was determined (see *4.3.5.8*).

4.3.5.3 Calcium

The calcium content of the dry ventricle tissue were 1735 ± 331, 1475 ± 310, 1284 ± 148, 1864 ± 275, and 1688 ± 429μg.g^{-1} (dry weight) for the control, AO control, 0.01 and 0.05% CO, 0.05% CO+AO groups, respectively. The 0.01% CO-treated hearts had a lower calcium content. No significant differences were found in the CO-treated hearts (± AO) relative to the control.

4.3.5.4 Magnesium

The level of magnesium (^{25}Mg) in the dry ventricle tissue were 1041 ± 146, 1053 ± 154, 817 ± 79, 983 ± 188, and 756 ± 137 μg.g^{-1} (dry weight) for the control, AO control, 0.01 and 0.05% CO, and 0.05% CO+AO groups, respectively. No significant differences were found in the CO-treated hearts ± AO relative to the control group. The analysis using ICP-MS also determined the levels of ^{26}Mg and these were similar to the ^{25}Mg levels (not shown).

4.3.5.5 Iron

The determined content of iron measured the total iron pool, i.e. Fe^{2+} (ferrous) and Fe^{3+} (ferric). The iron level in the dry tissue were 205 ± 73, 353 ± 101, 315 ± 42, 372 ± 95 and 349 ± 136 μg.g^{-1} (dry weight) for the control, AO control, 0.01 and 0.05% CO, and 0.05% CO+AO groups, respectively. The increased iron content of hearts exposed to CO had no significant difference relative to the control group. The control AO group also had an increase in iron content compared to the control group (with no AO), however, this increase was not statistically significant (at $P < 0.05$).

4.3.5.6 Copper

The copper content in the dry ventricle tissue were 63 ± 34, 27 ± 5, 34 ± 2, 31 ± 6, and 34 ± 13 μg.g^{-1} (dry weight) for the control, AO control, 0.01 and 0.05% CO, and 0.05%

CO+AO groups, respectively. The two CO exposed groups without AO had copper levels that were not significantly reduced compared to the control group. The control AO group had a lower copper content than the control group without AO (above). The AO appear to produce a similar change as CO for heart ventricle tissue, i.e. reduce the copper content.

4.3.5.7 Manganese

The level of manganese in the control group was unquantifiable (at -16 ± 9 $\mu g.g^{-1}$ dry weight). This level was considered negligible compared to the values for the AO control, 0.01%, 0.05% CO, and 0.05% CO+AO treated groups of 20 ± 6, 4 ± 3, 7 ± 3, and 12 ± 4 $\mu g.g^{-1}$ (dry weight), respectively. The antioxidant (AO) treatment increased the level of manganese in the heart ventricles, however, no significant difference were found between the treated groups relative to the control group

4.3.5.8 Sodium/potassium and calcium/magnesium ratios

A change in the ratio of sodium to potassium content may indicate cellular fluxes within the tissues. The ratio of sodium to potassium (Na^+/K^+) is determined from the tissue contents (above) and were 0.67 ± 0.01, 0.71 ± 0.04, 0.78 ± 0.12, 0.70 ± 0.14, and 1.00 ± 0.40 for the 0 (control), AO control, 0.01 and 0.05% CO, and 0.05% CO+AO groups, respectively. The CO ± AO-treatment showed an increased ratio, although this were not significant. The ratio of calcium to magnesium was determined similarly as for Na^+/K^+ to evaluate any fluxes of these ions in heart tissue following the exposure to CO. The ratio of calcium to magnesium ($Ca^{2+}/^{25}Mg^{2+}$) were 1.67 ± 0.28, 1.41 ± 0.28, 1.58 ± 0.18, 1.92 ± 0.23, and 2.39 ± 1.13 for the 0 (control), AO control, 0.01 and 0.05% CO, and 0.05% CO+AO groups, respectively. These values showed no significant difference between each treatment group compared to the controls and may suggest that no sodium or calcium loading occurred. The 0.05% CO+AO treatment increased this ratio, however, it were not signficant.

4.3.6 Osmolarity of Heart Perfusate

The osmolarity (a measure of the osmotic pressure) of the collected perfusate was determined (at a later date). The perfusate samples for each fraction were stored frozen prior to measuring its osmolarity. The mean osmolarity for each treatment group is shown in **Table 4.5*A***. No significant differences (in osmolarity) were found between the CO-treated and control groups, nor between the two CO exposed groups. The osmolarity of the perfusion buffer were also measured before the start and after the perfusions (**Table 4.5*B***). No significant differences were found in the osmolarity between the two groups. The perfusate osmolarity was not determined for hearts perfused with antioxidants.

A

Time (min)	***Osmolarity*** (mmol.kg^{-1})		
	0% CO (n = 3)	**0.01% CO** (n = 10)	**0.05% CO** (n = 6)
-12.5	257 ± 25	270 ± 17	275 ± 18
-7.5	283 ± 13	258 ± 19	278 ± 6
-2.5	261 ± 28	266 ± 22	278 ± 3
2.5	262 ± 39	268 ± 30	280 ± 7
7.5	255 ± 35	261 ± 24	279 ± 13
12.5	283 ± 5	270 ± 17	278 ± 5
17.5	282 ± 12	264 ± 15	275 ± 11
22.5	254 ± 28	253 ± 32	253 ± 36
27.5	266 ± 18	265 ± 18	277 ± 9
32.5	288 ± 5	259 ± 32	266 ± 20
40	262 ± 24	258 ± 35	260 ± 31
52.5	265 ± 33	269 ± 19	262 ± 23
70	264 ± 44	237 ± 47	274 ± 6
100	286 ± 2	260 ± 36	268 ± 11

B

Osmolarity of perfusing buffer (mmol.kg^{-1})	**0% CO** (n = 3)	**0.01% CO** (n = 10)	**0.05% CO** (n = 6)
Pre-perfusion	261 ± 43	269 ± 27	279 ± 4
Post-perfusion	278 ± 21	270 ± 22	280 ± 3

Table 4.5 Osmolarity in the coronary perfusate of rat hearts exposed to CO (***A***). The collected perfusate was stored at -20°C (after noting the necessary measurements and taking sample aliquots for biochemical analyses) prior to determining the osmolarity. The samples were thawed and kept on ice to minimise any evaporation to reduce inaccurate measurements. The osmolarity of the perfusion buffer before and after perfusing the hearts with CO is also shown in (***B***), pre- and post-perfusion, respectively. The data shown is the mean ± SD for the number of hearts (n) in each treatment group.

4.4 Discussion

The carbon monoxide (CO) treatment had a direct effect on the heart rate (HR) of isolated perfused rat hearts. CO exposure reduced the HR in the CO-treated hearts relative to the control group (not exposed to CO). A decrease in HR during CO exposure was also shown by McGrath (1984) using the Langendorff preparation. Their findings showed an immediate decline in HR during the CO-treatment, however, this decrease was significantly dose-dependent. Normoxic conditions were used during the experimental period using perfusion buffer equilibrated with 21% oxygen, whereas, McGrath (1984) used different levels of CO (0-95%) balanced with oxygen. The different level of oxygen used during CO exposure may have contributed to the reduced HR resulting from introducing hypoxia (as addressed previously in **3.4**). The HR post-CO exposure was not reported by McGrath (1984) to compare with our findings.

CO is shown to reduce the cerebral energy metabolism during CO exposure *in vitro* (Rogatsky *et al.*, 2002). This could suggest that a reduction in HR during and after CO exposure may be caused by a reduction in the cardiac energy metabolism. The further reduction in HR during the exposure to 0.05% CO with AO may be due to the inhibition of cytochrome *c* oxidase (or Complex IV). CO binds to the reduced haem centre of (ferro)cytochrome a_3 (Piantadosi, 1987) and could suggest a reduction of Complex IV by ascorbate (from the AO-treatment). CO can behave as a reductant, although this is reported to be 'far too slow' to inhibit cytochrome *c* oxidase (Morgan *et al.*, 1985). Early damage to mitochondrial function occurs during myocardial ischaemia (Kay *et al.*, 1997) and may contribute to the cardiotoxicity following severe CO poisoning *in vivo* where the incidence of hypoxia may be more likely than in the conditions used here. Whether any disruption in myocardial energetics occurred at the cytosolic or mitochondrial level needs further investigation to clarify the nature of events in CO poisoning and the heart rate.

An increased sodium content in heart tissue is shown to depress the contractile activity during anoxia (McGrath & Bullard, 1970). The decrease in HR could suggest that tissue

loading of sodium occurred in the heart during and/or following CO exposure. The contraction of rabbit papillary muscle disappear after ischaemia (Dekker *et al.*, 1996) suggesting that the CO-treatment may have resulted in ischaemia responsible for the decreased HR. This ischaemia-induced contracture is shown to increase the intracellular calcium. However, neither increase in sodium or calcium were found here (discussed below).

The intoxication of CO *in vivo* is shown to produce tachycardia to compensate for the depressed oxygen tension of the blood (1.4.1). However, bradycardia was observed in isolated hearts exposed to CO. The absence of these *in vivo* homeostatic mechanisms suggests a direct effect of CO in the isolated heart under normoxia. The bradycardia observed here is also shown in severe cases of CO poisoning where heart block is found to occur (Shafer *et al.*, 1965). Low levels of COHb (as low as 9% in blood) are associated with death following cardiac arrhythmia's (Dolan, 1985). The observed incidence of arrhythmia's in the hearts following CO-treatment (4.3.1) suggest that heart failure may have occurred if a longer period of CO-treatment and/or CO-free period were used. A major cause of death associated with acute CO intoxication is cardiac arrhythmia (Dolan, 1985). CO exposure produced an irregular beating of the heart similar to that described by Ehrich *et al.* (1944). These workers also found changes in the electrocardiogram (ECG) such as a decreased amplitude of the T-wave, inversion of the T-wave, elevation of the R-T segment, and later atrioventricular (AV) heart block. These ECG changes following CO poisoning are also found to occur during anoxia (Ehrich *et al.*, 1944; Middleton *et al.*, 1961). The observed irregular heart beats and fibrillation cannot conclude these were arrhythmia's as no ECG recordings of hearts were taken following CO exposure. However, it may be reasonable to associate those other findings with these found to suggest that CO may have resulted in anoxia (or ischaemia) within the myocardium. Although CO may have a different cardiotoxic mechanism than that of ischaemia/reperfusion (I/R) injury, the observed reduction in HR were similar to that of globally ischaemic hearts (over the same period as the CO exposure, i.e. 30 minutes). These findings may support a mechanism of I/R-like injury in the cardiotoxicity of CO.

However, ischaemia is not shown to reduce the HR in either isolated heart preparation (Chambers *et al.*, 1991).

In vivo a wide variation in blood PCO_2 and pH can affect the susceptibility of the heart to fibrillation (DeBias *et al.*, 1976). The altered HR in hearts (exposed to CO) cannot be explained by changes in PCO_2 (or pH) as these were constant throughout the perfusions. The chronotropic changes and arrhythmia's/fibrillation's may be due to an impaired coronary perfusion of the contractile components. The exposure to 0.01% CO in monkeys (for 6h) produced T-wave inversions that increased in incidence when infarcted monkeys were similarly exposed to CO. The energy requirements of the myocardium are greatest in the subendocardial layer of the LV, therefore, the inner wall is most likely to shown signs of reduced contractility in LV dysfunction (Einzig *et al.*, 1980). Patients with LV dysfunction (failure) have been shown to have an accompanying endothelial dysfunction and an early reduced myocardial perfusion during the transition from LV dysfunction to heart failure (de Jong *et al.*, 2003). The negative chronotropic response may indicate left ventricular dysfunction and the reduced coronary flow after CO exposure suggests that it could produce heart failure in this manner.

The *in vivo* positive chronotropic change following CO exposure arises from homeostatic changes to maintain adequate oxygenation of the tissues (above). Although an *in vivo* human study using humans exposed to high levels of CO (of 3.56%) found no significant increase in HR (Stewart *et al.*, 1973). The isolated heart may amplify any cardiac changes shown here as no other tissue compartments were present. Therefore, the findings from this study may be extrapolated to an *in vivo* setting associated with exposure to lethal levels of CO (**Chapter 3**). The use of a working heart preparation instead of the Langendorff preparation could have produced different results due to differences in myocardial energetics (3.1.2). Investigation of the change in HR during I/R (**1.6**) with both preparations of isolated rat heart has shown that the Langendorff preparation is more

resistant to ischaemia than working hearts in the time taken to attain a similar negative chronotropic level (Galinanes & Hearse, 1990b).

The observed order of events suggests that CO has a negative chronotropic effect that is produced before reducing the coronary flow (CF; 4.3.3). The permanently reduced HR following the 0.05% CO exposure suggests that some irreversible damage may have occurred in the contractile apparatus of the heart and account for the decreasing CF observed post-CO. Some hearts in the 0.01% CO-treated group had a recovery, whereas others showed a depressed HR with no sign of recovery post-CO suggesting an irreversible effect of CO. Histological change(s) in these tissues remain to be determined to identify any component(s) of the impaired heart activity. These changes appeared to be irreversible in this study, however, McMeekin & Finegan (1987) reported this impairment to be reversible using one patient.

The altered HR (following the CO exposure) may suggest an early causal role of heart failure by CO (Szatkowski *et al.*, 2001). The appearance of microscopic focal lesions were observed in the myocardium of adult rats exposed to 0.05% CO in air (Thomas & O'Flaherty, 1982) and supports the direct *in vivo* effect of CO that has been illustrated on several occasions in this study. Myocardial lesions as haemorrhage foci were observed in the ventricle tissue (4.3.1) and also shown in other cases of acute CO poisoning (Marek & Piejko, 1972). Necrosis is found in the reperfused canine myocardium after prolonged ischaemia (Becker *et al.*, 1999). Necrosis could have occurred in some of the myocytes and produced the contractile changes, however, this remains to be confirmed (in the ventricle tissue) using electron microscopy. These observed zones were predominantly found in the outer wall of the ventricle and support the transmural gradient driven by the perfusion through the coronary arteries (Griggs Jr. *et al.*, 1972). The lack of coronary obstruction (as found using coronary angiogram) in a patient exposed to acute CO (Marius-Nunez, 1990) appears to rule out the no-reflow phenomenon and supports the increased permeability of the coronary vasculature as a possible cause of the depressed coronary flow.

The change in HR by CO is similar to that reported for tobacco smoking and may be associated with the clinically recognised tobacco angina (Ayres *et al.*, 1970). Smoking tobacco produces initial tachycardia and shows a common flattening of the T-wave of the ECG trace (Oram & Sowton, 1963). The contractile changes described here may have resulted in an altered ECG recording during the period of CO exposure. Furthermore, the inverted T-waves found in ischaemia/reperfused hearts (Kolb, 2002) have been observed by other workers examining myocardial function following CO poisoning (McMeekin & Finegan, 1987). This may suggest that similar mechanisms and/or possible CO-induced ischaemia could be responsible for producing the CO-associated morbidity in heart tissue. Although the work of De Bias *et al.* (1976) showed an increased P-wave amplitude and T-inversion in the hearts of monkeys exposed to 0.012% CO (for 24 weeks), they concluded this was a result of non-specific myocardial stress rather than ischaemia (World Health Organization, 1979).

The changes in HR following the exposure to CO may be accounted for by ischaemia possibly at an intracellular level. Myocardial ischaemia as in a heart attack can kill cardiac cells (Sussman, 2001) and produce an altered ventricular function that can reside in two conditions: hibernating and stunned. The hibernating myocardium refers to viable myocardium that has become impaired following ischaemia, however, it is akinetic and may recover its function after adequate revascularisation (Siebelink *et al.*, 2000). The hibernating myocardium represents left ventricular dysfunction and can persist for several months to years (Kloner *et al.*, 1989). The stunned myocardium represents a prolonged myocardial depression (Lüss *et al.*, 2002). The myocardium can be salvaged by coronary reperfusion that exhibits transient postischaemic contractile dysfunction and lasts for hours to days (Patel *et al.*, 1988). The 0.05% CO-treated group had a depressed HR after CO exposure and may indicate a stunned myocardium. The 0.01% CO-treated group of hearts showed a recovery during the CO exposure, although the decline in the post-CO period may be attributed to a hibernating myocardium that may have shown recovery at a

later stage. Myocardial blood flow is impaired in the hibernating myocardium (Lüss *et al.*, 2002) and stunned myocytes show an impaired transport of calcium (Kloner *et al.*, 1989).

The coronary flow (CF) had a dose-dependent decline after the CO-treatment and suggests that CO may have direct effect(s) on the coronary vasculature. The coronary microvessels play a significant role in the determining the supply of oxygen and nutrients to the myocardium by regulating the CF conductance and substance transport (Komaru *et al.*, 2000). A similar reduction in blood flow is shown for rat lungs subjected to I/R (Vural & Oz, 2000). However, previous studies using whole animals exposed to CO, specifically rats, have shown that CF is increased in isolated perfused hearts taken from rats exposed to CO (Lin & McGrath, 1989; McFaul & McGrath, 1987). This is thought to be an adaptory response that occurs *in vivo* to maintain adequate oxygenation of the heart.

Heparin immediately increases the levels of hepatocyte growth factor (HGF) and this has a role in angiogenesis and growth of the collateral vessels (Matsumori *et al.*, 1998). Using heparin may have masked the changes observed in CF and could play a crucial role when examining the findings of other studies investigating the effects of ischaemia and CO *in vivo*. However, it is unlikely that any released HGF (if heparin had been administered) could evoke the described changes (above) in the period prior to perfusing the isolated heart.

The work by Bischoff *et al.* (1969) investigated the myocardial ultrastructure in dogs, rabbits and rats maintained at high altitude (4,300m for 5 months) and showed that the rat appeared to be the most resistant species by showing minor changes (Kjeldsen *et al.*, 1974). In light of this, the direct effect of CO on the coronary vasculature (found here) may be significant in man and could contribute to atherosclerosis, if not reversed before causing any myocardial damage. Work by McGrath & Smith (1984) showed that CF increased dose-dependently with CO levels below 50%, however, their study was limited by the introduction of hypoxia unlike here. Collateral flow is reduced following myocardial reperfusion after an ischaemic episode (Becker *et al.*, 1999), therefore, supporting the

notion (1.7) that intracellular hypoxia may have occurred following CO exposure in the presence of normoxia. Ischaemia has been shown to reduce the coronary vasodilator reserve (Fernández *et al.*, 2002) and suggests that the depressed CF after CO exposure may have arisen following intracellular ischaemia (produced from CO binding with myoglobin and limiting the diffusion of oxygen into the myocardial tissue). Isolated rat hearts subjected to global ischaemia (by clamping the aortic cannula for thirty minutes) have also shown a decline in CF after the ischaemic episode (Galinanes & Hearse, 1990a).

The arterioles, venules and capillaries of rabbit hearts exposed to 0.018% CO *in vivo* (for two weeks) showed similar swollen characteristics (Kjeldsen *et al.*, 1974). This swelling was possibly due to the absence of elastic membranes in the observed region. These workers also found a small accumulation of erythrocytes and thrombocytes and inferred petechiae, however, this tissue damage may have been caused by an inflammatory response. The autopsy of human hearts following acute CO poisoning showed acute circulatory failure in 45% of deaths (Marek & Piejko, 1972). Histological examination of these myocardial specimens showed similar changes of hyperaemia and lesions in the muscle fibres. Endothelial damage is attributed to the oedema found in the muscles of CO poisoned cases (Finley *et al.*, 1977). Endothelial cells may be sensitive to CO (Brenner & Wenzel, 1972) and the underperfusion of muscle tissue may contribute to the myonecrosis observed in CO poisoning.

The presented findings and evidence (above) suggest that CO intoxication may produce oedema. The vasodilatory effect of CO is possibly a direct effect and the normoxic conditions here rule out the involvement of any ischaemia-induced vasodilation. Rats that survived cyanide treatment showed no oedema, whereas CO produced significant cerebral oedema (Salkowski & Penney, 1995). The direct effect of CO must be considered in CO toxicity as oedema (produced through vasodilation) may be mistaken for cell swelling and indicate cell/tissue damage. The presence of a swollen vasculature appeared

to be largest for the 0.05% CO-treated hearts relative to the 0.01% CO group at the end of the perfusion period.

The profile of the CF post-CO may indicate some early damage prior to eventual heart failure through the cessation of myocardial perfusion. However, it has been shown that a reduced coronary blood flow of 60% lowered the oxygen consumption by 30%, accelerated the rate of glucose utilisation and increased the levels of lactate (Neely *et al.*, 1975). The reduction in CF could impair the oxygenation of the heart itself (depending on the dose and duration of CO exposure). The likely vasodilation following CO exposure found here may be mistaken *in vivo* as a compensatory mechanism to deliver more oxygen to the vasculature. The presence of excess fluid could limit the availability of oxygen (also other essential nutrients and impair the removal of waste) from the increased diffusion space produced between the capillary bed and localised tissues (Kjeldsen *et al.*, 1974). This may also be a protective measure to limit the delivery of CO within the myocardium. These myocardial changes are not restricted to the vasculature as other workers have also shown swelling in mitochondria and a complete disintegration of myofibril substructures. These swollen mitochondria have associated deposits of glycogen following acute CO poisoning and are suggested to represent myocardial cells incapable of using energy substrata (Tritapepe *et al.*, 1998). Blood flow determines the degree of oxygenation to the tissues *in vivo*. A reduction in oxygen availability in heart tissue could produce chemical ischaemia and may contribute to the no-reflow phenomenon or a myocardial infarct. One case has described an AMI attributed to acute CO poisoning (Ebisuno *et al.*, 1986). Serum enzymes, electrocardiogram and technetium-99m pyrophosphate scintigrams led these workers to conclude an AMI. In another similar case, the coronary arteriograms one week later showed no significant narrowing suggesting reversible coronary dysfunction (Marius-Nunez, 1990). It may be reasonable to state that CO may affect the coronary vessels to an extent that produces a direct AMI, although this needs further investigation (**Chapter 8**).

The work of Adams *et al.* (1973) showed that the coronary blood flow increased linearly with COHb in conscious dogs that were breathing 0.15% CO-air mixture for 30 minutes (through a tracheostomised preparation). These workers also found no significant changes in PO_2, PCO_2 and pH during the exposure to CO, therefore, the presence of the air mixture may have ensured chemical normoxia here. Despite the contrary change in CF by these workers compared to our findings, vasodilation was attributed to their observed increase in CF. Vasodilation of the coronary vasculature may be a direct effect of CO that continues to persist after the CO exposure and could be responsible for the oedema. Vascular CO is thought to inhibit vasoconstrictors by a mechanism involving stimulation of the tetraethylammonium (TEA)-sensitive potassium channel that is independent of cGMP (Kaide *et al.*, 2001). The likely vasodilation of the coronary vasculature by CO could account for the reduced perfusate flow. However, the dose-dependent effect of CO was present after CO exposure had ceased. The recently reported production of NO and the hydroxyl radical during reperfusion after ischaemia (Mączewski & Beręsewicz, 2003) may suggest that NO may produce vasodilation and/or endothelial damage (initiated by the hydroxyl radicals) in the coronary vasculature. The swelling of capillary endothelial cells is reputed to be a sign of ischaemic injury (Chiavarelli *et al.*, 1988) and suggests that the CO exposure in this study may have produced ischaemia (possibly by binding to intracellular myoglobin as no haemoglobin was present).

Based on the above evidence it is possible that CO exposure may produce a condition similar to myocardial infarction from the reduced delivery of oxygen to the myocardium that could eventually lead to heart failure. This highlights the severity of CO poisoning in people who have a cardiac disorder or are unknowingly predisposed to developing coronary disease (Scharf *et al.*, 1974). A primary factor in the rate of development and size of a myocardial infarct (produced from occlusion of the coronary artery) is the coronary collateral circulation (Maxwell *et al.*, 1987). The change in CF following the exposure to CO could account for the CO-induced AMI described in several cases (above).

Oedema is reported to occur when using perfusion buffers of low osmolarity and is also shown to decrease LV filling (Starr *et al.*, 1999). Using crystalloid solutions of low osmolarity to perfuse hearts can induce myocardial oedema (Starr *et al.*, 1999; Takoudes *et al.*, 1994), however, no significant differences were found in the osmolarity of the perfusing buffers of the CO-treated hearts relative to the control group. This suggests that the oedema characterised as swelling in the vasculature may be produced directly following the exposure to CO. Coronary constriction in open-chest dogs also showed no changes in the tissue water content relative to control animals that were cannulated without constriction (Griggs Jr. *et al.*, 1972). This suggests that the depressed CF could not be responsible for lowering the perfusate osmolarity and producing oedema. The normal osmolarity of the blood ranges from 290-310 $mOsm.l^{-1}$ and taking a normal level as 310 $mOsm.l^{-1}$ (Starr *et al.*, 1999) suggests that the osmolarity of the perfusion buffer used may be marginally lower here. This could account for the increase in wet weight (by inducing oedema) in hearts following their perfusion. However, the absence of oedema in the coronary vasculature of control hearts suggests that oedema may be a direct effect of CO. The water content (*ca.* 82%) of ventricle tissues were similar to that reported by Amirhamzeh *et al.* (1997) of 81% who used a perfusion buffer (called Plegisol; 289 $mOsm.l^{-1}$) with similar osmolarity. The nonsignificant increase in water content in all treated groups could suggest that this is attributed solely to the minor hypotonic nature of the perfusing buffer. This may have influenced the degree of oedema observed following the CO exposure and may suggest that the dietary intake of salt could influence the coronary response following CO exposure. The vasodilatory effect of CO may be responsible for the reported lowering of blood pressure (Penney & Chen, 1996). The decreased gain in HW following CO-treatment (relative to control group) may suggest some loss of the myocardial content occurred and could indicate tissue damage produced by CO (**Chapter 5**). An increase in water content was anticipated in light of the swelling observed in hearts exposed to CO, however, this absence may suggest that the loss of myocardial content following CO could be greater than the gain in HW (4.3.4).

In brief, a significant flux of one electrolyte will produce other electrolyte fluxes, especially when specific cellular ion exchange mechanism(s) are impaired such as in heart failure (Schwinger *et al.*, 2003). Some changes that occur in ischaemia/reperfused hearts have been mentioned here to discuss the findings (4.3.5) in light of the hypothesis (**1.7**). The electrolyte content was determined using inductively coupled plasma mass spectrometry (ICP-MS) to examine any flux(es) in heart tissue exposed to CO. The electrolytes measured are of physiological importance and by comparing suitable ratios it is possible to determine any ion fluxes due to CO poisoning.

The work of Gélinas *et al.* (1992) also used ICP-MS to determine the normal inorganic content (of various elements) in several tissues of Sprague-Dawley rats including the heart. Although their work did not cite the type of heart tissue used in determining the electrolyte distribution, i.e. heart ventricle tissue was used here; and their values were used as a comparison to identify any ion flux(es) following the exposure to CO in the isolated heart. The electrolyte content determined by Gélinas *et al.* (1992) for the heart was 68, 220, 0.13, 12, 5, 0.25 and 0.02 $mg.g^{-1}$ (*wet tissue*) for sodium, potassium, calcium, magnesium, iron, copper, manganese, respectively. The results (shown in 4.3.5) were expressed per gram of dry tissue with the water content of the tissues *ca.* 82% (4.3.4). The electrolyte levels were proportional to those of Gélinas *et al.* (1992).

The major electrolytes in heart tissue are sodium (Na), potassium (K), magnesium (Mg) and calcium (Ca). These metal ions play a vital role in the contractile function of the heart. The sodium/potassium ratio can provide information regarding the conduction of action potentials responsible for regular contractions in heart tissue. It has been shown that potassium levels declined significantly in the LV tissue (of hearts) from cadavers of CO poisoning (Marek & Piejko, 1972). This loss of potassium from the myocardium is found in failing hearts following ischaemia, whereas hearts that recovered from total ischaemia showed a gain of potassium (Humphrey *et al.*, 1987). The magnesium/calcium ratio can provide useful data on excitation-contraction coupling in myocardial performance. Many studies have reported that the plasma concentration of magnesium is associated with

cardiovascular diseases such as ischaemia, arrhythmias and hypertension (Murphy *et al.*, 1991). A low intracellular [magnesium] can cause significant dysfunction in cardiac myocytes in the absence of any other disease state(s) (Griffiths, 2000).

An increased sodium and calcium content and decreased potassium and magnesium content is established in the myocardium of isolated rat hearts subjected to ischaemia/reperfusion (I/R) injury (Kamiyama *et al.*, 1996; Humphrey *et al.*, 1987; McGrath & Martin, 1978). Similar fluxes are found in the serum following the exposure to high-altitude for simulating hypoxia (Christensen *et al.*, 1975). However, their study failed to consider the effects of dehydration in their electrolyte changes. The depletion of magnesium and potassium (with sodium loading) is shown to be responsible for cardiac arrhythmia's and death (Guideri *et al.*, 1975). The findings here do not substantiate the observed arrhythmia's in some CO-treated hearts. High intracellular levels of sodium can inhibit some enzyme systems, particularly those involved in glycolysis (Lockwood, 1963). Intracellular loading of sodium during anoxia can depress cardiac activity (Hercus *et al.*, 1955; McDowall *et al.*, 1955). The extrusion of cellular sodium is increased in the presence of extracellular bicarbonate. The perfusion buffer contained 20mM sodium bicarbonate (4.2.1) that may have reduced any sodium loading of the tissues. However, the role of bicarbonate in reperfusion-induced arrhythmias is ruled out (Zhu *et al.*, 2003). The absence of these changes (as described for I/R injury) do not support the *in vitro* cardiotoxicity of CO to proceed through an I/R *like* injury (mechanism) under the conditions used here. A lack of change in the sodium content is shown in hearts taken from victims of CO poisoning at autopsy (Marek & Piejko, 1972). Myocardial levels of potassium are reduced from 61.5 ± 4.9 to 50.0 ± 7.0 mequiv.kg^{-1} (to *ca.* 80%) in lethal cases of acute CO poisoning (Marek & Piejko, 1972). Much of the potassium is transported into cardiac myocytes across their membranes by an active transport mechanism involving the membrane bound Na^+-K^+-ATPase (Mousa *et al.*, 1987). However, no increase in potassium were found here following CO exposure (relative to the control group).

However, any electrolyte changes may have taken place during and/or immediately following the exposure to CO, and then returned to control levels by the end of the experimental period (4.6.3). Furthermore, these electrolyte changes may only occur in infarcted tissue with minor changes in the surrounding boundary tissue relative to normal tissue in infarcted hearts (Polimeni & Al-Sadir, 1975). The appearance of haemorrhaged zones (petechiae) found in some hearts treated with 0.05% CO could suggest that these zones had some tissue damage produced by CO. Regions of myocardial necrosis appear as diffuse haemorrhages in the peri- and endocardium following CO exposure (Ehrich *et al.*, 1944) along with degenerative changes in individual fibres (World Health Organization, 1979). These degenerative changes in individual fibres may arise due to the functional arrangement and architecture involving gap junctions that relay the transient electrolyte changes along the muscle fibres and compromise the contractile activity in this way (Ruiz-Meana *et al.*, 1999). Any electrolyte changes in these damaged zones remain to be determined. Myocardial scar tissue (verified by histology) has shown that the electrolyte changes described for I/R injury occur in these damaged region(s) and these changes could alter the overall myocardial picture (Lehr *et al.*, 1972). The observed focal zones (possibly of necrosis) may have been too small (in size and distribution) to have produced any significant changes found similar to I/R injury, however, these findings indicate a direct cytotoxic effect of CO on the myocardium for the 0.05% CO dose. The absence of minor change(s) in sodium content may be due to other means of passive transport that could have compensated for any electrolyte changes.

The influx of calcium (as established in I/R injury) was not found here. This influx is shown to occur during the reperfusion phase following ischaemia (Kloner *et al.*, 1989). Although rats administered verapamil (a Ca^{2+}-channel blocker) before and/or during acute exposure to 0.24% CO showed no protective effect (Penney & Chen, 1996). The influx of calcium is also shown to produce nitric oxide in aortic endothelium (Koyama *et al.*, 2002). However, the presence of low levels of calcium (1mM) has been shown to be detrimental in the stability of the isolated working heart preparation (Chambers *et al.*, 1991) with the optimal [calcium] being 1.2mM (Yamamoto *et al.*, 1984). Elevated intracellular calcium levels have

been found to occur after overloading the blood with salt (Mahboob *et al.*, 1997). Therefore, using a slightly hypotonic perfusing media (discussed above) may have had a protective effect by possibly reducing the influx of calcium as found in I/R injury (Haigney *et al.*, 1994). The intracellular loading of calcium into the tissue surrounding the coronary arteries and vasculature cannot be ruled out as the sodium/calcium exchanger (NCX) is also present in the tissues of the coronary arteries (Budel & Beny, 2000). If calcium loading were found to occur (following CO-treatment) in the coronary arteries it could suggest a tissue-specific effect of CO within the heart. Whether an intracellular overloading of calcium precedes or contributes to the CO-induced vasodilation remains to be determined. The possibility of calcium loading in the coronary arteries may suggest that an I/R injury could have occurred in light of the depressed coronary flow after CO exposure through an altered contraction of the arteries. These findings may also suggest that intracellular hypoxia occurred here. However, if any electrolyte changes did occur during the early CO-free period and returned to the control values found at the end of the experimental perfusion period, this may suggest that a reversible transition in these changes was evoked by CO. The decline in perfusate flow (after the exposure to CO) failed to show any recovery and may suggest that a reversible loading of calcium is not possible and/or may not be involved in the observed coronary dysfunction.

The iron (Fe), copper (Cu) and manganese (Mn) content showed changes that may be attributed to the CO-treatment. The iron content in the rat heart ventricle tissue was elevated whereas the copper content was lowered (both in a dose dependent manner). The manganese content followed a similar trend as the iron. The changes are discussed in light of their role(s) in heart tissue.

Iron is the most abundant transition metal in the human body (*ca.* 4-5 grams) and is essential for oxygen transport, DNA synthesis and electron transport (Conrad *et al.*, 1999). However, it is tightly regulated *in vivo* to ensure no free iron exists (Balagopalakrishna *et al.*, 1999). Iron is stored as ferritin and complexed with other biomolecules, and only a small portion of iron (*ca.* 1μM) is loosely bound and detachable

(Higson *et al.*, 1988). Although iron deficiency may upregulate certain inflammatory mediators such as COX-2 (Tanji *et al.*, 2001), it is also involved in cardiac hypertrophy and mitochondrial enlargement with copper deficiency (Goodman *et al.*, 1970). The increase in iron content could precede oxidative stress (and possibly contribute to releasing the cellular content from the tissues as described in **Chapter 7**). The role of iron as a cofactor for nitric oxide synthase (NOS; Halliwell & Gutteridge, 1999) may produce an upregulation of NOS and could have contributed to the decline in perfusate flow (via NO mediated vasodilation and/or coronary dysfunction through oxidative stress). The elevated iron may precede an increased production of NO, however, the reason(s) for this increase in iron by CO remains unknown. Iron and copper can produce the hydroxyl radical via the Haber-Weiss reactions in the presence of the reduced (cationic) forms, Fe^{II} (ferrous) and Cu^{I}, respectively. Iron overload is a well-known cause of myocardial failure and is thought to occur through iron-catalysed free radical generation that damages the myocardium and alters cardiac function (Bartfay *et al.*, 1999) suggesting that acute exposure to CO may initiate heart failure through oxidative stress (**1.7**). Significantly elevated levels of cytotoxic aldehydes is found in heart tissue of iron-loaded mice (Bartfay *et al.*, 1999), however, as no other significant electrolyte fluxes were found the likely cause of iron overload by CO remains undetermined.

Copper is required to maintain normal iron metabolism and can increase the oxidation of Hb and Mb (Halliwell & Gutteridge, 1999). It is also required as a cofactor for cytochrome *c* oxidase and copper zinc superoxide dismutase (CuZnSOD). The chelating property of copper ions for the intracellular antioxidant (or reductant) glutathione (GSH) could suggest that more GSH may be available for cellular protection. This could also suggest that oxidative stress occurred as a result of the CO exposure (**Chapter 5**). Conversely, the decline in copper content could suggest that oxidative stress occurred and depleted the intracellular content of copper-bound GSH. Coincidentally, iron and copper have an inverse relationship in which these two metals depend on each other in normal metabolism (Harris, 1994). An increase in iron content and a fall in the copper content of the hearts exposed to CO were found here.

No difference in the manganese content of the CO-treated groups and the controls were found here. However, the level of manganese in the control group was negative and suggests it was below the detection limit of the analytical method used (ICP-MS). The manganese content appeared to rise in the CO-treated hearts and is visible if the control group is assigned a level of zero and the other values adjusted accordingly (*ca.* 20 and 23 $\mu g.g^{-1}$ for 0.01 and 0.05% CO hearts, respectively). Manganese in cells and tissues is predominantly localised in mitochondria as a cofactor for superoxide dismutase (SOD). SOD catalyses the dismutation of superoxide to hydrogen peroxide (Halliwell & Gutteridge, 1999). When the supply of copper ion falls the activity of copper zinc SOD (CuZnSOD; cytosolic SOD) is reduced, however, more manganese SOD (MnSOD) is synthesised to maintain a similar total activity of SOD. Coincidentally, the copper content had fallen in ventricle tissue following CO exposure, whereas the manganese levels appeared to rise relative to the control group. This may suggest an incidence of oxidative stress in the ventricles of CO-treated hearts. Oxidative stress through the production of ROS can disrupt ion channels and cell/tissue homeostasis (Kuzuya *et al.*, 1993; Ziegelstein *et al.*, 1992), however, no ion fluxes were found here.

4.5 Conclusions

Perfusing isolated rat hearts with CO altered the heart rate (relative to the control group) and produced an incidence of arrhythmias. The presence of 21% oxygen (in the perfusing buffer may rule out systemic hypoxia and could suggest a direct effect of CO in the heart. The decline in coronary (or perfusate) flow in a dose-dependent manner after CO exposure under these conditions suggests that an I/R-like injury may have resulted from CO exposure. The reduced heart rate and the swelling of the vasculature may indicate different effect(s) of CO in the ventricle and vasculature tissues, respectively. Therefore, the cardiotoxicity of CO may arise through several effects from tissues within the heart, and these may contribute to the CO-associated morbidity that arise following CO poisoning. The lack of electrolyte fluxes that are found in other studies examining I/R injury in heart tissue appears to rule out the hypothesis that the cardiotoxicity of CO precedes through an I/R *like* injury (**1.7**). However, other changes do occur in hearts subjected to I/R and an absence of the electrolyte fluxes do not rule out the postulated hypothesis. Further measurements to evaluate the possible production of intracellular hypoxia and oxidative stress are necessary to test the postulated hypothesis (**1.7**).

4.6 Further Work

4.6.1 Osmolarity

A hyperosmotic perfusing media (infused during reperfusion) has been shown to improve the stunned myocardium arising after ischaemia in isolated rat hearts and reduce the intracellular calcium overload (Sun & Lin, 1990). The uses of hypo- and hypertonic intravenous cocktails remain to be investigated in light of preventing further cardiac damage following acute CO poisoning. This may ensure satisfactory reduction in the degree or incidences of the cardiac morbidity(s) associated after acute exposure to CO and determine whether dietary factors such as salt intake could influence CO poisoning.

4.6.2 Changes in tissue electrolyte content

The possibility of electrolyte changes occurring in heart ventricle tissue during the CO exposure remains to be investigated. This issue could be addressed by using the same isolated heart preparation and removing these perfused hearts at various times during and after following the exposure to CO. This would determine any electrolyte flux(es) that may have normalised (to control levels) after the 90 minute CO-free period and confirm whether I/R *like* changes took place to support the hypothesis. The electrolyte contents could be determined in the perfusate samples to identify any fluxes within the tissues that may have appeared as nonsignificant.

Chapter Five:

Cardiotoxicity of CO During Normoxia - Biochemical Changes

5.1 Background

The work described (in **Chapter 4**) showed physiological changes consistent with ischaemia/reperfusion (I/R) injury and other findings that were not. The physiological changes were found using isolated hearts exposed to CO in the presence of normoxia. These changes show a direct effect of CO that may not otherwise be observed *in vivo*. The underlying biochemistry is important to identify any changes within the myocardium to fulfil the aims of the study (**1.7**). This chapter presents the biochemical findings and ascertain whether intracellular hypoxia (hypoxia$_i$) and/or oxidative stress occurred in the heart during CO exposure.

5.2 Materials & Methods

5.2.1 Isolation & Langendorff Preparation

The isolation of rat hearts and their Langendorff preparation was performed as described previously (4.2.1 and 4.2.2, respectively). No heparin was administered to the (unconscious) animals as heparin decreases the levels of assayed troponin I (Gerhardt *et al.*, 2000; and 5.2.3).

5.2.2 Experimental Protocol

This was performed as described in the previous chapter (4.2.2).

5.2.3 Cardiac Markers of Viability

The perfusate samples (for determining the tissue viability) were kept in heparin-free tubes placed on ice during the experiments. The samples were delivered for analysis ≤1h after completing the perfusion. Lactate dehydrogenase (LDH), creatine kinase (CK) and troponin I (TnI) are used as markers in cardiology. LDH and CK activities in the collected perfusate fractions were assayed on the day by Triphasic Laboratories (Plymouth, UK) using commercial diagnostic kits and automated analysers (Beckman). The control hearts with or without AO (n = 3) and 0.05% CO-treated group with AO had their LDH and CK activity determined *in house* using a commercial diagnostic kit from Sigma. The perfusate from the CO-treated groups (0.01, 0.05 and 0.10% CO) were assayed by Triphasic Ltd. The use of LDH and CK to determine tissue viability were described in 3.2.4. Heparin is not compatible with the LDH and CK assays used (personal communication with Jane Dickinson, Triphasic Laboratories, Plymouth, UK). No heparin was administered to the animal following anaesthesia as it has also been shown to contribute in angiogenesis and increase collateral blood flow (4.4; Matsumori *et al.*, 1998; Shulman, 2000).

Troponin I (TnI) is a very specific marker in assessing cardiac function, and its activity was determined in four perfusate fractions: pretreatment period (in the –5 to 0 min fraction), during CO exposure (10 to 15 min), post-CO (30 to 35 min) and post-recovery treatment (80 to 120 min). Only one determination per sample (from each fully perfused heart) was assayed for TnI. The assay was conducted by Triphasic on samples that had been frozen at -80°C (prior to thawing on ice) before being assayed as described above. The samples for troponin analysis were frozen before their determination as a prerequisite of the diagnostic assay (personal communication with Jane Dickinson, Triphasic Laboratories Ltd, Plymouth, UK). A study by Heeschen *et al.* (2000) has shown no significant reduction in troponin concentrations for plasma samples anticoagulated with sodium heparin, however, this is contradictory to the findings by another study (Gerhardt *et al.*, 2000).

5.2.4 Preparation of Rat Heart Ventricle Tissue Homogenate

The preweighed frozen rat heart ventricle tissue was homogenised as described by Rouet-Benzineb *et al.* (1999); although tetrahydrobiopterin were not included in the homogenisation buffer (HB). Briefly, the 10% (w/v) homogenates were prepared by mincing the tissue in HB using scissors, followed by a Potter-Elvehjem glass-Teflon homogeniser (5 passes per gram of wet weight tissue). Ice-cold HB comprised 250mM Tris-HCl, 1mM EGTA, 1mM phenylmethylsulphonylfluoride (PMSF), 10μg/ml pepstatin A and 10μg/ml leupeptin (to pH 7.4 using 4M KOH). The homogenate (10% w/v) was collected and centrifuged at 1500*g* (5min). The supernatant was centrifuged (1500*g*, 5min) and at 8000*g* for 15 minutes. The supernatant (homogenate) was immediately deproteinised for later analysis (5.2.5).

5.2.5 Deproteinisation of Homogenates

This was performed immediately on freshly prepared homogenate samples (5.2.4) to prevent the oxidation of various analytes by neutral pH, air and cellular proteins. Care

must be taken as perchloric acid ($HClO_4$) is a strong oxidising acid. Two volumes of ice-cold 8% $HClO_4$ (analytical reagent; BDH Chemicals Ltd, Poole) were added to one volume of homogenate. The contents were gently mixed by inversion and kept on ice (15min) with occasional mixing prior to centrifugation (2000*g* for 10 min) to remove the protein. The supernatant was snap-frozen and stored (at -80°C) in 0.5-1ml aliquots (in microeppendorf tubes) for later analysis (5.2.7). Any exposure of the deproteinised extracts to air were kept to a minimum to prevent the oxidation of some analytes. Samples for glutathione analysis had 2µl of 2-vinylpyridine (VP) added to 100µl of deproteinised extract. These were mixed by vortexing before storing at -80°C for later analysis (*5.2.7.5*).

5.2.6 Protein Determination

The protein content of the perfusates (5.2.3) and tissue homogenates (5.2.4) were determined (to express the enzyme activity in specific units of activity). The protein assay kit was supplied by BioRad Laboratories (Hemel Hampstead), and used according to the manufacturers instructions based on the method of Wiechelman *et al.* (1988).

5.2.7 Other Biochemical Measurements In Heart Tissue

The following assays (below) were performed using a spectrophotometer (3.2.5).

5.2.7.1 Lactate

The lactate content was determined in deproteinised homogenate extracts (5.2.5) using a commercial Sigma Diagnostic kit (Lactate 826-B; Lot 120K6030) according to the manufacturers instructions based on the method of Gloster & Harris (1962).

5.2.7.2 Pyruvate

The pyruvate content was determined in deproteinised homogenate extracts (5.2.5) using commercial Sigma Diagnostic kit (Pyruvate 726; Lot 031K6054) according to the manufacturers instructions based on the method of Bucher *et al.* (1963).

5.2.7.3 Glucose

The glucose content was determined in deproteinised homogenate extracts (5.2.5) using commercial Sigma Diagnostic kit (115-A; Lot 100K6112) according to the manufacturers instructions based on the method of Bergmeyer (1963).

5.2.7.4 Glycogen

The glycogen levels in deproteinised homogenate extracts (5.2.5) were determined by measuring the glucose content (*5.2.7.3*) after hydrolysing the glycogen according to the method of Keppler & Decker (19xx). In brief, the pH of the deproteinised extract was adjusted to pH 4.5 for optimal activity of the amyloglucosidase (as pH > 4.8 would inhibit the hydrolysis) and incubated at 40°C for 2h (final activity of 24 $U.ml^{-1}$) prior to stopping the enzyme by adding perchloric acid.

5.2.7.5 Glutathione

Deproteinised rat heart ventricle homogenates (5.2.5) were used to determine the tissue glutathione levels. The assays were performed using a modified method described by Griffith (1980). The acidic deproteinised extracts (500µl) were neutralized with ice-cold 16% KOH in 0.12M Tris-base buffer (52µl), centifuged at 13,000*g* (2min) to remove the insoluble potassium perchlorate. The supernatant was carefully removed and placed on ice prior to analysis. A working assay reagent was freshly prepared prior to use by mixing reagents A, B and C (at room temperature). All assay reagents were prepared in 100mM Na^+-phosphate buffer with 1mM Na^+-EDTA (pH 7.4 at 25°C) and stored in the fridge

before use: A, 0.4mM NADPH; B, 4mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB); and C, 50 $U.ml^{-1}$ glutathione reductase (GR). Standards of reduced and oxidized glutathione (GSH and GSSG) were prepared in 5.3% perchloric acid prior to use and assayed as described. Final assay concentrations of reagents (in 1ml final volume) were 0.2mM NADPH, 0.4mM DTNB, and 0.5 $U.ml^{-1}$ GR. The working range of the assay was determined as 0-25μM from the linear range of the standards used (200μl samples). All samples were kept on ice prior to analysis. Appendix A gives details of the assay method and other tests performed to implement the assay.

5.2.8 Data analysis

Performed as described in 4.2.9.

5.3 Results

5.3.1 Tissue Viability

The viability of the isolated perfused hearts were assessed by measuring the activities of markers released into the perfusate. Three markers were used to assess the viability of heart tissue: lactate dehydrogenase (LDH), creatine kinase (CK) and troponin I (TnI). The activity of LDH was determined in the collected perfusate fractions (after measuring the volume to determine the coronary flow; *4.2.3.2*) and normalised to the wet weight of the tissue (**Table 5.1*A***). To allow comparisons to be made between the groups; the mean of the three pretreatment (basal) values were taken as 100% and the activity of the other fractions were expressed relative to the mean basal level. The mean data from each treatment group is shown in **Figure 5.1**. No significant difference in LDH activity was found at $P < 0.05$ in any of the groups relative to control levels, although the CO-treatment did increase the loss of LDH from the heart. The activity of CK was determined in the same perfusate samples that LDH was assayed. The pretreatment CK activity (normalised) are shown in **Table 5.1*B***; and shown as a mean percentage of the pretreatment level as performed for the LDH data to compare between groups (**Figure 5.2**). The CO-treated hearts had an increased activity of CK compared to the controls, however, no significant differences were found. The enzyme activities in the perfusates of the start-stop experiments (where hearts were perfused for a specified duration and CO exposure) were not determined.

Table 5.1 *(overleaf)* The basal activity of lactate dehydrogenase (LDH) and creatine kinase (CK) in the perfusate of rat hearts exposed to carbon monoxide (CO) in the presence of antioxidants (**AO**). The perfusate was collected and stored on ice before measuring the activity of LDH on the same day. Isolated hearts were perfused in a retrograde manner through the aorta using a Langendorff perfusion apparatus at 6ml.min^{-1}.g^{-1} (wet weight). The hearts were perfused with buffer at 36.9°C (see Materials & Methods for the composition of the perfusion buffer). The buffer was equilibrated with a certified gas mixture containing 21% oxygen (O_2), 5% carbon dioxide (CO_2), balanced with nitrogen (N_2) and gassed constantly with this mixture for the entire perfusion period to establish pH 7.4. Hearts were perfused for 15min to wash out the remaining blood and allow the organ to stabilise from the excision procedure. The basal (or pretreatment) activity of LDH (***A***) and CK (***B***) were then determined over three five-minute fractions (over 15min period) prior to commencing the carbon monoxide (CO) treatment. The CO exposure (for 30min) used *normoxic* conditions with 0% (n = 3 hearts; controls), 0% with **AO** (n = 3 hearts), 0.01% (n = 10 hearts), 0.05% (n = 6 hearts) and 0.05% CO with **AO** (n = 5 hearts). Normoxic conditions were used throughout the entire perfusion period using gas mixtures with varying nitrogen levels to compensate for the different CO levels (0-0.05%). Hearts were treated by perfusing with buffer that was pre-equilibrated with CO. The time to attain this switch-over was *ca.* 2min. After the 30min CO exposure hearts were perfused for 90min to assess any functional recovery post-CO. The data shown is the mean ± SD for n hearts in each group.

A

Time	***LDH activity*** (units.l^{-1}.g^{-1} wet weight of heart)				
(min)	**0% CO** (n = 3)	**0% CO with AO** (n = 3)	**0.01% CO** (n = 10)	**0.05% CO** (n = 6)	**0.05% CO with AO** (n = 5)
-12.5	0.22 ± 0.04	0.05 ± 0.02	45 ± 16	22 ± 16	0.06 ± 0.02
-7.5	0.22 ± 0.04	0.04 ± 0.01	40 ± 17	18 ± 11	0.06 ± 0.02
-2.5	0.19 ± 0.04	0.04 ± 0.01	39 ± 18	14 ± 6	0.06 ± 0.05

B

Time	***CK activity*** (units.l^{-1}.g^{-1} wet weight of heart)				
(min)	**0% CO** (n = 3)	**0% CO with AO** (n = 3)	**0.01% CO** (n = 10)	**0.05% CO** (n = 6)	**0.05% CO with AO** (n = 5)
-12.5	30 ± 5	35 ± 19	30 ± 16	10 ± 7	29 ± 18
-7.5	26 ± 7	29 ± 14	29 ± 15	11 ± 7	36 ± 20
-2.5	26 ± 7	27 ± 12	28 ± 14	10 ± 8	36 ± 26

Table 5.1 *The table legend is on the previous page.*

Figure 5.1 *(overleaf)* Normalised activity of lactate dehydrogenase (LDH) and creatine kinase (CK) in the perfusate (coronary effluent) from isolated hearts perfused with carbon monoxide (CO). Hearts were perfused in a retrograde manner through the aorta using a Langendorff apparatus. The hearts were exposed to 0, 0.01 and 0.05% CO and 21% O_2 (balanced with N_2) for 30 minutes followed by a CO-free gas mixture (90min) by perfusion. Some hearts were treated with antioxidants (**AO**): TroloxC and sodium ascorbate were included in the perfusion buffer (at 0.2 and 1mM, respectively) during the 15min pretreatment (basal) period and during the CO exposure. The CO exposure (for 30min) used *normoxic* conditions with 0% CO (n = 3 hearts; controls), 0% CO + AO (n = 3 hearts; antioxidant controls), 0.01% CO (n = 10 hearts), 0.05% CO (n = 6 hearts) and 0.05% CO + AO (n = 5 hearts). The data points show the activity of LDH (*top panel*) and CK (*bottom panel*) in the collected perfusate fractions. The black and blue bar denote the period that hearts were exposed to CO (for 30min) and AO (45min), respectively. Same conditions as described in the legend for Table 5.1. The mean enzyme activity was determined from the three pretreatment fractions and taken as 100%, with the activity of LDH and CK for the other perfusate fractions expressed as a percentage of the mean basal activity for each heart. The data shown is the mean ± SEM with the number of hearts (n) used in each group shown in the figure. The timepoints overleaf (in minutes) represent the midpoint of the perfusate fractions that were collected during each perfusion to determine the tissue viability: -15 to -10, -10 to -5, -5 to 0, 0-5, 5-10, 10-15, 15-20, 20-25, 25-30, 30-35, 35-45, 45-60, 60-80 and 80-120 minutes.

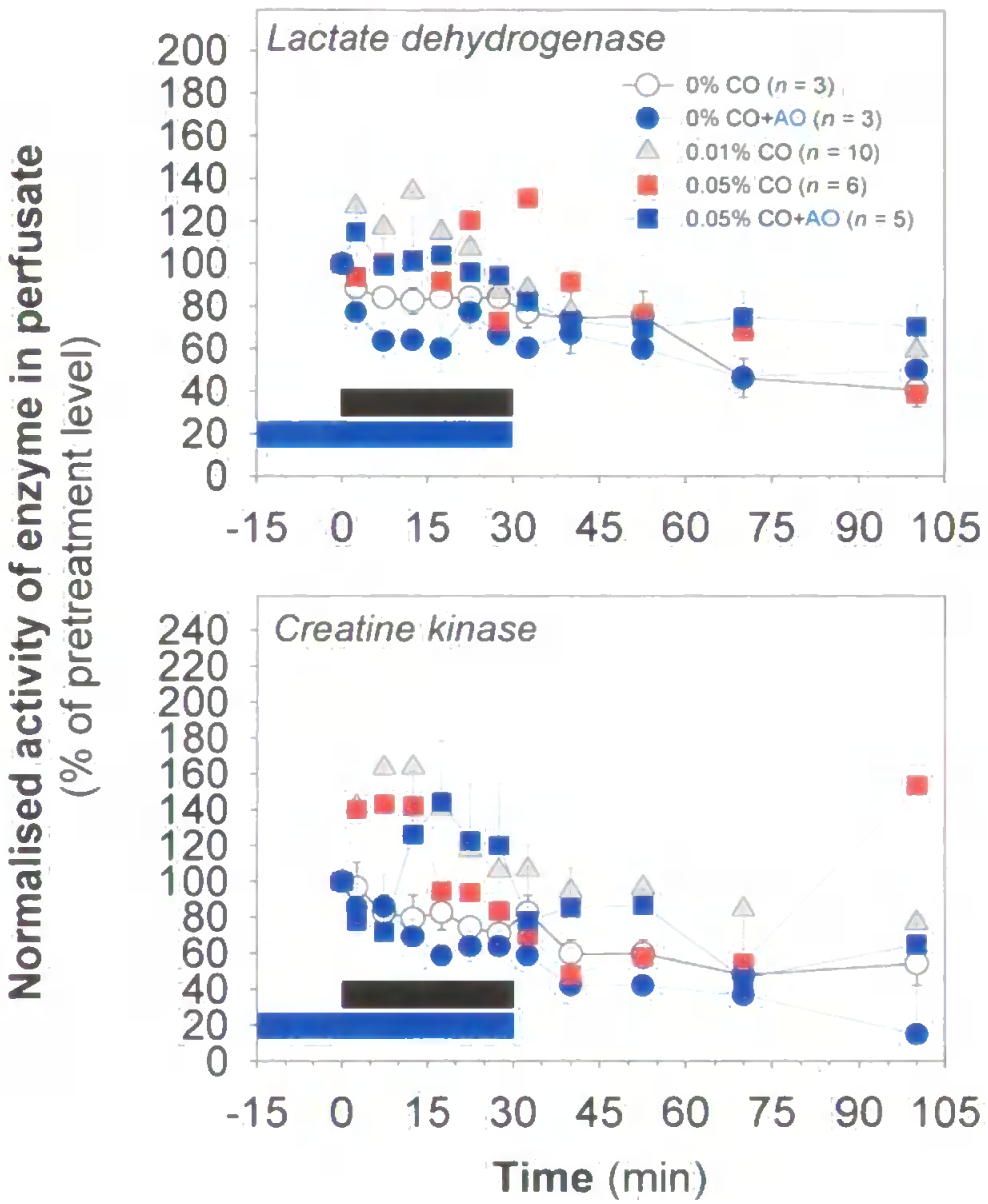

Figure 5.1 *The figure legend is on the previous page.*

The troponin I (TnI) activity was determined in the same samples (after storage for *ca.* 6.5 weeks at -80°C prior to their measurement; *5.2.3.2*). The normalised pretreatment data is presented in **Table 5.2**. The rate of release of TnI (**Table 5.2**) was determined by calculating the total amount of TnI in each perfusate fraction and expressing the amount released per minute. No significant differences were found between the CO-treated hearts and the controls. The activity of TnI for the 0.05% CO-treated group has shown the mean data from 5 hearts (compared to the 6 hearts cited for the LDH and CK data) as the samples for one heart were misplaced (by the laboratory performing the enzyme assays).

Perfusate fraction (min)	***Troponin I (TnI) activity*** in ng.l^{-1}.g^{-1} (wet weight) (***Rate of TnI release*** in ng.min^{-1}.g^{-1})		
	0.00% CO (n = 3)	**0.01% CO** (n = 10)	**0.05% CO** (n = 5)
-5 to 0	5.42 ± 0.83	3.43 ± 1.08	0.72 ± 0.72
	(*0.041 ± 0.005*)	(*0.030 ± 0.009*)	(*0.005 ± 0.005*)
10 to 15	5.58 ± 2.09	9.04 ± 2.86	18.70 ± 14.35
	(*0.043 ± 0.016*)	(*0.079 ± 0.025*)	(*0.132 ± 0.101*)
30 to 35	2.68 ± 0.24	12.26 ± 3.88	0.85 ± 0.85
	(*0.020 ± 0.002*)	(*0.105 ± 0.033*)	(*0.007 ± 0.007*)
80 to 120	3.55 ± 1.27	9.25 ± 2.92	0.55 ± 0.34
	(*0.028 ± 0.011*)	(*0.077 ± 0.024*)	(*0.004 ± 0.002*)

Table 5.2 The activity of troponin I (TnI) as a marker of tissue viability were determined in perfusate samples of hearts perfused with CO. Four determinations were made for each heart perfused: pretreatment (-5 to 0min), mid-exposure to CO (10 to 15min), immediately post-CO exposure (30 to 35min) and at the end of the perfusion (in the 80 to 120min fraction). The activity of TnI was normalised to gram of heart tissue (wet weight) in the treated hearts. The rate of TnI release was determined by deriving the normalised absolute amount in the specified time fraction and dividing this value by the duration (in minutes) of the fraction to obtain a final rate. The time in **bold** text denotes the period that hearts were exposed to CO, i.e. 0 to 30 minutes. The data shown is the mean ± SEM with the number of hearts (n) used in each group.

The rate of release of the markers were determined to assess when the CO exposure caused a loss of tissue viability. This was determined similarly (as performed above for the troponin I data) for LDH and CK and is shown in **Table 5.3** and **5.4**, respectively. The profiles for the rate of release mirror the activity of the markers in the perfusate fractions (not shown) and appeared to be increased with CO-treatment, however, no significant differences were found.

Time	***Rate of LDH release*** (units.min^{-1}.g^{-1} wet weight of heart)				
(min)	**0.00% CO** (n = 3)	**0.00% CO with AO** (n = 3)	**0.01% CO** (n = 10)	**0.05% CO** (n = 6)	**0.05% CO with AO** (n = 5)
-12.5	0.0017 ± 0.0004	0.00044 ± 0.00019	0.39 ± 0.14	0.18 ± 0.14	0.00064 ± 0.00022
-7.5	0.0017 ± 0.0004	0.00040 ± 0.00007	0.35 ± 0.15	0.14 ± 0.09	0.00064 ± 0.00030
-2.5	0.0014 ± 0.0004	0.00034 ± 0.00009	0.34 ± 0.16	0.12 ± 0.06	0.00063 ± 0.00055
2.5	0.0014 ± 0.0004	0.00029 ± 0.00011	0.46 ± 0.30	0.14 ± 0.14	0.00084 ± 0.00054
7.5	0.0013 ± 0.0003	0.00024 ± 0.00011	0.44 ± 0.32	0.14 ± 0.16	0.00079 ± 0.00055
12.5	0.0013 ± 0.0005	0.00024 ± 0.00010	0.49 ± 0.32	0.16 ± 0.21	0.00084 ± 0.00056
17.5	0.0013 ± 0.0003	0.00023 ± 0.00012	0.42 ± 0.27	0.13 ± 0.15	0.00085 ± 0.00056
22.5	0.0013 ± 0.0003	0.00029 ± 0.00012	0.38 ± 0.24	0.17 ± 0.11	0.00078 ± 0.00053
27.5	0.0013 ± 0.0003	0.00024 ± 0.00007	0.32 ± 0.26	0.11 ± 0.10	0.00073 ± 0.00043
32.5	0.0012 ± 0.0004	0.00023 ± 0.00009	0.29 ± 0.19	0.25 ± 0.43	0.00066 ± 0.00042
40	0.0011 ± 0.0004	0.00024 ± 0.00005	0.25 ± 0.18	0.10 ± 0.07	0.00059 ± 0.00038
52.5	0.0011 ± 0.0001	0.00021 ± 0.00003	0.23 ± 0.14	0.08 ± 0.06	0.00051 ± 0.00031
70	0.0007 ± 0.0002	0.00017 ± 0.00003	0.21 ± 0.11	0.08 ± 0.04	0.00047 ± 0.00023
100	0.0006 ± 0.0001	0.00018 ± 0.00005	0.18 ± 0.10	0.06 ± 0.07	0.00045 ± 0.00025

Table 5.3 Rate of lactate dehydrogenase (LDH) release from CO-treated hearts perfused with or without antioxidants (**AO**; same conditions as previously described). The times show the midpoint of the collected perfusate fractions (as given in **Figure 5.1**). The time in **bold** text denotes the period that hearts were exposed to CO, i.e. 0 to 30 minutes.

Time	***Rate of CK release*** (units.min^{-1}.g^{-1} wet weight of heart)				
(min)	**0% CO** (n = 3)	**0% CO with AO** (n = 3)	**0.01% CO** (n = 10)	**0.05% CO** (n = 6)	**0.05% CO with AO** (n = 5)
-12.5	0.23 ± 0.05	0.32 ± 0.18	0.25 ± 0.13	0.08 ± 0.06	0.35 ± 0.23
-7.5	0.20 ± 0.07	0.26 ± 0.13	0.25 ± 0.12	0.09 ± 0.06	0.43 ± 0.28
-2.5	0.20 ± 0.07	0.24 ± 0.12	0.25 ± 0.12	0.08 ± 0.07	0.45 ± 0.36
2.5	0.20 ± 0.05	0.23 ± 0.10	0.36 ± 0.25	0.10 ± 0.09	0.40 ± 0.35
7.5	0.17 ± 0.02	0.22 ± 0.10	0.43 ± 0.36	0.12 ± 0.14	0.45 ± 0.49
12.5	0.17 ± 0.07	0.21 ± 0.13	0.44 ± 0.41	0.14 ± 0.21	0.57 ± 0.43
17.5	0.17 ± 0.02	0.17 ± 0.10	0.37 ± 0.36	0.10 ± 0.13	0.58 ± 0.42
22.5	0.15 ± 0.03	0.19 ± 0.12	0.30 ± 0.29	0.08 ± 0.08	0.58 ± 0.44
27.5	0.15 ± 0.07	0.19 ± 0.12	0.26± 0.28	0.06 ± 0.06	0.53 ± 0.39
32.5	0.17 ± 0.02	0.17 ± 0.10	0.25 ± 0.26	0.05 ± 0.05	0.39 ± 0.43
40	0.12 ± 0.02	0.15 ± 0.13	0.20 ± 0.21	0.05 ± 0.05	0.40 ± 0.31
52.5	0.12 ± 0.02	0.15 ± 0.13	0.21 ± 0.17	0.05 ± 0.03	0.38 ± 0.30
70	0.10 ± 0.07	0.13 ± 0.12	0.18 ±0.15	0.04 ± 0.05	0.23 ± 0.30
100	0.11 ± 0.02	0.09 ± 0.14	0.17 ± 0.13	0.06 ± 0.09	0.31 ± 0.25

Table 5.4 Rate of creatine kinase (CK) released from hearts treated with CO in the presence of antioxidants (**AO**). The times show the midpoint of the collected perfusate fractions (as given in **Figure 5.1**). The time in **bold** text denotes the period that hearts were exposed to CO, i.e. 0 to 30 minutes.

The cumulative activities of LDH and CK in the perfusate fractions were determined. The figures were expressed as a percentage of the mean pretreatment level to allow comparisons to be made (as performed previously). The control hearts showed an exponential decline in the activities of LDH and CK during the perfusion (**Figure 5.2**). The hearts treated with CO showed an elevated level, although this was not significant and the 0.01% CO group appeared to release more LDH and CK than the 0.05% CO-treated hearts. The AO control group had a lower loss of LDH and CK and these were not significantly different to the control group. Hearts treated with 0.05% CO in the presence of AO showed a similar loss in cumulative activity for both LDH and CK relative to the 0.05% CO-treatment without AO. However, the CO-treatment increased the loss of LDH and CK from hearts during CO exposure, whereas the AO-treatment reduced the loss (of LDH and CK) after the CO-treatment. None of the described changes were significant. The cumulative activity of troponin I (TnI) were not determined using the four perfusate fractions.

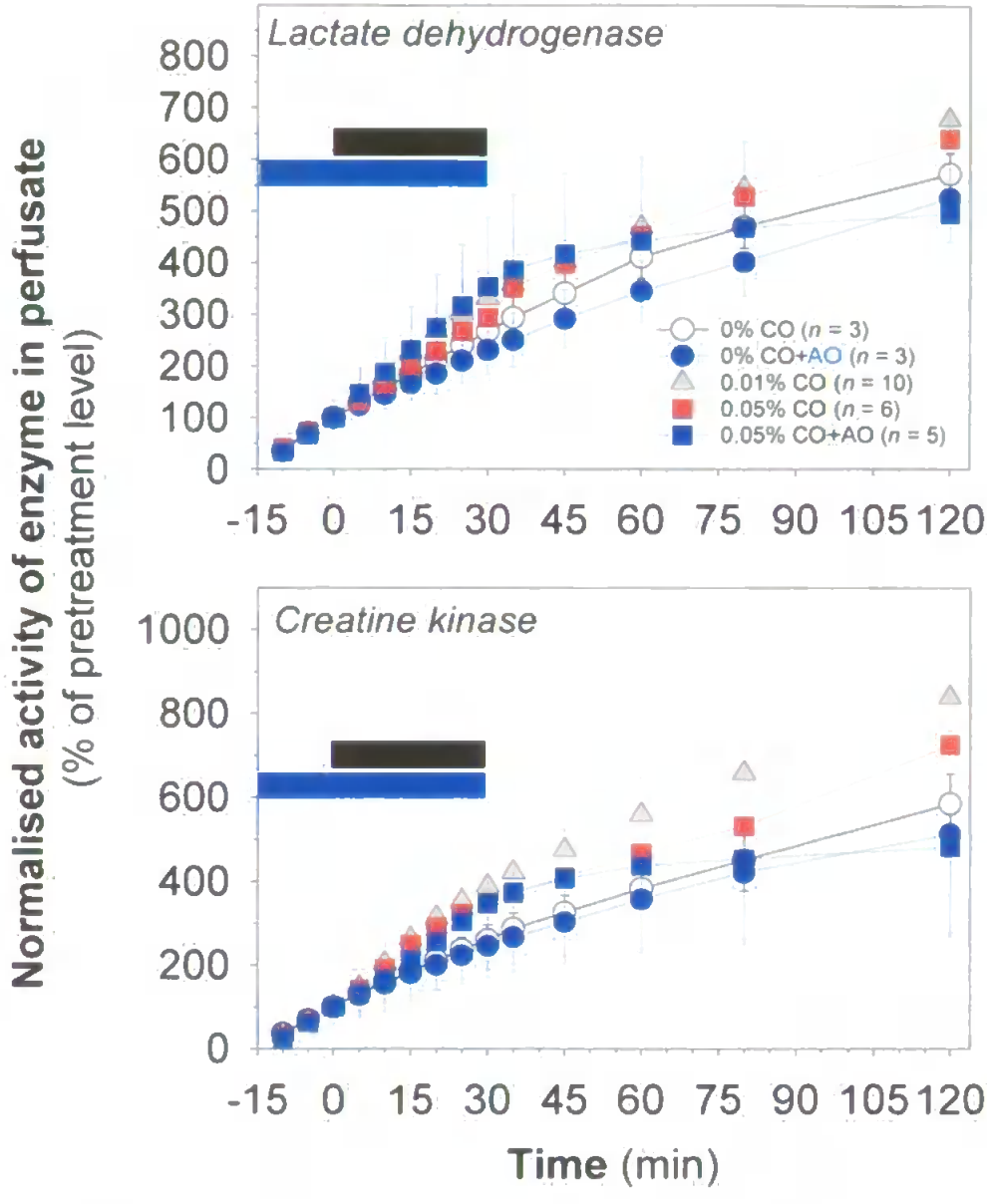

Figure 5.2 Cumulative activities of lactate dehydrogenase (*top panel*) and creatine kinase (*bottom panel*) in the perfusates of CO-treated hearts perfused with or without antioxidants (AO). The data shown is expressed as a percentage of the mean pretreatment level (determined from the -15 to 0 minute fractions). The data shown is the mean ± SEM with the number of hearts (*n*) used in each group shown in the figure. The black and blue bar denote the period that hearts were exposed to CO (for 30min) and AO (45min), respectively.

5.3.2 Other Tissue Measurements

5.3.2.1 Lactate & Pyruvate

The lactate and pyruvate content were determined in the homogenates from ventricle tissue of isolated hearts exposed to CO. Lactate and pyruvate levels ranged from 0.061-0.83 μmoles.mg^{-1} protein and from 0-1.43 nmoles.mg^{-1} protein in 10% ventricle homogenates (with no significant differences), respectively **(Table 5.5)**. The lactate/pyruvate ratio (L/P) expressed as the mean ± SD were 343 ± 101, -1987 ± 929, 285 ± 725, 563 ± 237, and 409 ± 800 for 0% (control; *n* = 2), 0% + AO (AO control; *n* = 3), 0.01% (*n* = 10), 0.05% CO (*n* = 4), and 0.05% CO+AO (*n* = 4) groups, respectively. The L/P ratio showed no significance differences between treated hearts relative to the controls. The negative L/P ratio for the AO control may suggest some depletion of pyruvate by the antioxidants. The *n* numbers differ here as some ratios could not be determined due to a couple of zero values for the pyruvate content (one heart from the 0%, 0.05% CO, and 0.05% CO + AO groups).

The start-stop perfusions had some hearts immediately removed from the perfusion apparatus before and after the 30min perfusion with 0.01% CO. The lactate content before any treatment (at *t* = 0min) were 43 ± 6 μmoles.g^{-1} (mean ± SD); and the content after 30min of perfusion with 0 and 0.01% CO were 39 ± 2 and 39 ± 3 μmoles.g^{-1}, respectively. There were no significant difference between these values; and also for the pyruvate data (below). The pyruvate content before any treatment (at *t* = 0min) were 0.26 ± 0.16 μmoles.g^{-1} (mean ± SD) and the content after 30min of perfusion with 0 and 0.01% CO were 0.31 ± 0.06 and 0.36 ± 0.04 μmoles.g^{-1}, respectively. The specific content of lactate and pyruvate from these start-stop perfusions is shown in **Figure 5.3**. The pyruvate content increased over the 30min treatment period, then declined over the next 90min similar to the lactate profile that had an almost linear relationship with time.

Treatment (*n*)	*Lactate* (μmoles.g^{-1} wet tissue)	*Pyruvate* (μmoles.g^{-1} wet tissue)
0.00% CO (*3*)	18.83 ± 0.98	0.038 ± 0.020
0.00% CO + **AO** (*3*)	18.59 ± 0.71	-0.012 ± 0.05
0.01% CO (*10*)	17.68 ± 2.00	0.022 ± 0.011
0.05% CO (*5*)	21.80 ± 1.37	0.033 ± 0.010
0.05% CO + **AO** (*5*)	10.61 ± 3.58	-0.001 ± 0.011

Table 5.5 The contents of lactate and pyruvate were determined in the homogenates of ventricle tissue prepared from isolated rat hearts exposed to CO with or without antioxidants (AO; under the same conditions as described previously). The absolute amount of lactate and pyruvate (in μmoles) is expressed per gram of tissue (wet weight). The data shown is the mean ± SEM and no significant differences were found.

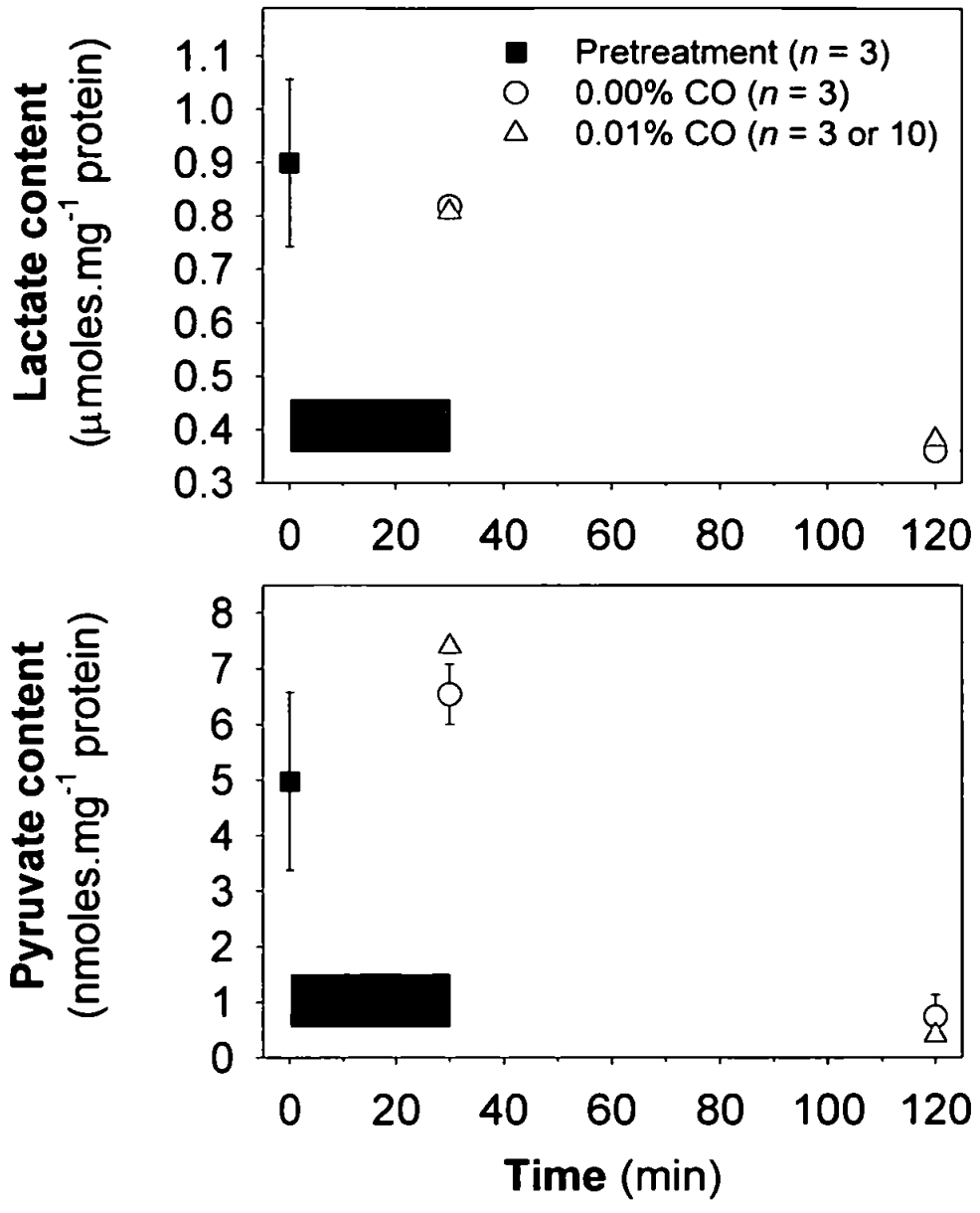

Figure 5.3 Time course of the lactate and pyruvate content in ventricle tissue homogenates from isolated rat hearts treated with CO. The black bar denotes the period that hearts were exposed to CO (for 30min). The data shown is the mean ± SEM.

5.3.2.2 Glucose

The glucose content was determined in the deproteinised ventricle homogenate extract and expressed per mg of protein in the tissue homogenate (**Table 5.6**). The AO in the 0.05% CO-treated group significantly increased the glucose content compared to the AO control group (**Table 5.6**).

5.3.2.3 Glycogen

The glycogen content is expressed as the equivalent unit of liberated glucose. This was determined in samples (as above; in *5.3.2.2*) by hydrolysing the glycogen to glucose units (using amyloglucosidase) and subtracting the initial glucose level that was present before hydrolysis (*5.2.7.4*). No significant differences were found between the groups, however, the antioxidants appeared to lower the glycogen content (**Table 5.6**).

Treatment (*n*)	***Glucose*** (μmoles.g^{-1} wet tissue)	***Glycogen*** (μmoles glucose.g^{-1} wet tissue)
0.00% CO (*3*)	4.33 ± 1.78	33.52 ± 2.49
0.00% CO + **AO** (*3*)	3.09 ± 0.41	23.11 ± 5.58
0.01% CO (*10*)	3.75 ± 0.99	26.31 ± 5.07
0.05% CO (*5*)	4.49 ± 1.32	35.52 ± 9.38
0.05% CO + **AO** (*5*)	*8.26 ± 1.26	23.63 ± 2.20

Table 5.6 The absolute content of glucose and glycogen determined in homogenates of ventricle tissue from rat hearts exposed to CO with or without antioxidants (**AO**). The glucose content was determined in acidic deproteinised samples and the glycogen content was expressed as an equivalent glucose content following its hydrolysis with amyloglucosidase (see Materials and Methods for details). The data shown is the mean ± SEM with the number of hearts (*n*) used in each group shown in the figure. * The asterick indicates a significant difference (at $P < 0.05$) between the marked group and the control AO group, i.e. treated with 0% CO with antioxidants.

5.3.2.4 Glutathione

The glutathione levels were determined in deproteinised samples of rat heart ventricle homogenate prepared from isolated hearts perfused with CO ± antioxidants (AO). The CO-treated hearts had a significantly decreased content of glutathione and the AO showed a protective effect (by preserving some of the CO-induced depletion of glutathione). These results were published (Patel *et al.*, 2003) and are shown (on pages 293-297). The depletion of glutathione was found to occur after the CO exposure from the start-stop perfusions (**Table 5.7**).

A

Treatment (*n*)	***GSH*** (μmoles.g^{-1} wet tissue)	***GSSG*** (μmoles.g^{-1} wet tissue)	***Reduced/Oxidised ratio*** (GSH/GSSG)
0.00% CO (*3*)	0.375 ± 0.007	0.075 ± 0.003	5.01 ± 0.30
0.01% CO (*10*)	0.070 ± 0.007	0.027 ± 0.005	3.97 ± 1.19
0.05% CO (*5*)	0.050 ± 0.010	0.015 ± 0.002	3.82 ± 1.19

B

Time and Treatment (*n* = 3)	***GSH*** (μmoles.g^{-1} wet tissue)	***GSSG*** (μmoles.g^{-1} wet tissue)	***Reduced/Oxidised ratio*** (GSH/GSSG)
t = 0min; 0% CO	0.591 ± 0.070	0.036 ± 0.016	31.59 ± 5.43
t = 30min; 0% CO	0.406 ± 0.082	0.033 ± 0.021	13.92 ± 9.15
t = 30min; 0.01% CO	0.431 ± 0.071	0.003 ± 0.003	73.17 ± 18.49

Table 5.7 The absolute content (in μmoles per gram of wet tissue) of the reduced (GSH) and oxidised glutathione (GSSG) was determined in rat heart homogenates prepared from isolated perfused hearts treated with CO under normoxia (*top part*, ***A***). The *bottom part*, ***B***, is the glutathione content in homogenates taken from ('start-stop') hearts that were removed from the Langendorff apparatus at the stated time and CO-treatment (under *normoxic conditions* used throughout). The data shown is the mean ± SEM with the number of hearts (*n*) used in each group shown in the figure. The reduced to oxidised ratio was determined for each heart and is expressed as the mean ± SEM for each treatment group. No significant differences were found.

5.3.3 Protein Concentration of Rat Heart Ventricle Homogenate

The protein concentration in the tissue homogenates prepared from isolated perfused rat hearts exposed to CO were determined to assess any intracellular loss from heart tissue and CO-treatment. The protein concentrations were measured (using a commercial diagnostic kit) and is shown in **Table 5.8** (*top part*, ***A***). No significant difference was found between the treated and control groups, although the 0.01% CO-treated group had a lower protein content. The protein concentration for the start-stop perfusion is included in **Table 5.8** (*bottom part*, ***B***).

A

Treatment (*n*)	***Protein concentration*** ($mg.ml^{-1}$ homogenate)
0.00% CO (*3*)	5.24 ± 0.22
0.01% CO (*10*)	4.74 ± 0.15
0.05% CO (*5*)	5.36 ± 0.21

B

Time (*n*)	***Protein concentration*** ($mg.ml^{-1}$ of homogenate)
0.00% 0min (*3*)	4.95 ± 0.51
0.00% 30min (*3*)	4.75 ± 0.21
0.01% 30min (*3*)	5.03 ± 0.67

Table 5.8 The protein concentrations of ventricle homogenates prepared from isolated perfused rat hearts exposed to CO. The ventricle tissue was taken from the experimental heart and homogenised prior to determining the protein in the 10% homogenates (see Materials and Methods). The protein concentrations shown are from (***A***) the CO-treated groups and (***B***) the start-stop perfusions. The data shown is the mean ± SEM with the number of hearts (*n*) used in each group shown in the figure. No significant differences were found.

5.4 Discussion

The activities of lactate dehydrogenase (LDH), creatine kinase (CK) and troponin I (TnI) in the perfusate of isolated perfused rat hearts were measured to assess the viability of heart tissue exposed to CO. The activities of CK and LDH were determined in fresh perfusate samples kept on ice prior to their determination as storage at 20°C results in a significant loss of CK activity (Swaanenburg *et al.*, 1998). The high sensitivity of the troponins (and myoglobin) make them useful for detecting early acute myocardial infarction relative to LDH and CK (Stokke *et al.*, 1998). The activities of LDH, CK and TnI were increased following the CO-treatment in the perfusate of control hearts. The level of activities (of these measured markers) mirrored their rate of release into the perfusate (out of the heart) and declined over time suggesting that no significant damage occurred in the control hearts (under the conditions used). It may be reasonable to suggest that CO exposure produced subtle tissue damage, otherwise no increase in enzyme activity may have been found.

The decreasing activity of LDH and CK may indicate some washout produced from early injury (in control hearts). This injury could arise during the removal of the heart (from the rat) prior to its perfusion (Rodríguez-Sinovas *et al.*, 2003). Excision of the heart results in ischaemia of the organ from the temporary loss of blood supply prior to oxygenation (by perfusion). It is possible that this may produce oxidative stress (**1.5**) as a result of some ischaemia/reperfusion (I/R) injury (**1.6**) induced whilst preparing this model. The activities of LDH and CK were highest in the pretreatment perfusate than in later fractions (**Figure 5.1**). Some readers may disagree with using the isolated Langendorff preparation in favour of a working heart. However, no significant differences were shown in the leakage of CK between the two preparations when used to examine ischaemia (Galinanes & Hearse, 1990a). The degree of CO-induced ischaemia here (if any) is anticipated to be less than the ischaemia produced by global ischaemia. Therefore, the levels of CK and LDH activities cannot compare the extent of the tissue damage by either treatment. The different levels of oxygen used in other studies (95% O_2) compared to this study (21% O_2)

could contribute to some of the differences found and cannot offer suitable enzymic data for comparison to evaluate whether the injury arises following indirect CO-induced ischaemia and/or solely from CO.

Young adult rats exposed to 0.15% CO in air for two hours show elevated activities of LDH and CK (in serum) of 117 and 132% above control at two hours postexposure, respectively (Penney & Maziarka, 1976). Their study also found different rates and periods of enzyme release. An immediate release of CK were shown and the release of LDH was delayed post-CO. The elevated activities of LDH and CK (in the collected perfusate samples here) could not be compared quantitively to other studies as these used blood samples (Barrabés *et al.*, 2000; Dolan, 1985; Penney & Maziarka, 1976). Their elevated activities cannot be attributed solely to heart tissue as the blood compartment could indicate damage from other tissues/organ(s), whereas we examined heart tissue. The immediate elevated activity of LDH (and CK) during CO exposure does not support the hypothesis as the reperfusion period is shown to induce the release of LDH from rat hearts (Rodríguez-Sinovas *et al.*, 2003). The increased perfusate activity of CK may suggest that myocardial necrosis occurred during CO exposure. Myonecrosis is reported in patients of CO poisoning (Shapiro *et al.*, 1989). However, no correlation is found between the levels of CO exposure and CK determined in the serum (Shapiro *et al.*, 1989), similar to the observed activities of CK (and LDH) in the perfusate of 0.01 and 0.05% CO-treated hearts. Glucose is shown to reduce the release of cytosolic enzymes from heart tissues following ischaemia (Hearse & Humphrey, 1975). The inclusion of glucose in the perfusion buffer may have offered some myocardial protection here. The lack of association in the loss of tissue viability with dose of CO is discussed below.

The established role of free radicals (**1.5**) in I/R injury (**1.6**), particularly the reactive oxygen species (ROS) may blind the true extent of tissue damage as some ROS can lower the activity of CK. The activity of cytosolic CK (in rat heart) is decreased by ROS and is thought to arise from the oxidation of a cysteine residue involved in substrate binding at the active site (Genet *et al.*, 2000). It is possible that any intracellular production

of ROS may precede the loss of cellular viability and that some CK measured in the perfusate could be inactivated (described above). This could account for the unexpected low activity of CK in perfusate of rat hearts exposed to 0.05% CO relative to the 0.01% CO-treated group. However, ROS production is shown to occur predominantly in the reperfusion period of I/R injury (Grupp *et al.*, 1999; Halliwell & Gutteridge, 1999). Therefore, the rise in CK (and LDH) activity in the perfusates taken during CO exposure could be from the direct effect(s) of CO, possibly at some other cellular target(s) involved in maintaining cellular integrity. CO is shown to depress the specific activity of LDH and glucose-6-phosphate in cultured heart cells (Brenner & Wenzel, 1972). Chronic CO exposure increased the M-subunit of LDH in adult rats (Penney *et al.*, 1974), however, Brenner & Wenzel (1972) found a decrease in this subunit. The use of serum in culture by Brenner & Wenzel (1972; above) may account for some of these differences, as serum is shown to alter the isoforms of cultured heart cells (Van Der Laarse *et al.*, 1979). The exposure of adult male rats to 0.05 and 0.1% CO in air produced an elevated activity of CK (MB isoform) in the plasma (Thomas & O'Flaherty, 1982). In the heart, the mitochondrial CK activity arises from the sarcomeric isoform (Hammerschmidt *et al.*, 2000). The activity of several isoforms of myocardial CK is shown to be altered following β-adrenergic stimulation and this is thought to precede the failing heart. The observed loss of CK may impair myocardial energy metabolism and lead to heart failure (morbidity) in this manner.

The release of CK and LDH did not reflect or associate with any of the trends found in the physiological changes following CO exposure (**Chapter 4**). Fortunately, for this study another specific marker of cardiac viability was used, i.e. troponin I (TnI). In hindsight, the author suggests measuring the troponin levels in all the perfusate fractions (TnT or TnI; Ooi & Maddock, 2000) and the activity of LDH/CK in the ventricle homogenates. *In vivo* TnI is primarily released into the plasma as a binary complex with troponin C and later occurs as a distribution of several forms (Gerhardt *et al.*, 2000; Ueda *et al.*, 2001). The activities (and rates of release) of perfusate LDH and CK were maximal during the 10-15 minute period of CO exposure (**Figures 5.1**). Therefore, the 10-15min fraction were used

for TnI analysis, with the following fractions: pretreatment fraction (-5 to 0min); fraction of observed maximal CO damage (10 to 15min); immediately post-CO (30 to 35min); and at the end of the perfusion, i.e. final perfusate fraction (at 80 to 120min).

The troponins (Tn) comprise of three sub-groups: I, C and T (Sato *et al.*, 1998; Zhao *et al.*, 2000). These are currently the best markers in clinically diagnosing any muscle damage in heart tissue using blood samples taken from patients. Troponin I is found in the serum of *ca.* 30% of patients with severe heart failure (Benamer *et al.*, 1999; La Vecchia *et al.*, 2000). Although, perfusate samples were used to determine the level of TnI, serum must be free from plasma otherwise falsely elevated TnI concentrations can arise (Nosanchuk, 1999). Troponin T (TnT) is regarded as the most reliable marker out of the three in establishing cardiovascular damage (Johansen *et al.*, 1998) and analytically costs more than measuring TnI levels (personal communication with Jane Dickinson, Triphasic Laboratories, Plymouth, UK). The higher sensitivity of TnT is thought to be due to a difference in the cross-reactivity of antibodies to TnI and TnT (Bertinchant *et al.*, 2003; Heeschen *et al.*, 2000). However, TnI may have a similar prognostic value as TnT (Möckel *et al.*, 1998). However, TnI may not always suggest myocyte injury in patients of congestive heart failure (Kamblock, 2002). The extrapolation to the human heart is possible as the troponin assays have been validated in rats using an isolated heart model (Fredericks *et al.*, 2001).

The loss of intracellular material from perfused hearts exposed to CO were difficult to associate with the overall loss of measured markers (i.e. LDH, CK and TnI). The CO-treated groups showed greater activity in the perfusate and rates of release relative to the control group, however, the 0.01% CO-treated group had a greater loss of viability than the 0.05% CO group (result not significant). This may account for the lower protein content in ventricle tissue from 0.01% CO-treated hearts (5.3.3) and could suggest that most damage may have occurred in this group.

Both groups of CO-treated hearts had an increased level of TnI in perfusate samples during and immediately after exposure to CO (compared to controls). These results support the LDH and CK data and suggest that CO exposure (under normoxic conditions) produced cadiac damage as shown by the loss of intracellular material (or viability) compared to controls. This is supported by the increased rate of TnI released from CO exposed hearts relative to controls. The rate of release during CO exposure, immediate post-CO period and in the last CO-free perfused fraction were elevated relative to the basal levels. These rates of release were greater than the time-matched decline found for the control hearts and indicate that CO toxicity *in vivo* may not be caused solely from a lack of oxygen (from COHb formed during CO exposure). CO may have its own cardiotoxic effect(s) that may be potentiated by CO-induced hypoxia *in vivo*. The release of TnI (and presumably TnT) could predict an adverse cardiac outcome in the long-term (de Filippi *et al.*, 2000) and may suggest that cardiac morbidity occurs in hearts exposed to CO. The dose-dependent decline in coronary flow (shown in **Chapter 4**) may be associated with the loss of TnI following CO exposure (**Table 5.3**) and could suggest diffuse coronary disease and/or a worse outcome to follow the initial damage found (Fuchs *et al.*, 2002).

Experimental ischaemia producing intracellular hypoxia (hypoxia$_i$) is shown to produce a loss of troponins from the myocardial infarcted zones, with the greatest loss occurring in the periphery of the infarcted zones (Fishbein *et al.*, 2003). This loss of troponin (produced by ischaemia) preceded tissue necrosis and could suggest that ischaemia may have occurred in the hearts exposed to CO. The troponins are involved in the contractile function of the heart. The deletion of TnT can lead to diastolic dysfunction and ventricular arrhythmias (Frey *et al.*, 2000). The loss of TnI could account for the depressed HR (shown in **Chapter 4**). Cases of reported myocardial infarctions (MI) produced following the exposure to CO were described in **Chapter 4** (Ebisuno *et al.*, 1986). The troponins correlate closely with the size of infarct (Metzler *et al.*, 2002). The elevated levels of TnI could suggest a direct effect of CO inducing a MI. Furthermore, this may be potentiated through the reduced coronary perfusion (shown by the depressed perfusate flow after CO

exposure in **Chapter 4**). The zone(s) of focal haemmorhaging found in the ventricle tissue following 0.05% CO-treatment (**Chapter 4**) also showed an increased release of TnI, and may highlight a possible region of infarcted tissue. However, the increase was not significant to the other 0.05% CO-treated hearts that showed no zones of damaged tissue.

The degree of intracellular hypoxia (by COMb formation) is unlikely from the ratios of the gases that the hearts were exposed to. The dissolved diatomic oxygen utilised by cytochrome *c* oxidase (in the mitochondrial ETC) is delivered by the diffusive gradient between the blood and mitochondra. Myoglobin facilitates this delivery of oxygen to the mitochondria and this is inhibited in the presence of CO, however, this inhibition requires lethal levels of CO (Wittenberg & Wittenberg, 1993). A ratio of the partial pressure of CO (P_{CO}) to the partial pressure of oxygen (P_{O_2}) of at least six can inhibit the diffusion of O_2 from Mb to cytochrome *c* oxidase (Piantadosi, 1996); whereas, a P_{CO}/P_{O_2} of one would bind CO to all the Mb. Whether hypoxia$_i$ was partly responsible for the described changes in **Chapter 4** has to be determined (discussed below). The reduced myocardial steady-state O_2 consumption by NO is comparable to CO suggesting that CO may stimulate cGMP production within the myocardium (Gong *et al.*, 1999). The possible production of cGMP may have a role in the chronotropic responses observed through its inotropic modulating properties. Myoglobin is also thought to be involved directly in mitochondrial respiration by the 'myoglobin-mediated oxidative phosphorylation' and somehow participates in respiration without facilitating the diffusion of oxygen to the mitochondria (Wittenberg & Wittenberg, 1993). These direct effects of CO suggest that CO may have a widespread role within the myocardium than once thought. The tissue(s) of the heart damaged by CO-treatment remain to be identified. However, in light of the discussed changes (in **Chapters 4** and here) the endothelial cells may be a possible target of CO. The direct toxicity of CO towards endothelial cells were shown in bovine pulmonary artery endothelial cells exposed to 0.001 to 0.01% CO (Thom *et al.*, 2000).

The lactate and pyruvate content of the tissues were determined to examine whether intracellular hypoxia (hypoxia$_i$) had occurred in hearts exposed to CO. The measurements

of myocardial lactate and pyruvate serve as indicators of oxidative metabolism (Ayres *et al.*, 1970). During glycolysis, a flux from pyruvate to lactate occurs under anaerobic conditions, whereas pyruvate is channeled into the Kreb's cycle under normal conditions, i.e. normoxia. Hypoxia$_i$ and the associated increase in intracellular [lactate] can lower the binding affinity of myoglobin for oxygen under moderately acidic pH (Giardina *et al.*, 1996). A reduced myocardial oxygen consumption and/or anaerobic metabolism follows lactate production. The measured pyruvate content were not as effective in evaluating the intracellular conditions relative to the lactate measurements. The lactate to pyruvate (L/P) ratio is a more reliable indicator of the degree of intracellular oxygenation than the pyruvate content. The L/P ratio were compared using the determined content of each analyte (*5.3.2.1*). The L/P ratios support the above and also suggest that no oxygen limiting conditions occurred inside cells/tissues from the normoxic CO-treatment used here.

Lactate levels in the blood mirror the tissue concentration *in vivo*, therefore, the lactate levels were expected to fall in the perfused organ following washout occuring to equilibrate with the perfusion buffer. The results (in **Table 5.5**) showed that the level of lactate in ventricle tissue were slightly elevated with increasing CO exposure, however, no significant differences suggest that no hypoxia$_i$ occurred in CO-treated groups at the end of the perfusion (*5.3.2.1*). This is in contrast to the elevated blood lactate found in rats exposed to 0.24% CO that occurred during and after the CO exposure (Penney & Chen, 1996). Therefore, the lactate content was determined in ventricle tissue from hearts used in the start-stop perfusions (**Figure 5.3**). The start-stop heart perfusions (exposed to 0.01% CO) showed no elevation in the lactate content of ventricle tissue. This may suggest that the CO exposure to isolated rat hearts during normoxia did not produce intracellular hypoxia in the ventricles. The intracellular partition coefficient between CO and O_2 is shown to be 36 from NMR signals (Glabe *et al.*, 1998). Under the assumption that the levels of COMb and MbO_2 is proportional to the partial pressures of CO and O_2 (P_{CO} and P_{O_2}, respectively); and considering the partition coefficient (taken as 36) gives 0.017 and 0.086 for the intracellular COMb:MbO_2 ratio for 0.01 and 0.05% CO,

respectively. Glabe *et al.* (1998) found an increase in the level of lactate to suggest inhibition of the mitochondrial ETC occurred when COMb:MbO_2 ≥1.5.

Other workers have reported that the effects of CO are not solely attributed to systemic hypoxia through the formation of COHb. Basset & Fisher (1976) showed that lactate and pyruvate production increased when isolated rat lungs were ventilated with CO (Lin & McGrath, 1989). Their increase in lactate suggests that intracellular hypoxia may be produced by the formation of COMb in the presence of normoxia. However, the ratio of lactate to pyruvate in the blood was unchanged in conscious dogs exposed to 0.15% CO through a permanent tracheostomy (Cramlet *et al.*, 1975). Any increase in the level(s) of lactate could be masked during and/or after the exposure to CO as lactate (and amino acids) can be used in the synthesis of glycogen to maintain glycolysis by glycogenolysis (Fournier *et al.*, 2002). However, glucose can maintain a linear anaerobic increase in lactate (Hohl *et al.*, 1982). Using the changes in lactate content to measure the degree of $hypoxia_i$ may not rule out $hypoxia_i$ in the atria (not determined), and could implicate $hypoxia_i$ for the changes in coronary flow (in **Chapter 4**). An increased or decreased P_{O_2} is shown to elevate the lactate levels in tachycardic hearts (Neill *et al.*, 1973) suggesting that hypoxia may not increase the level of tissue lactate, and that the contractile state of the tissues may determine such biochemical changes (Salkowski & Penney, 1995).

The production of reactive oxygen species (ROS) is established in I/R injury (described previously in **1.5** and **1.6**). The inactivation of glycolysis (in P388D1 cells) by hydrogen peroxide also inhibited the uptake of glucose and the production of lactate (Hyslop *et al.*, 1988). The absence of any change in ventricle lactate levels could suggest that oxidative stress may have occurred following the exposure to CO in isolated hearts (see later). The role of ROS producing myocardial injury is established from many studies using antioxidants (AO). Many chemicals having antioxidant properties (by indirectly increasing the endogenous AO capacity and/or directly participating in scavenging ROS) and were identified from studies using cell culture and isolated hearts (Chen & Tappel, 1995; Facino *et al.*, 1999; Massey & Burton, 1990; Yim & Ko, 1999). Some obvious points are not

discussed here as they were previously mentioned elsewhere (Patel *et al.*, 2003; shown on pages 293-297). The perfusion of AO before and during CO-treatment were used for two reasons. Firstly, if oxidative stress were involved in the cardiotoxicity of CO, the model used should not be subjected to oxidative stress following organ isolation (as it would not be suitable due to the reduced tissue AO capacity). Secondly, preconditioning may arise in the hearts after their excision and this may not provide an accurate picture of events in CO-treated tissues, particularly if oxidative stress were involved in the cardiotoxicity of CO. The isolated hearts were treated with AO before and during the CO exposure as this period also appeared to produce the cardiac changes observed after CO exposure, for example, the depressed coronary flow (**Chapter 4**). Similarly, ketamine (a NMDA-receptor antagonist) administered before and during acute 0.24% CO exposure had a protective effect in reducing blood lactate in female rats (Penney & Chen, 1996). The period of AO intervention (used here) were similar to other studies examining I/R injury in the heart (Persoon-Rothert *et al.*, 1990; Walker *et al.*, 1998). Vitamin C and TroloxC were selected as the antioxidants as they are water soluble analogue and established in the literature (Arrigoni & De Tullio, 2002; Niki, 1987; and Shang *et al.*, 2003). Glutathione contributes significantly to cellular/tissue antioxidant defence (Krieter *et al.*, 1994; Reiners Jr. *et al.*, 2000). The glutathione levels were determined to examine for oxidative stress in the heart tissue exposed to CO. Other markers of lipid peroxidation (to support CO-induced oxidative stress) such as malondialdehyde were not determined due to its artifactual production during homogenisation (Cordis *et al.*, 1995; Gutteridge, 1981; Verbunt *et al.*, 1996). The glutathione levels in rat heart ventricle tissue following CO exposure had a significant depletion (shown on pages 293-297) suggesting that oxidative stress occurred after CO exposure (Patel *et al.*, 2003; and **Table 5.7**). The depletion of cellular antioxidants can also indicate anoxia (Blokhina *et al.*, 2000), however, this is unlikely under our experimental conditions and our biochemical data suggests no hypoxia occurred (**Table 5.5** and **Figure 5.3**).

The depletion of other myocardial antioxidants cannot be ruled out, particularly as a hierarchy of antioxidants is shown to exist (Dhalla *et al.*, 2000; Haramaki *et al.*, 1998;

Palace *et al.*, 1999). Ascorbate is more effective than TroloxC as an antioxidant (AO) in the cardiac microvasculature (Molyneux *et al.*, 2002). Furthermore, ascorbate is the primary AO in myocardial injury (Haramaki *et al.*, 1998) and this could suggest that ascorbic acid (vitamin C) may be the key AO that preserved the glutathione depletion, by supplementing the endogenous antioxidant content (of ascorbic acid). If this were shown, it may suggest that ROS were responsible for depleting the glutathione content. Additionally, using HEPES buffer (with the AO perfusions) could have prevented or contributed in preserving the glutathione content, as HEPES is shown to scavenge hydroxyl radicals (Grootveld & Halliwell, 1986). Some depletion of glutathione could have occurred through leakage as shown by a minor loss of tissue viability, however, the loss of glutathione found here far exceeded that of the tissue viability (above). The likelihood of ROS being responsible in reducing the activities of LDH and CK were not determined and may not account for the difference between loss of glutathione and tissue viability. Therefore, the CO-treatment could have depleted the glutathione (possibly with the production of ROS) and/or by other unknown mechanism(s) through direct or indirect mode(s).

The role of NO in CO poisoning could produce RNS (**Chapter 1**) that may account for the oxidative stress found here. Nitric oxide is shown to deplete GSH by forming *S*-nitrosoglutathione (Miglani *et al.*, 2003). This may be likely as the production of NO after CO exposure is shown (**Chapter 1**). The reaction of NO with thiol-containing compounds (producing *S*-nitrosothiols; Foresti *et al.*, 1997) could have depleted the myocardial glutathione (possibly after the production of NO following CO exposure). Therefore, the inclusion of ascorbate (in the AO-treatment) may have indirectly preserved glutathione depletion in this manner and not necessaily by scavenging ROS. ROS are shown to promote intercellular endothelial gap formation (Schäfer *et al.*, 2003) and could be responsible for the irreversible swelling (found after CO-treatment in **Chapter 4**). Taken together, these could suggest an additive role of NO and ROS in the cardiotoxicity of CO. TroloxC can scavenge the hydroxyl radical and superoxide species with a reported IC_{50} of 1.15 and 1mM, respectively (Walker *et al.*, 1998) and suggests that ascorbate may have

significantly contributed to the AO effect as 0.2mM TroloxC was used here (i.e. $\leq IC_{50}$). The depletion of glutathione could not be responsible for the depressed HR as the physiological change in HR occurred before the oxidative stress. In contrast, Ganafa *et al.* (2002) showed the depletion of glutathione increased the HR of male Sprague-Dawley rats.

The regulation of extracellular calcium by glycolysis is shown (Aasum *et al.*, 1998), however, any altered glycolysis (by oxidative stress following CO exposure) did not produce any intracellular changes in calcium content (**Chapter 4**). Although any changes in the calcium content that occurred during the CO exposure may have returned to the control levels found after the 90 minute CO-free (recovery period). Lactate is shown to modulate the production of superoxide in bovine cardiac myocytes (Mohazzab-H. *et al.*, 1997). Although the use of intracellular lactate as a measure of $hypoxia_i$ has been addressed, an early study by Huckabee (1958) showed that the production of lactate by tissues is not attributed to hypoxia. His study showed that the changes in blood lactate were due to the equilibria between pyruvate and lactate in the tissues.

Huckabee (1958) showed that the infusion of glucose or pyruvate into the blood produced a secondary increase in the lactate levels of the tissues before changes in the blood (when hypoxia was absent). Their study also showed that the inhibition of glycolysis (with iodoacetate) produced a rise in blood pyruvate. This may suggest that the lactate content should have been determined in the coronary effluent of the perfused isolated hearts exposed to CO (5.6.1) to provide an accurate account of the changes. The increase in pyruvate following the exposure to 0.01% CO could suggest that some glycolysis may have been impaired by oxidative stress (above). However, the rise in [pyruvate] was not significant to the time-matched control level and may not be fully attributed to the inhibition of some component(s) of the Kreb's cycle resulting in the accumulation of pyruvate from glycolysis. The α-ketoglutarate dehydrogenase complex (in the Kreb's cycle) regulates mitochondrial function and cellular calcium (Huang *et al.*, 2003) and its inhibition (by CO) may be ruled out as no changes in calcium content were found (in **Chapter 4**).

Furthermore, the absence of glutathione depletion during the CO exposure period supports the evidence indicating an absence of hypoxia$_i$ (above); as hypoxia is shown to decrease the tissue content of glutathione in rat hearts (Park *et al.*, 1991).

The content of glucose and glycogen were determined to examine any glycolytic fluxes that may have occurred in the ventricle tissue of hearts exposed to CO. Glycolysis supports cellular function by the production of ATP under anaerobic conditions and the glycolytic end-products (such as lactate) are thought to determine the extent of ischaemic cell damage (Bradamante *et al.*, 2000). The blood glucose level is reported to correlate with the HR (Iwakura *et al.*, 2003). Therefore, this may be expected to show a lower level of glucose (in light of the depressed HR) in tissues exposed to CO (**Chapter 4**). However, no differences in the level of glucose or glycogen were found here in the ventricle tissue between CO-treated and control hearts. TroloxC (at 0.2mM) is shown to increase the HR and CF in perfused isolated rat hearts (Belló-Klein *et al.*, 1997). However, these contradictory findings were not found here in the control AO group of hearts, similarly to the findings of Walker *et al.* (1998) and cannot be explained. The positive chronotropic effect of TroloxC also reduced the glycogen content in their hearts from a higher consumption of energy (Belló-Klein *et al.*, 1997). The AO-treated groups (here) showed a similar level of glycogen that appeared to fall (**Table 5.6**), and may suggest a higher consumption of glycogen without any concomitant increase in HR.

Ischaemia produces an elevated level of glucose in porcine hearts subjected to an hour of regional ischaemia (Elvenes *et al.*, 2002) possibly to maintain anaerobic metabolism. As the depletion of glucose was not observed between the CO-treated and control hearts, it is reasonable to assume that glycogenolysis may have occurred to provide glucose for glycolysis. However, glycogenolysis did not appear to have occurred as no glycogen depletion were found in CO-treated hearts compared to the control group (*5.3.2.3*). The perfusion buffer contained 11mM glucose during the whole perfusion period. This could have provided a sufficient level of glucose to maintain the adequate production of ATP by glycolysis if intracellular hypoxia was produced by CO (via the formation of COMb). This

may be possible as perfusing glucose is shown to protect isolated rat hearts subjected to anoxia (Hearse & Humphrey, 1975; Hohl *et al.*, 1982). Mild to moderate ischaemia produces a marked increase in glucose uptake and usage by inducing the translocation of a glucose transporter using protein kinases, however, severely ischaemic myocardium loses its ability to extract more glucose from the blood (Egert *et al.*, 1997; Sun *et al.*, 1994). These changes are protective and associated with preserved myocardial function, a decreased release of myocardial enzymes and an improved recovery during reperfusion. The inclusion of glucose may account for the unchanged levels of glucose found in the ventricles of CO-treated hearts compared to controls. This may also explain the lack of glycogenolysis following CO exposure. Further work could repeat this study in the absence of glucose in the perfusion buffer of hearts exposed to CO and examine any differences in the degree of tissue damage (see 5.6.2). No difference in the tissue levels of glucose are anticipated to be found between the hearts of different ages treated with CO as ageing shows no change in the myocardial utilisation of glucose (Kates *et al.*, 2003). However, the age difference in the cardiotoxicity of CO may reveal some significant changes and remains to be determined. Rats exposed to 4% CO (for 4 minutes) showed no change in the plasma level of glucose (Hattori *et al.*, 1990). However, their level of CO is expected to be lower than the levels delivered solely to the heart, and may be expected to produce no significant change despite their reduced ATP content in the myocardium. No change in the glycogen content was found and could suggest that CO may interfere with the glycolytic pathway in a subtle manner to produce other changes not measured here.

The role of glucose in preserving myocardial function following I/R was reported (above) to have a protective effect (Hearse & Humphrey, 1975). However, the activity of glutathione reductase (GR) in isolated rat hearts perfused with glucose is decreased during ischaemia (DeGroot *et al.*, 1995). Oxidative stress produced after CO exposure (in the heart ventricle tissue) could indicate a reduced activity of GR that may result in the depletion in glutathione (through loss of oxidised glutathione and/or other glutathione conjugates), however, this would have to be determined. The lactate and pyruvate data

suggested that no intracellular hypoxia occurred during CO exposure, therefore, it may suggest that CO in the presence of glucose could directly affect GR.

A heart with limited energy can pump up to the limits of its energy supply (Wittenberg & Wittenberg, 1993), therefore, it would be expected to be observed as a diminished maximum rate. These workers also showed that greater than 40% COMb in isolated rat cardiac myocytes decreased the mean steady-state respiratory uptake of oxygen in these heart cells. Contractile activity is directly related to glycolytic ATP synthesis (Bradamante *et al.*, 2000), although myocardial recovery is possible when the depletion of ATP and creatine phosphate (≥ 95%) occurs in myocytes (Bonz *et al.*, 1998). No change in the content of glucose and glycogen found for the CO-treated hearts (relative to the control group) may suggest that the glycolytic activity was not altered, therefore indicating that $hypoxia_i$ may not be responsible for the reduced HR (shown in **Chapter 4**). The influence of the glycolytic pathways could be investigated using glycogen-depleted hearts, although this may show glycogenolysis and it could also produce metabolic hibernation. Glycogenolysis may be related to the contractile recovery of postischaemic myocardium, however, this requires glycolysis during early reperfusion to support the cellular house keeping activities (Bradamante *et al.*, 2000).

5.5 Conclusions

The use of normoxic conditions, i.e. isolated hearts perfused with a buffer equilibrated with 21% O_2 may exceed the oxygen content delivered to the heart *in vivo* resulting in findings that indicate an absence of intracellular hypoxia. The isolated rat heart preparation has demonstrated some direct effects of CO that could be masked at the *in vivo* level. The effects of CO shown (here and the previous chapter) suggest that the changes in heart rate and coronary flow (in **Chapter 4**), and the loss of tissue viability may be produced directly by CO. The discussion here raised findings that suggest no intracellular hypoxia had occurred, however, the *in vivo* scenario may be different and would have to consider the blood compartment. Hypoxia in the blood (following the *in vivo* exposure to CO) could possibly contribute to an elevated intracellular P_{CO} to P_{O_2} ratio (**5.4**) and potentiate the *in situ* formation of carboxymyoglobin (COMb). This may shift the intracellular COMb:MbO_2 to exceed 1.5 indicating hypoxia$_i$ (Glabe *et al.*, 1998), therefore, possibly decreasing the heart rate of the heart through an impaired mitochondrial ETC. The role of oxidative stress in the heart following CO poisoning may indicate that a different mechanism (versus an I/R-like injury) occurs as no ischaemia (intracellular hypoxia) were found.

5.6 Further Work

5.6.1 Intracellular Hypoxia & Lactate

To examine the degree of intracellular hypoxia by measuring the levels of carboxymyoglobin (COMb) in isolated perfused rat hearts exposed to CO (under our experimental conditions). The COMb content may be determined in the stored ventricle tissue homogenates as CO is not lost from frozen tissue (Sokal *et al.*, 1984). The COMb content could be determined using spectroscopy under the assumption that the COMb complex is stable as carboxyhaemoglobin (COHb) by blocking the dissociation of CO by oxygen using sodium hydrosulphite ($Na_2S_2O_4$; Rodkey *et al.*, 1979). However, Wittenberg & Wittenberg (1993) showed that CO dissociated rapidly from myoglobin, particularly when CO is removed from the ambient solution. If this is the case, the degree of intracellular hypoxia (by the formation of COMb) may not be determined so easily and could consider using fresh tissue homogenates exposed to CO. It is possible that no CO may bind in tissues (complexed to Mb), although this could be tested by measuring the level of CO in the tissue homogenate samples (Harper Jr., 1952). CO exposure produced the release of LDH, CK and TnI (5.4.1), however, any loss of myocardial myoglobin (Mb) could limit the degree of oxygen delivered to the mitochondria. Therefore, it may be useful to determine the Mb content of the homogenate samples (also in the atrial tissue) to evaluate any loss of Mb following CO poisoning (Reynafarje, 1963).

The activity of monoamine oxidase (MAO, using phenylethylamine as the substrate) can be used as a probe for examining the oxygen gradients in heart cells (Katz *et al.*, 1984). Intracellular hypoxia (by a limited diffusion of oxygen to the mitochondria via the possible formation of intracellular COMb) during CO intoxication could be determined indirectly in the CO-treated heart tissue by rapid homogenisation prior to measuring the MAO activity.

The lactate content was not determined in the atrial tissue. Any hypoxia in the atrial tissue was not determined due to discarding the tissues (in **5.4**). In hindsight, the author would propose measuring the lactate content in the atrial tissue from rat hearts exposed to CO.

The lactate and pyruvate content may also be determined in fresh perfusate to evaluate any changes in the (ef)flux of the tissue content.

5.6.2 Substrate Supplementation For Isolated Hearts Exposed To CO

The presence of glucose may limit the cardiotoxicity of CO. Glucose could be omitted during the perfusion with CO and/or supplemented in the CO-free period to evaluate its role in CO poisoning.

Chapter Six:

Mitochondria & Oxidative Stress in CO Poisoning

6.1 Introduction

Hyperbaric oxygen therapy/treatment (HBOT) is widely used in clinical medicine and is important in the treatment of CO intoxication (**1.2** and **1.3**). HBOT is highly effective in treating patients after CO poisoning; it can produce an elevated clearance of CO from the blood, with a rapid loss of symptoms of CO toxicity. However, this treatment results in oxidative stress as shown by the elevation in markers of oxidative damage (Bearden *et al.*, 1999; Dennog *et al.*, 1999). The role of reactive oxygen species (ROS) in I/R injury is established (Becker & Ambrosio, 1987; Entman *et al.*, 1992; Maulik *et al.*, 1998). In general, a longer duration of ischaemia produces more oxidative stress during reperfusion resulting in a greater degree of reperfusion-injury. The precise nature of events occurring under these conditions is not clear due to the involvement of many cellular components.

Mitochondria are organelles whose 'power house' role is to produce ATP for cellular work via the electron transfer chain (ETC; Cadenas & Davies, 2000). In heart tissue, virtually all the ATP is produced by mitochondria (Borutaite *et al.*, 1996). Mitochondria are the major sites of ROS production via the ETC (Staniek & Nohl, 2000). The role of mitochondria in initiating cell death by releasing mitochondrial pro-apoptotic factors also makes this organelle of particular interest in tissue dysfunction. With this in mind (pro-apoptotic factors) and the high abundance of mitochondria in heart tissue, it is reasonable to assume a possible role of mitochondria in producing heart dysfunction following CO poisoning. The binding of CO to cytochrome *c* oxidase (or Complex IV) in the ETC is another reason for investigating this organelle. The role of mitochondria under hypoxic conditions is not fully understood and mitochondrial dysfunction is found in ischaemic myocardial tissue (Kay *et al.*, 1997). Previous studies have shown that CO binding to cytochrome *c* oxidase results in the inhibition of the respiratory chain (**5.4**). This inhibition of the ETC can result in ROS production when oxygen is present, from oxygen interacting with reduced electron carriers.

6.2 Aim & Objectives

The role of mitochondria in CO toxicity is not fully known. Inhibition of cytochrome *c* oxidase is well established, but its catalytic cycle during CO poisoning in the presence of oxygen is not known (Morgan *et al.*, 1985). The aim were to investigate the effect of CO in the presence of oxygen, on possible ROS production from mitochondria during and/or after CO exposure and normoxia (as hypothesised in **1.7**). Another aim was to investigate the role of mitochondria in possible ROS production during (re)oxygenation and HBOT following CO poisoning. Mitochondria from rat heart ventricles were chosen to associate the findings of the previous work (shown in **Chapters 4 and 5**).

Specific objectives include:

(i) the isolation of coupled mitochondria from fresh rat heart ventricle tissue;

(ii) the use of hydrogen peroxide (H_2O_2), the dismutation product of superoxide ($O_2^{\bullet-}$), as an indicator of superoxide radical production from mitochondria to evaluate if ROS production occurs during/after CO exposure; and

(iii) the assessment of the effect of hyperbaric oxygenation on ROS production using different levels of oxygen after CO exposure.

The hyperbaric conditions may be simulated using custom made pressure vessels (2.3.4). However, it is possible that the elevated pressure(s) may have a direct effect on the mitochondria. We therefore used different oxygen fractions (at normobaric pressure) to simulate the increased partial pressure of oxygen (P_{O_2}) in the blood that occurs during HBO exposure (as described in 1.3.1).

6.3 Materials & Methods

6.3.1 Isolation of Rat Heart Mitochondria (RHM)

The isolation procedure was performed as described by Staniek & Nohl (2000; who used a modified method described originally by Mela & Seitz, 1979). Briefly, rat heart mitochondria (RHM) were isolated from male Sprague-Dawley rats (250-270 grams) following an acclimatization period ≥24h. The beating heart was rapidly excised after being anaesthesized (4.2.1). Hearts were immediately transferred into ice-cold isolation buffer prior to removal of unwanted tissue (thymus, lungs and atria). The isolation buffer (IB) contained 0.3M sucrose, 20mM triethanolamine (TEA) and 1mM EGTA (adjusted to pH 7.4 with KOH). The remaining ventricle tissue were chopped into small pieces with scissors and washed with buffer several times to reduce the contaminating blood and kept in *ca.* 15ml IB prior to homogenisation. To obtain coupled mitochondria, this stage should be performed quickly, taking *ca.* 30s following the excision of the heart (Mela & Seitz, 1979).

The chopped tissue was homogenised at *ca.* 500rpm with 3-4 up and down strokes. During homogenisation the vessel was kept in an ice-bath. The homogenate was transferred into two 15ml centrifuge tubes and spun at 480*g* for 5 minutes. The supernatant were collected carefully, then filtered through two layers of cheesecloth into two clean centrifuge tubes, and immediately centrifuged at 7700*g* (10min). The resulting solid light-brown pellets were rinsed carefully with IB. Using a small volume of buffer (2-3ml), the fluffy layer above the pellets were gently shaken loose and discarded. Care must be taken during gentle agitation, as vigorous shaking may disrupt the pellet and result in a loss of mitochondria. The pellet was resuspended carefully via aspiration using a 1ml Gilson pipette with a cut tip. Alternatively, the pellet may be resuspended very gently using a smooth surface or ground-glass rod, rotating around the edges of the dry pellet with no pressure, slowly moving towards the centre of the pellet. Buffer (15ml) was added to one tube of resuspended mitochondria and pooled with the remaining tube prior to

centrifugation at 7700*g* (5min). The washing step comprised of rinsing the mitochondrial pellet surface, resuspending the mitochondria, and the centrifugation step was repeated.

The mitochondrial fraction was suspended in incubation buffer (at 0.5ml.g^{-1} of tissue) containing 0.3M sucrose, 20mM triethanolamine, 1mM diethylenetriaminepentaacetic acid (DTPA), and 0.5g.l^{-1} fatty-acid free BSA (pH 7.4 on ice) to produce a suspension containing *ca.* 20mg.ml^{-1} of mitochondrial protein (Mela & Seitz, 1979). The suspension was left on ice to 'rest' for an hour before commencing any experiments. All experiments were then performed at 25°C.

6.3.2 Isolation of Rat Liver Mitochondria (RLM)

Rat liver mitochondria (RLM) were isolated using a hybrid method adapted from Rickwood *et al.* (1987), Staniek & Nohl (2000), and Trounce *et al.* (1996). Briefly, RLM were isolated from male Sprague-Dawley rats (280-320 grams), after being starved for *ca.* 24h to deplete the tissue glycogen and free fatty acids (FFA). Livers were removed from two anaesthesized animals and placed into ice-cold mitochondrial isolation buffer (MIB). Ice-cold MIB was used throughout unless otherwise stated. MIB comprised of 275mM mannitol (analytical grade), 1mM EGTA, 5mM HEPES and 0.1% BSA (pH 7.4 using 4M KOH). The livers were rinsed and the lobes were cut off and weighed. The lobes were cut into smaller pieces with scissors and homogenised with three 5s bursts. The homogenate (15% w/v) was then centrifuged at 1500*g* (5min) and the supernatant were centrifuged under the same conditions (pellets were discarded). The supernatant was then centrifuged at 8000*g* (15min). The pellets were washed to remove the fluffly white layer (by gentle aspiration; 6.3.1) and resuspended in a smaller volume of MIB (*ca.* 60% of initial volume). The resuspended pellet was centrifuged (8000*g* for 15min) before gently resuspending in ice-cold respiration buffer (RB) at 0.1ml.g^{-1} (wet weight of tissue). Ice-cold RB (pH 7.4) comprised 5mM HEPES, 5mM KH_2PO_4, 275mM mannitol, 1mM diethylenetriaminepentaacetic acid (DTPA) and 0.1% BSA (fatty acid free). The RLM were left on ice for *ca.* one hour to 'rest' prior to starting any experiments.

6.3.3 Mitochondrial ROS Production by CO and/or Hyperoxia

Rat liver mitochondria (RLM) were incubated (for 5min) in a normoxic environment (21% O_2; control group), with 0.05% CO (CO group), hyperoxia (95% O_2; HPO group), CO (5min) followed by HPO (5 min; CO+HPO group). RLM were equilibrated by gentle bubbling (*ca.* 1 bubble.s^{-1}) with the appropriate gas into the mitochondrial suspension held in a sealed glass cuvette whilst being stirred. Following each treatment the RLM suspensions were incubated at 25°C whilst stirred for 10min. The mitochondrial suspensions were then centrifuged at 8,000*g* (15min) and the supernatant was carefully removed and stored at -20°C (for later determination of hydrogen peroxide; 6.3.5). The pellets of RLM were resuspended and stored at -20°C (for determining the protein content; 6.3.6). A positive control for the mitochondrial production of H_2O_2 were performed by incubating RLM with 2μg.ml^{-1} antimycin A (final concentration). The antimycin A from Streptomyces sp. (Sigma; Lot 117H4102) was prepared as a stock solution at 2mg.ml^{-1} in ethanol (EtOH; analytical grade) and used at a final concentration of 0.1% EtOH in the RLM suspensions. Suitable experiments with RLM in 0.1% EtOH served as a blank for the antimycin A (positive control) group.

6.3.4 Respiratory Control Ratio (RCR)

Mitochondrial respiration was measured using a Clarke type oxygen electrode using the method described by Moody (1983; PhD Thesis). The platinum electrode was maintained at -0.6 volts (with respect to the silver:silver chloride reference). 2ml of respiration buffer (pH 7.4 at 25°C; 6.3.2) were pipetted into the electrode chamber and 200μl of RLM (in respiration buffer) were added to the stirring contents before placing the stopper. The background rate of oxygen uptake was measured for 3-4min. State 4 substrate (25μl 0.4M succinate) were added using a 50μl Hamilton syringe and the rate (of oxygen uptake) was measured over 3-4min. Next, the state 3 substrate (ADP; 10μl 40mM stock) was added

and the rate of oxygen uptake recorded. The RCR is determined from the ratio of state 3 to state 4 respiration. The state 3 and 4 respiratory rates were performed at least three times for each mitochondrial batch.

6.3.5 Detection of H_2O_2

The assay for H_2O_2 was performed as described by Staniek & Nohl (2000) using homovanillic acid (HVA). The fluorescence measurements were performed using a LS50-B fluorimeter (Perkin Elmer) with 5nm slit widths for both excitation and emission. HVA was prepared fresh prior to use. The assays were performed at 25°C in a 3ml fluorimeter quartz cuvette and stirred with a paddle after adding the sample (100µl). The calibration was performed using samples of known concentrations of H_2O_2 (prepared in respiration buffer; 6.3.2) to quantify the level of H_2O_2. All samples were kept on ice prior to analysis and assayed in triplicate.

6.3.6 Mitochondrial Protein Content

The protein content of the isolated mitochondria were determined using the Biuret method with BSA as standards.

6.4 Results

6.4.1 CO and Hyperoxic Exposure of Mitochondria

Using chemical reagents to specifically detect superoxide radicals formed within intact mitochondria is restricted by the permeability of the outer mitochondrial membrane (OMM). Therefore, the rapid dismutation of superoxide along with the mitochondrial matrix bound superoxide dismutase (SOD), both yield H_2O_2 that easily diffuses out of the organelle into the extra-mitochondrial space. The method of Staniek & Nohl (2000) was used to quantify the level of H_2O_2 using fluorescence spectroscopy (after removing mitochondria to minimise interference by light scattering). In the presence of H_2O_2, a horse-radish peroxidase (HRP)-catalysed the decrease in fluorescence (using scopoletin as substrate), or increase in fluorescence (using homovanillic acid) was recorded.

Hyperoxia did not appear to produce mitochondrial superoxide and neither did the hyperoxia treatment following CO exposure (**Table 6.1**). The production of superoxide was confirmed in all the isolated mitochondrial batches with the positive control using antimycin A (**Table 6.1**). Carbon monoxide and/or hyperoxia appears to decrease the production of superoxide (and/or hydrogen peroxide), although, ths reduction was not significantly different to the normoxic treatment (control). No significant changes in the state 3 and state 4 respiration for the RLM were found following the CO and/or hyperoxia treatment (**Table 6.2**), however, CO and hyperoxia appeared to lower the rate of state 3 respiration. The positive control (with antimycin A) inhibited both mitochondrial states to show a zero RCR.

Experimental condition (n = *4 or 5)	Rate of production of H_2O_2 (nmoles.mg^{-1} protein.min^{-1})	RCR
Positive control (using antimycin A prepared in EtOH)	1171.50 ± 88.65	0
EtOH blank	38.81 ± 20.98	4.16 ± 1.79
Normoxic treatment; control (21% O_2, 5 minutes)	18.40 ± 10.18	2.48 ± 0.42
Carbon monoxide treatment (0.05% CO, 5 minutes)	1.28 ± 19.71	4.90 ± 1.47
Hyperoxia treatment (95% O_2, 5 minutes)	-16.60 ± 15.68	5.05 ± 1.87
*CO (0.05%, 5 minutes) followed by hyperoxia (95% O_2, 5 minutes)	-5.48 ± 8.24	2.05 ± 0.62

Table 6.1 Rate of mitochondrial production of hydrogen peroxide (H_2O_2), the dismutation product of superoxide, following CO exposure *during normoxia* and/or hyperoxia treatment (to simulate hyperbaric oxygen treatment). Mitochondria were isolated from fasted (overnight) adult male Sprague-Dawley rat livers and 'rested' on ice for *ca.* one hour prior to any experiments. The rat liver mitochondria (RLM) had its respiratory control ratio (RCR) determined prior to any experimental treatment (as described in the Materials & Methods). The mitochondria were treated in a glass cuvette with a stirrer under the conditions before removing the RLM and freezing the supernatent for the later analysis of H_2O_2 (using the method of Staniek & Nohl, 2000). The positive control was tested using antimycin prepared in ethanol (EtOH) and a suitable blank to rule out any effect of EtOH. The RLM had their RCR determined after the experiments were conducted and showed no significant change in RCR prior to performing the experiments (see Table 6.2). The data shown is the mean ± SEM for n hearts in each group.

Experimental condition (n = 3 or *2)	State 3 (nmol $O_2.min^{-1}$ per mg protein)	State 4 (nmol $O_2.min^{-1}$ per mg protein)	RCR (State 3/State 4)
'Rested' mitochondria	0.052 ± 0.003	0.014 ± 0.006	5.42 ± 2.07
Positive control (using antimycin A prepared in EtOH)	0	0.001 ± 0.001	0
EtOH control	0.048 ± 0.002	0.015 ± 0.005	4.16 ± 1.79
Normoxic treatment (21% O_2, 5 min)	0.065 ± 0.029	0.026 ± 0.012	2.48 ± 0.42
Carbon monoxide treatment (0.05% CO, 5 min)	0.052 ± 0.009	0.014 ± 0.006	4.90 ± 1.47
Hyperoxia treatment (95% O_2, 5 min)	0.050 ± 0.009	0.012 ± 0.004	5.05 ± 1.87
*CO (0.05%, 5 minutes) followed by hyperoxia (95% O_2, 5min)	0.036 ± 0.009	0.018 ± 0.000	2.05 ± 0.62
Mitochondria 'at end' (after completing the above treatments)	0.045 ± 0.013	0.013 ± 0.006	5.00 ± 1.61

Table 6.2 The respiratory control ratio (RCR) of rat liver mitochondria (RLM) following exposure to CO and/or hyperoxia. The RLM were isolated from animals that had been fasted overnight as described in 6.3.2. The isolated mitochondria were 'rested' on ice for *ca.* one hour before commencing any measurements to determine the RCR. The data shown is the mean ± SEM for *n* hearts in each group. The RCR was determined for each treated batch of mitochondria and expressed as the mean ± SEM for each treatment group.

6.5 Discussion

The lack of hypoxia during CO exposure here rules out any reperfusion-injury in producing the oxidative stress (shown in **Chapter 5**). Therefore, this naturally led us to examine the source(s) and type(s) of ROS that may be produced after CO exposure, and/or other mechanism(s) responsible for the CO-induced depletion of glutathione. Mitochondria are shown to produce ROS and contribute in reperfusion-induced tissue damage (Fannin *et al.*, 1999). Isolated mitochondria were used to examine whether the production of ROS (after CO exposure) were responsible for the oxidative stress (in **Chapter 5**). Using intact mitochondria from rat heart tissue would have complemented the isolated rat heart model; however, this was not possible and rat liver mitochondria (RLM) were used. The (liver) mitochondria were isolated from rats that were fasted for 24h to utilise glycogen and free fatty acids and so minimise uncoupling (Casolo *et al.*, 2000). In contrast, the isolated hearts were perfused with glucose during the entire period (in **Chapters 4** and **5**). The role(s) of glucose in CO poisoning have not been investigated, although it may involve glucose oxidase in producing ROS after CO exposure (below).

Isolated RLM were used to determine the mitochondrial production of superoxide by measuring the levels of its dismutation product, hydrogen peroxide. RLM showed no production of H_2O_2 suggesting that mitochondria were not the source of CO-induced ROS production (either superoxide or H_2O_2 species). The absence of ROS production could be due to the lack of hypoxia (Davies, 1989) under the conditions used, therefore, the role of mitochondria in ROS production following CO exposure cannot be ruled out *in vivo*. The lack of a CO-free period following CO-treatment in the isolated mitochodria (as performed for the isolated hearts) is not attributed for inducing ROS production, as no evidence of ROS production were found following the hyperoxic treatment after CO exposure (**Table 6.1**). However, mitochondria are damaged following CO poisoning (Tritapepe *et al.*, 1998) and this could arise following direct ROS production and/or following secondary cellular damage. The mitochondrial volume density, heart rate (HR) and rate of oxygen consumption has a close correlation (Barth *et al.*, 1992). Therefore, any change in

mitochondrial volume (from oxidative damage) may impair mitochondrial functioning and alter the oxidative capacity to produce more ROS (Smith *et al.*, 1996).

In contrast to our findings, RLM is shown to produce superoxide anion (Du *et al.*, 1998). However, their animals were not fasted before isolating the mitochondria and could indicate a possible role of glucose oxidase in CO-induced oxidative stress. The greater antioxidant capacity of RLM relative to RHM (Evelson *et al.*, 2001; Venditti *et al.*, 2001) and/or using sucrose in the mitochondrial isolation buffer may account for the lack of H_2O_2 production. Sucrose can form highly stable soluble complexes with metal ions, especially iron (Konstantinova *et al.*, 2000), and this may have removed functional metal ions from some mitochondrial components involved in CO-induced ROS production. The 'intactness' (coupled state) of isolated mitochondria may have a role in ROS production after CO exposure. The isolated RLM were coupled, however, a higher quality preparation (with greater coupled mitochondria) could have provided a more accurate conclusion regarding mitochondria as a potential source of ROS production (in CO poisoning). The inclusion of bovine serum albumin (BSA) in experiments using RLM may have prevented some appearance of ROS, as albumin has antioxidant properties particularly in binding peroxynitrite (Watts & Maiorano, 1999).

Tissue differences could play a role in ROS production, as rat brain mitochondria are shown to produce hydroxyl radicals after CO poisoning *in vivo* (Piantadosi *et al.*, 1995; Zhang & Piantadosi, 1992). RHM also have a higher oxygen affinity in the ADP-stimulated state (or state 3) compared to RLM, and is proportional to their higher content of cytochrome *c* oxidase (Gnaiger *et al.*, 1998). Furthermore, CO may inhibit Complex I as it is shown in Rhodospirillum rubrum to inhibit the transcriptional activation of CO hydrogenase by binding to protein subunits homologous to those found in eukaryotic Complex I (Fox *et al.*, 1996). Complex I could be impaired following CO exposure (by oxidative stress) as it is sensitive to attack by ROS (Borutaite *et al.*, 1996). The impaired activity of Complex I may alter other metabolic pathways within the mitochondrion

(Weinberg *et al.*, 2000) and lead to inefficient bioenergetics that compromise myocardial function.

The hyperoxic treatment were performed to simulate the elevated blood level of dissolved oxygen to that found during hyperbaric oxygen therapy (HBOT). The isolated mitochondria treated with hyperoxic conditions showed no increase in the production of H_2O_2 (**Table 6.1**). However, hyperbaric oxygen treatment (HBOT) is shown to increase the production of H_2O_2 in RLM (Boveris & Chance, 1973). This could suggest that some pressure effect may be responsible for mitochondrial oxidative stress and warrants further attention.

Cytochrome *c* oxidase requires cardiolipin (a polyunsaturated phospholipid localised in mitochondrial membranes) for maximal activity and function (Jiang *et al.*, 2000). The absence of cardiolipin leads to defects in protein import and other mitochondrial functions. Cardiolipin is tightly bound to cytochrome *c* oxidase and its removal during enzyme purification leads to a loss in the activity of cytochrome *c* oxidase (Lenaz *et al.*, 1999; Soussi *et al.*, 1990). The oxidative stress found in hearts after CO exposure may initiate lipid peroxidation (LPO) that could disrupt the stability of cytochrome *c* oxidase through oxidation of cardiolipin. The high content of polyunsaturated phospholipids in mitochondrial membranes makes the mitochondrion susceptible to LPO, and LPO is shown to occur following CO exposure (Miro *et al.*, 1999; Kudo *et al.*, 2001). Whether any LPO occurred here (following the CO exposure) remains to be determined, however, any impaired activity of complex IV disturbs proton translocation (Karpefors *et al.*, 1998) and could affect mitochondria at a respiratory level within the myocardium (Kadenbach *et al.*, 2000). The disruption in complex IV may produce an altered state(s) of other respiratory complexes within the mitochondria (to compensate for the affected complex IV) and this may eventually lead to an altered metabolic state and/or execute apoptosis (Villani & Attardi, 2000). The compensatory changes of the mitochondrial respiratory carriers following CO exposure are protective mechanisms (Chance *et al.*, 1970). Any loss of cytochrome *c* oxidase activity could potentiate the incomplete reduction of oxygen and augment the formation of ROS within the myocardium. Following this, the oxidative stress

(following the CO exposure) could lead to myocardial failure responsible for CO-associated cardiac morbidity. The activity of cytochrome *c* oxidase in the mitochondrial fraction (from homogenate prepared from perfused hearts) were not determined as its activity is significantly lost during storage (Prohaska, 1983).

CO-induced oxidative stress is thought to involve RNS (derived from NO production) and involves many other organs *in vivo* that show oxidative stress following CO exposure (Thom *et al.*, 1997). The production of cardiac NO is mostly found within the endothelial cells of the vasculature (**Chapter 4**). This nitrative stress (by RNS) could be responsible for the depressed coronary flow after CO exposure and/or inhibition of cytochrome *c* oxidase (Cassina & Radi, 1996). Ascorbate enhances the *S*-nitrosation of glutathione by peroxynitrite ten-fold (Schrammel *et al.*, 2003), however, this does not account for the preserved content of glutathione. One possible site of oxidative stress suggested by Thom *et al.* (1997) following CO exposure are the erythrocytes and this could suggest that myocardial haemoprotein(s) may have a role in the production of ROS. The absence of haemoglobin here suggests that another haemoprotein, myoglobin (Mb) could be a source of ROS production in CO poisoning (**Chapter 7**). The type(s) of reactive species were not determined, however, many ROS (or RNS) could be produced following CO exposure (Ischiropoulos *et al.*, 1996; Piantadosi *et al.*, 1995). The production of the hydroxyl radical (Piantadosi *et al.*, 1997) and NO (Thom *et al.*, 1997) following CO poisoning may produce other reactive species through Fenton chemistry-based reactions within the cell (Bartfay *et al.*, 1999).

Rat brains show LPO following CO exposure *in vivo* that were suggested to arise from xantine oxidase-derived ROS (Thom, 1992). Rat hearts have a relatively high activity of xanthine oxidoreductase compared to human hearts, however, they also have a higher content of SOD compared to human hearts (Janssen *et al.*, 1993). Xanthine oxidase (XO) is also involved in I/R injury (Halliwell & Gutteridge, 1985). However, the evidence rules out tissue ischaemia here (**Chapter 5**) and could suggest that XO is not involved in ROS production (under our *in vitro* conditions). The elevated Po_2 responsible for exarcerbating

the tissue insult following LPO does not require reperfusion (Fisher *et al.*, 1991) and may arise *in vivo* after some loss of CO (under normobaric conditions). CO-induced hypoxia *in vivo* may deplete ATP and result in hypoxanthine to accumulate and become oxidised by XO to generate superoxide and hydrogen peroxide (Halliwell & Gutteridge, 1985).

Another cellular source of CO-induced oxidative stress could be the NADH/NADPH oxidase. Changes in Po_2 are shown to produce superoxide in the vasculature via NADH oxidase (Mohazzab-H. *et al.*, 1997). Pretreating myocytes with antioxidants or an inhibitor of NADH/NADPH oxidase reduced the increase in ROS production (Tanaka *et al.*, 2001). This could suggest that NADH/NADPH oxidase may be inhibited by the AO-treatment, and/or that ROS production in the isolated hearts were initiated in the vasculature tissues before disturbing other tissues, e.g. ventricle tissue. TroloxC accelerates the reaction of GPX *in vivo* (Le *et al.*, 1992) and this could utilise NADPH, and so limit the production of ROS by NADH/NADPH oxidase in this manner, and reduce the loss of glutathione (as found with the AO-treatment). *In vivo* hypoxia is shown to reduce the rate of supply of NADPH (Tribble & Jones, 1990) and measuring the myocardial content of NADPH could prove very valuable (not determined here). Glucose stimulates the production of NADPH (Le *et al.*, 1992) and this could have a role in the CO-induced oxidative stress by accelerating GR in a protective role (Le *et al.*, 1995). Catalase (CAT) has a high affinity for CO (Coburn, 1979; Hattori *et al.*, 1990) and this may affect any detoxifying role during oxidative stress following CO poisoning. The absence of CAT in heart and liver mitochondria (Scholz *et al.*, 1997) may potentiate cellular/tissue damage from exogenous superoxide. The binding of CO to Hb may reduce the ability of Hb to remove H_2O_2 *in vivo* (Masuoka *et al.*, 2003) and this could potentiate CO-induced oxidative stress *in vivo*.

6.6 Conclusions

Rat liver mitochondria did not show any production of superoxide (under our conditions) and this could suggest that mitochondria are not a source of CO-induced oxidative stress. However, tissue differences may have to be considered before any conclusions can be made regarding the source of oxidative stress shown to occur in rat hearts following CO exposure.

6.7 Further Work

6.7.1 Determination of Mitochondrial Calcium & Glutathione

To exclusively rule out rat heart mitochondria (RHM) in producing ROS reposnsible for depleting glutathione, further work could use isolated RHM to perform the experiments (carried out here) examining superoxide production by determining the levels of H_2O_2. The mitochondrial fractions were isolated here following homogenisation of the ventricle tissue (from isolated rat hearts perfused with CO). It is proposed to determine the calcium content (using ICP-MS; 4.2.6) and levels of reduced and oxidised glutathione (GSH and GSSG, respectively). The contents of other thiol proteins (non-glutathione proteins) could be measured to accurately determine the redox conditions within the cytosol and mitochondria (Lesnefsky *et al.*, 1991). However, the prolonged storage (at -80°C) with protein and the lack of 2-VP (5.2.5 and 6.3.4) may produce inaccurate data for the mitochondrial content of GSH and GSSG, unless fresh mitochondria from perfused hearts treated with CO are used. Conversion of xanthine dehydrogenase to xanthine oxidase upon storage of frozen heart tissue could affect the results (Schoutsen *et al.* 1983). It may also be possible to determine the activities of several components of the ETC, however, the reader is requested to review the stability of these prior to any determination. The mitochondrial membrane potential could be investigated in isolated hearts (Fuchs *et al.*, 1990) to determine whether CO-induced oxidative stress affects the MPT pore and disrupts mitochondrial function after CO poisoning.

6.7.2 Cytochrome c Content in Rat Heart Ventricle Homogenate & Apoptosis

The homogenate (of ventricles from rat hearts exposed to CO) could be used to determine the content of cytochrome *c* to evaluate the possible loss from mitochondria. Cytochrome *c* is a prooxidant component that is localised between the inner and outer mitochondrial membranes and its loss results in initiating of the caspases responsible for producing cell death by apoptosis (programmed cell death; PCD). Also, other markers of PCD could be determined to evaluate its contribution (if any) to the cardiotoxicty of CO; particularly, in

light of the cardiac morbidity associated with CO poisoning (**Chapter 1**). The levels of haem oxygenase (HO) could also be determined (Ryter *et al.*, 1998) to predict any other myocardial role(s) of oxidative stress induced after CO poisoning.

6.7.3 Other Methods To Investigate Oxidative Stress Further

Other compounds mentioned in the discussion could be perfused (as performed using antioxidants here) to identify the mode(s) of oxidative stress within the heart after CO exposure. Such compunds could include catalase, glutathione peroxidase and/or desferrioxamine (iron chelator) amongst many others. These could be administered to the hearts at different stages of the perfusion to rule out or identify likely ROS contributing to the cardiotoxicity of CO. The myocardial content of ATP (Juengling & Kammermeier, 1980) and overall antioxidant capacity of tissue homogenates (Kohen *et al.*, 2000) could also be determined to determine the *in vitro* effect of CO in hearts for possible extrapolation to the *in vivo* setting. The activity of xanthine oxidase could be measured to determine its contribution (if any) during CO poisoning (Terada *et al.*, 1990).

6.7.4 Cytochrome c Oxidase and CO Poisoning

Purified cytochrome *c* oxidase could be used to determine the extent of its reduction by ascorbate in order to comment whether this was responsible for the further depressed heart rate in hearts treated with CO and antioxidants. These experiments would be valid regarding its functional similarity to whole mitochondria (Takahashi & Ogura, 2002).

Chapter Seven:

Myoglobin & Oxidative Stress

7.1 Background

Oxidative stress is postulated to occur in numerous disorders (Becker & Ambrosio, 1987; Brömme & Holtz, 1996; Entman *et al.*, 1992; Maulik *et al.*, 1998). Evidence for this comes from markers of oxidative damage, e.g. lipid peroxidation (**1.5**). The lack of association of clinical outcome with blood COHb levels (**Chapter 1**) warrants a need for accurate marker(s) of CO poisoning. The role of oxidative stress in CO poisoning was presented earlier (in **Chapter 5**) and may be exploited here to find a suitable marker of cardiac damage. The current status in clinical biomarkers of oxidative damage is limited to carbon monoxide (Paredi *et al.*, 2002), heat shock proteins (Peng *et al.*, 2000), DNA-adducts and protein carbonyl groups (Dalle-Donne *et al.*, 2003). Therefore, the emerging role of oxidative stress in initiating and/or causing further tissue/organ dysfunction calls for novel biomarkers of oxidative stress (with the potential to allow rapid measurements to be made).

Myoglobin (Mb) is widely found in muscle tissue where its primary role is to facilitate the diffusion of intracellular oxygen (Cole, 1983, Wyman, 1966). The difference in oxygen-dissociation curves for haemoglobin (Hb) and Mb are principally the shapes, sigmoidal and hyperbolic, respectively, thus showing the functional differences in oxygen dissociation between the two proteins. There is also a possible role in CO toxicity as Mb can bind CO, with evidence in the literature suggesting that Mb binds oxygen 2-3 times more strongly than Hb (Kjeldsen *et al.*, 1974; Piantadosi, 1996).

At present Mb is used as a marker to diagnose early myocardial infarction in conjunction with other markers such as the creatine kinase isoforms and cardiac troponin T (Panteghini, 2000; Penttilä *et al.*, 2002) and may also indicate damage to skeletal muscle. The oxidant hydrogen peroxide, H_2O_2, is known to covalently modify Mb in three ways: modification of the prosthetic haem group; alteration of the protein itself; and oxidative cross-linking of the haem to the polypeptide (Mb-H). Mb modified in the third manner, i.e. covalent cross-linking of the haem to the polypeptide, can be detected via its peroxidatic

activity on polyacrylamide gels (Osawa & Pohl, 1989; Vuletich & Osawa, 1998). This suggests that Mb-H could be a useful marker of oxidative damage.

7.2 Aim & Objectives

The potential role of oxidative stress in a CO-induced I/R-like injury (**1.7** and **Chapter 5**) and the possible oxidative modification of Mb led us to investigate this protein as a novel marker of oxidative stress. The hypothesis is that ROS produced during oxidative stress could alter Mb (as described in **7.1**) and that this change (if found) could be an index to assess oxidative damage.

The main aim was to show if exogenous oxidative stress can modify Mb *in situ*, and to evaluate if Mb-H can be detected via its peroxidatic activity on SDS-PAGE. Specific objectives were:

(i) to perfuse isolated rat hearts with hydrogen peroxide to provide exogenous oxidative stress;

(ii) to use SDS-PAGE to isolate Mb from ventricle homogenate; and

(iii) to use an enhanced chemiluminescence (ECL) assay to detect peroxidatic activity in the resolved Mb.

A second aim was to examine the role of various antioxidants in preventing the oxidation of Mb from oxidative stress.

7.3 Materials & Methods

7.3.1 Isolated Heart Preparation

Rat hearts were isolated by rapid excision following anaesthesia and were then perfused with buffer containing HEPES and oxygenated with medical oxygen (100% oxygen) as described in **Chapter 2**. Bicarbonate buffer was unnecessary here since CO_2 was absent. The hearts were oxidatively stressed by including 430μM hydrogen peroxide in the perfusion buffer. Control hearts were perfused in an identical manner (for 90min) in the absence of oxidant. Those hearts which failed to resume a regular sinus rhythm within ten minutes were discarded from the study (Palace *et al.*, 1999).

7.3.2 Biochemical Assays

7.3.2.1 Lactate dehydrogenase (LDH)

Viability of the perfused organ was assessed by measuring the LDH activity from spot samples of perfusate taken at various time points. The assay method was performed as described by Bergmeyer & Bernt (1974) with minor modifications.

7.3.2.2 Griess assay for nitrite

Nitrite in the perfusate was detected as described by Yamamoto *et al.* (1998).

7.3.2.3 Ferrozine assay for iron

Free iron was detected in the perfusate samples as described by Stookey (1970) with modifications to work on a 96-well microtitre plate reader.

7.3.3 Ventricle Homogenate

Rat heart ventricles were prepared as described by Rouet-Benzineb *et al.* (1999).

7.3.4 Peroxidatic Activity of Myoglobin (Mb)

Isolated myoglobin (Mb) was resolved from ventricle homogenate constituents using SDS-PAGE (as described by Schägger *et al.*, 1988). The protein was not renatured as we were examining the peroxidatic activity derived from the haem group covalently bound to myoglobin (Mb-H). The isolated protein was electroblotted overnight onto nitrocellulose membranes and peroxidatic activity was detected as described by Vuletich & Osawa (1998) using an enhanced chemiluminesence (ECL) reagent (Super Signal from Pierce).

7.3.5 HPLC Analysis For Mb-H

The perfusate taken at 15min from perfused hearts (7.3.1) were analysed for the haem-protein cross-linked myoglobin species (Mb-H) using reverse-phase high-performance liquid chromatography (RP-HPLC). This analysis was performed by Dr. Brandon Reeder (University Of Essex, UK) using their own *in house* method (Reeder *et al.*, 2002). Briefly, the perfusate samples were concentrated using a 10kDa MW cut-off filter and this concentrate was injected for HPLC analysis (below). The low MW wash was also analysed under the same conditions. The HPLC analysis were performed using an Agilent HP1100 HPLC using diode array detection. The column used was a Zorbax StableBond 300Å C3 packing material (250 x 4.6mm) fitted with a guard column (12 x 4.6mm). Solvent A was 0.1% trifluoroacetic acid (TFA) and solvent B (B) was 0.1% TFA in acetonitrile. A gradient system was used starting at 35% solvent B (for 10min) that increased to 37% B over 5min; to 40% over 1min and then to 43% B over a further 10min. The flow rate was 1ml.min^{-1} and the temperature was 25°C.

7.3.6 Antioxidant Protection of Mb in vitro

Various non-enzymatic antioxidants were tested to assess their role in protecting the degradation/oxidation of Mb during oxidative conditions (i.e. in the presence of H_2O_2).

Briefly the antioxidants were incubated with Mb for 10min; then oxidative stress was induced by the addition of H_2O_2. Changes in Mb were assessed by following the spectral changes over time. All incubations were carried out in 50mM sodium phosphate buffer (pH 7.4) at 25°C and performed in triplicate.

7.3.7 Data Analysis

Each point in the perfusion study measurements was made in triplicate before averaging for each animal. The data shown is the mean ± SEM for three animals. Statistical analysis was performed on data where stated.

7.4 Results

7.4.1 Perfusions

Control hearts were perfused with perfusion buffer (PB) containing no hydrogen peroxide (H_2O_2); these showed no change in colour during the 90min experimental period, and continued to beat regularly for the entire perfusion. Treated hearts were perfused with PB containing 430μM H_2O_2 after an initial washout/stabilisation period (*ca.* 15min). The amount of oxidant required to modify isolated myoglobin to Mb-H is a three-fold excess (Vuletich & Osawa, 1998). An excess of oxidant, i.e. 100-fold, is shown to degrade the haem group and therefore yield no peroxidatic activity. The level of Mb in skeletal muscle is 0.2mM (Galaris *et al.*, 1989a) and is expected to be higher in heart tissue (*ca.* 0.3-0.4mM; Arai *et al.*, 1999). We therefore chose 430μM H_2O_2 to compensate for the organ's antioxidant capacity. This level of oxidant is similar to that shown previously to cause considerable structural and functional damage (Harrison *et al.*, 1994; Onodera *et al.*, 1992). The 90min duration was chosen as any covalent cross-linking that occurred would remain permanent as it were considered an irreversible process.

All treated hearts had stopped beating by 15min of perfusion. However, a distinct change in colour relative to the control hearts was observed after 5min of perfusion. Control hearts remained red-brown in colour during the entire perfusion, whereas, treated hearts gradually changed to an off-green colour after 5min of H_2O_2 perfusion. At 60min of H_2O_2 perfusion, the upper part of all the treated hearts (comprising the atria) were relatively pale in colour compared to the rest of the organ, and devoid of the green colouration observed earlier. This loss of colour had spread to the entire organ by 90min of H_2O_2 perfusion resulting in yellow-brown hearts. All treated hearts showed increasing back pressure at *ca.* 15min as observed by an increased volume of PB in the bubble trap used. The observed loss of contractile function in H_2O_2-treated hearts occurred before any loss in tissue viability (below).

7.4.2 Tissue Viability

The viability of the heart tissue was assessed by monitoring the release of lactate dehydrogenase (LDH) into the perfusate. **Figure 7.1** shows the LDH release from control and treated hearts per gram of heart tissue. Control hearts showed none or little LDH activity in the perfusate samples over the entire 90min measurement period. Treated hearts showed a similar trend up to 15 minutes followed by a significant increase in LDH release reaching a maximum at *ca.* 30min. LDH activity in perfusate from treated hearts decreased after 30min, however, it remained elevated above that of the control hearts. There is clear evidence of severe oxidative damage to the tissue as observed previously (Dulchavsky *et al.*, 1996).

The perfusates from control and treated hearts at 30min were run on a gel and showed the presence of only Mb in treated heart perfusate. This finding supports the role of Mb as a marker of cardiac damage, due to its negligible levels in the blood, it is used as a cardiac-specific marker in assessing the development/regression of myocardial infarctions (Ebisuno *et al.*, 1986).

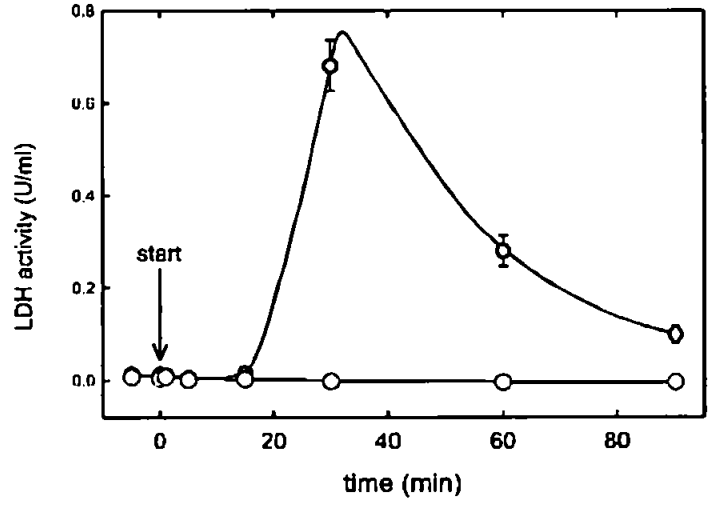

Figure 7.1 Activity of LDH in rat heart perfusates from control (open circles) and hydrogen peroxide (filled circles) perfused hearts. Data shown is the mean ± SEM (n = 3).

7.4.3 Nitric Oxide & Iron Release Into Perfusate

The production of reactive oxygen species (ROS) induces type 2 nitric oxide synthase (iNOS), thus resulting in nitric oxide (NO) production, which serves as a signalling molecule in a number of tissues especially of vascular and endothelial origin (Joe *et al.*, 1998; Sherman *et al.*, 1997). These findings from other studies suggest that the peroxynitrite radical may be responsible for the delayed underlying tissue dysfunction observed (Singh *et al.*, 1996). We investigated NO for a possible role in early-immediate damage from constitutive sources of NO such as endothelial NO synthase. Nitrite is used as an indirect measure of NO production as NO is short-lived and nitrite is a breakdown product of NO (Yamamoto *et al.*, 1998). No differences in the nitrite levels were observed in perfusates from control and H_2O_2-treated hearts (results not shown). However, in both groups, nitrite levels increased steadily throughout the perfusion.

Perfusate iron levels were determined because of evidence of iron release from Mb as indicated by the colour changes observed during perfusion with hydrogen peroxide (7.4.1). **Figure 7.2** shows the iron levels in the perfusates from the two groups over the 90min period. Similar iron release was observed up to 15min for both groups of hearts. The control hearts showed a fall in iron release after 15min, however, the treated hearts showed a significant rise which reached a maximum at *ca.* 30min that declined over the remaining period. The iron released after 15min in treated hearts remained elevated during the entire time course relative to the controls.

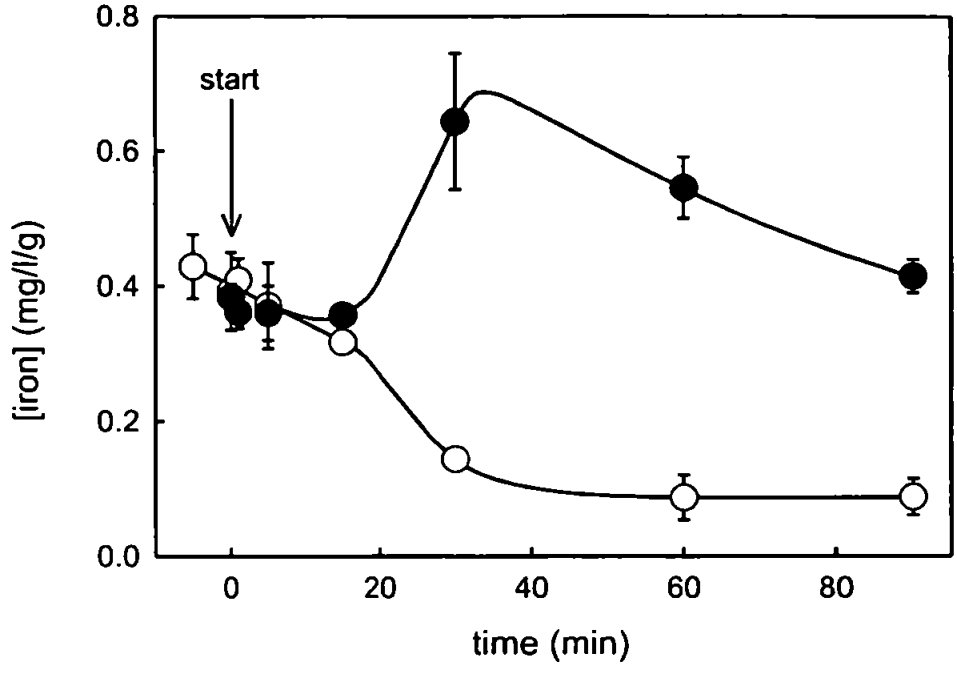

Figure 7.2 Iron content in perfusate samples from control (open circles) and H_2O_2-treated hearts (filled circles). Data shown is the mean ± SEM (n = 3).

7.4.4 Oxidative Modification of Myoglobin

The supernatant fraction containing myoglobin (Mb) from ventricle homogenates were resolved using SDS-PAGE (**Figure 7.3**) and transferred onto nitrocellulose (NC) membranes. SDS-PAGE had successfully resolved the myoglobin as identified with the myoglobin standard. The NC membranes were then exposed to the ECL reagent. Any peroxidatic protein bands would produce luminescence which was detected after exposure to X-ray film (**Figure 7.4**). The ECL assay were repeated three times and produced similar results each time. HPLC analysis of the perfusate samples (by Dr. Brandon Reeder) taken at 15min showed no appearance of Mb-H (results not shown).

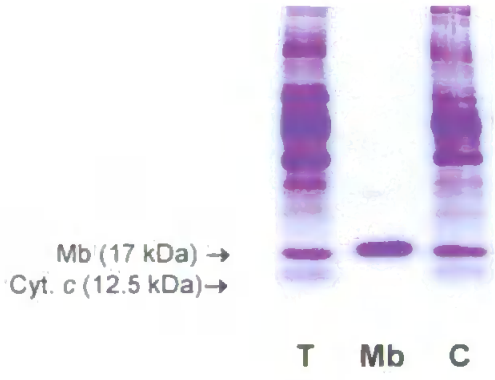

Figure 7.3 (*above*) SDS-PAGE shows myoglobin (Mb) in control (C) and H_2O_2-treated (T) heart ventricle homogenate.

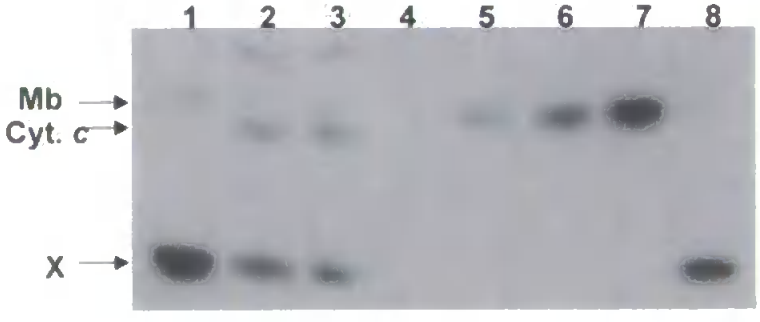

Figure 7.4 (*above*) Detection of peroxidatic activity on nitrocellulose membranes using the enhanced chemiluminescence (ECL) assay. Dark bands (following 2min exposure to X-ray film) correspond to luminescent signals from peroxidatic material: myoglobin (**Mb**), cytochrome *c* (**Cyt. c**) and unknown low molecular-weight (**X**). Lanes correspond to polyacrylamide gel: 1μg untreated Mb (**1** and **8**); 5μg protein from control and H_2O_2-treated heart ventricle homogenate (lanes **2** and **3**, respectively); concentrated perfusate from treated hearts at 30 min (**4**); 0.25, 0.5 and 1.0μg of treated Mb (lanes **5**, **6** and **7**, respectively).

7.4.5 Antioxidants, Myoglobin and Oxidative Stress

The likely role of oxidative stress in CO toxicity suggests that the protective effect of exogenous and endogenous antioxidants may clearly be significant in protecting intracellular Mb. The work of Vuletich & Osawa (1998) showed the *in vitro* modification of Mb by hydrogen peroxide. Therefore, the maintenance of functional Mb in the cell may be altered under conditions of oxidative stress, where its oxygen storage/transfer function could be compromised by oxidative damage. There has been much work in heart tissue on the protective effect of various antioxidants against I/R injury (Ladilov *et al.*, 1998; Nakamura *et al.*, 1998). It was therefore decided, concurrently with the perfusion study, to examine the protective effect of antioxidants against the degradation/modification of Mb *in vitro* by hydrogen peroxide.

The non-enzymatic antioxidants tested were the hydroxyl radical scavenger dimethylthiourea (DMTU), *N*-acetyl-L-cysteine (NAC) and ascorbic acid (vitamin C). In the absence of oxidant, none of the antioxidants tested resulted in the loss of Mb (at 11μM) signal over 90min (not shown). The effect of oxidant on Mb was examined by adding a three-fold molar excess of hydrogen peroxide (H_2O_2). A three-fold molar excess of H_2O_2 (relative to Mb) were used as this level of oxidant is shown to cause modification of Mb to produce Mb-H without the degradation of any haem (Vuletich & Osawa, 1998). The level of oxidant used was found to result in significant loss of the haem signal and a shift in the absorption maximum of the γ (Soret) absorption band (**Figure 7.5**). An approximate 55-fold molar excess of H_2O_2 to Mb resulted in complete loss of the haem signal (**Figure 7.5** *inset*).

Each antioxidant was incubated with Mb for 10 minutes before the addition of H_2O_2 (resulting in a three-fold excess relative to the final Mb concentration). The absorption spectra of the Mb was then examined. DMTU did not preserve the haem signal after the addition of oxidant when used at *ca.* 5- and 50-fold excess to Mb (**Figure 7.6**). Similar results were obtained when NAC were tested using the same concentrations of DMTU (results not shown). Ascorbate (at a molar equivalent to Mb) showed some protective role

by the delay in loss of signal compared to the other two antioxidants tested (not shown). The higher level of ascorbate tested (*ca.* 10-fold relative to Mb) showed more protection relative to the equivalent dose used to Mb under the same conditions (**Figure 7.7**).

The antioxidants were also tested in combination for any synergistic activity in protecting Mb. No protection of Mb was observed when DMTU and NAC were used together each in 55-fold molar excess to Mb (treated as before with a three-fold excess of oxidant; results not shown). Ascorbate with DMTU and/or NAC showed similar results to that observed with ascorbate alone (results not shown).

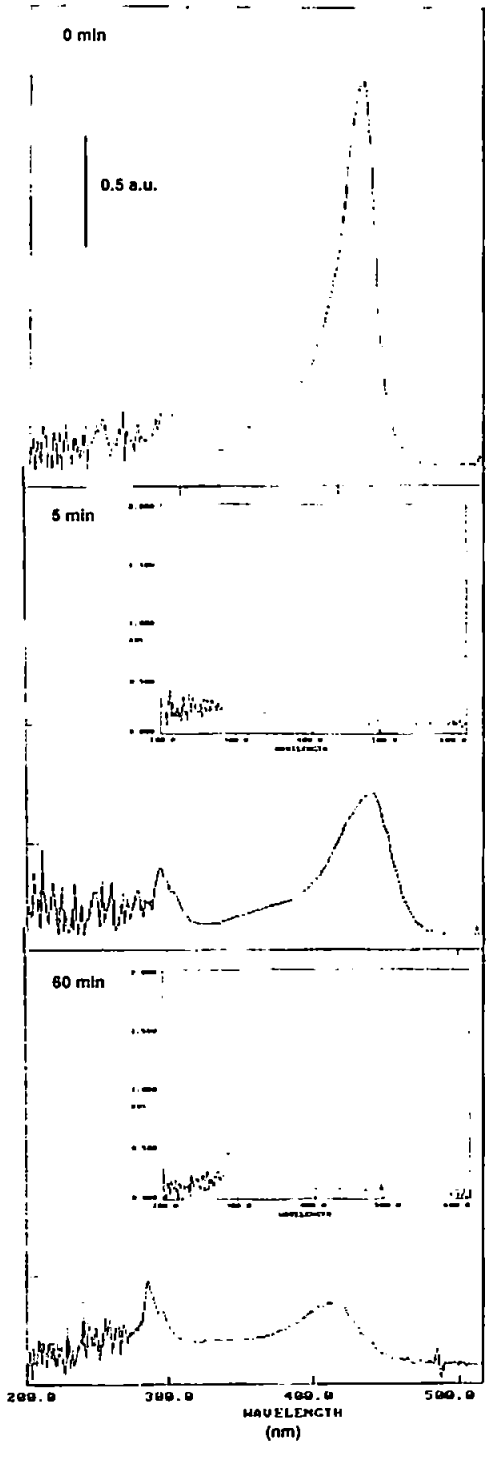

Figure 7.5 Change in absorption spectrum of Mb (11μM) treated with *ca.* three-fold excess of hydrogen peroxide (35μM H_2O_2). The *inset* shows the effect of a higher level of oxidant (600μM). Concentrations are final levels in 50mM sodium phosphate buffer (pH 7.4) at 25°C.

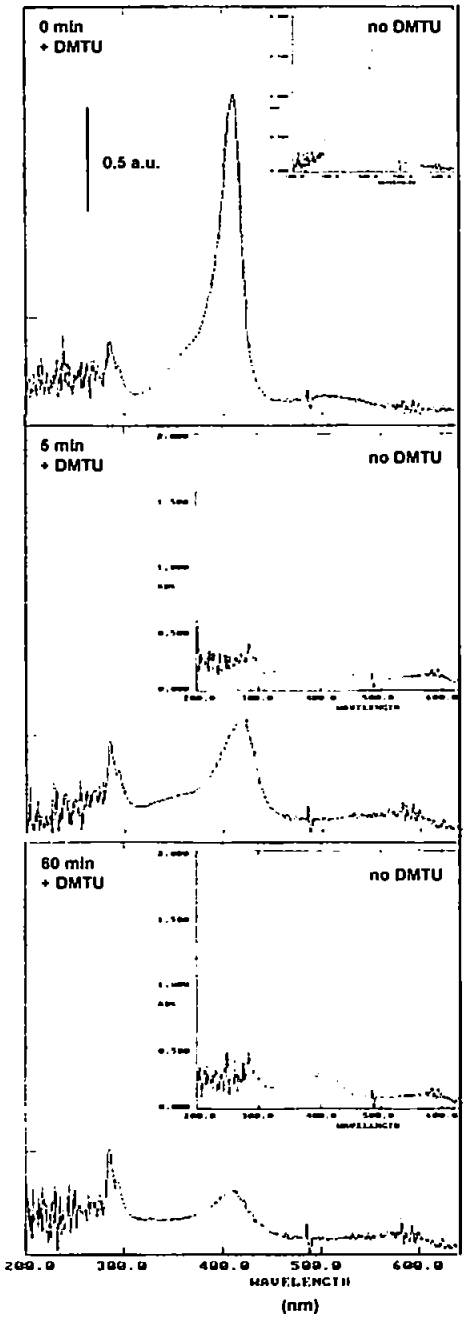

Figure 7.6 The effect of 570μM dimethylthiourea (DMTU), a hydroxyl radical scavenger, on the bleaching of Mb by H_2O_2 (same conditions as given in Figure 7.5).

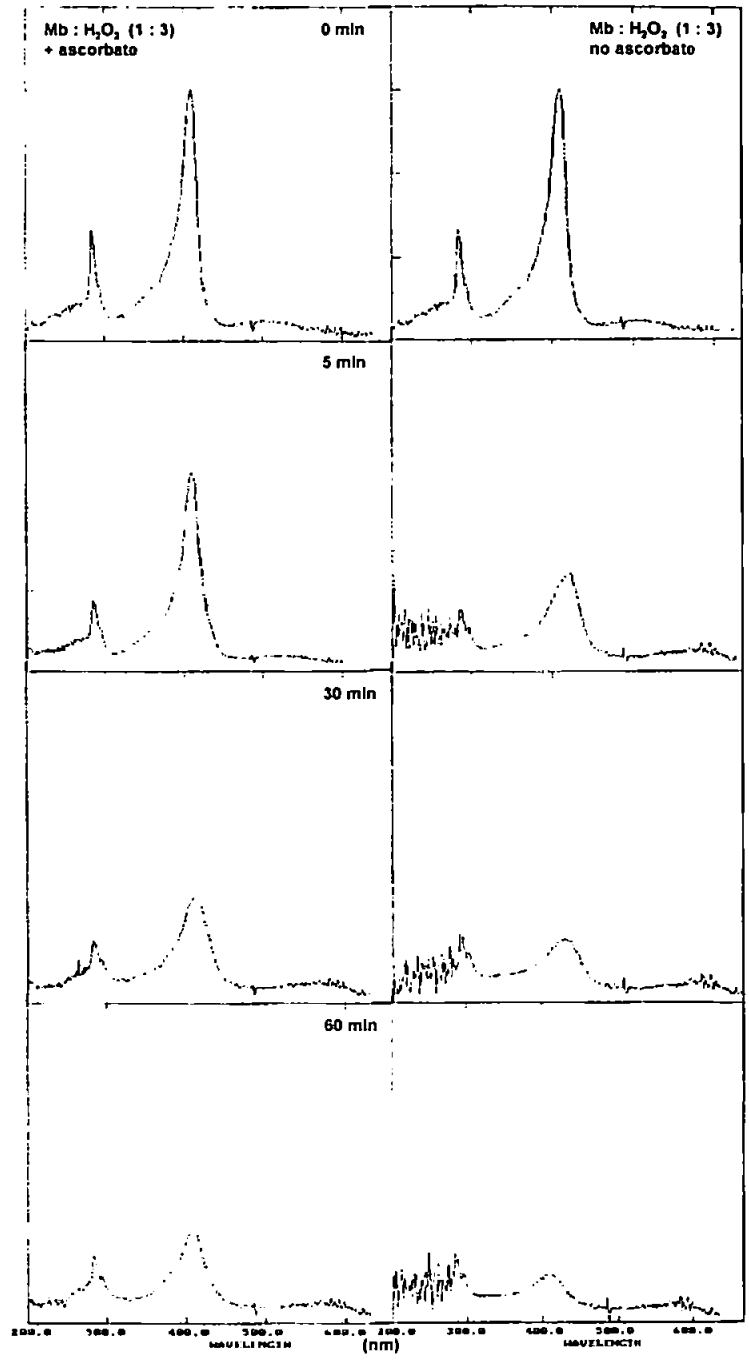

Figure 7.7 The protective role of ascorbate (100μM) against bleaching of Mb by H_2O_2.

7.5 Discussion

Oxidative stress is implicated in cardiac dysfunction associated with various pathologies and ischaemia-reperfusion (I/R) injury (Abete *et al.*, 1999; Brömme & Holtz, 1996; Hess & Manson, 1984; Janssen *et al.*, 1993; Maulik *et al.*, 1998). Reactive oxygen species (ROS) including free radicals are shown to be generated under these conditions. Oxidative stress is shown to occur after hyperbaric oxygen therapy (HBOT; mentioned in **2.1**). HBOT is routinely administered to victims of fire-smoke inhalation to aid the clearance of carbon monoxide from the body and also to promote wound healing (1.3.1). At present, biomarkers of oxidative stress are limited and are not tissue-specific. The present study was performed to investigate whether covalently modified myoglobin (Mb-H) could be induced in the perfused rat heart model under oxidative conditions. Hence, the aim was to determine whether Mb-H could be used as a novel biomarker of oxidative stress in the myocardium.

Myoglobin (Mb) is a haemoprotein that is abundant in the heart; this protein contributes to the dark reddish-brown colour of this organ (Carlez *et al.*, 1995). Prolonged exposure to CO is shown to increase the myocardial concentration of Mb (Pankow & Ponsold, 1979) in the right ventricle (Turek *et al.*, 1973). Mb is shown to form a predominant protein radical species in heart tissue under oxidizing conditions (Detweiler *et al.*, 2002). The role of Mb appears to be an oxygen storing/supplying intermediate, passing oxygen from the blood to the mitochondria, so that cardiac tissue can function using the ATP formed from oxidative (aerobic) respiration (Cole, 1983; Wang *et al.*, 1997; **Chapter 5**). The haem in native Mb is non-covalently bound (Vuletich & Osawa, 1998). Oxidative damage to Mb can occur in three ways: modification of the haem group, modification of the polypeptide, and the covalent linking of the haem group to the polypeptide (Mb-H) (Ortiz de Montellano, 1990; Osawa & Pohl, 1989; Vuletich & Osawa, 1998). The third modification is the basis of the current investigation. We thought that Mb-H may be formed during oxidative stress, therefore, allowing this species to be used as a diagnostic indicator to assess the degree of oxidative stress within the myocardium.

Oxidative stress were induced in the isolated heart preparations by perfusing with hydrogen peroxide (H_2O_2) (Abete *et al.*, 1999). The *in vitro* modification of purified Mb to Mb-H occurred using H_2O_2, however, the purified Mb used was predominantly in the met form when tested by us (results not shown). The two electron oxidation of ferric Mb (metmyoglobin) results in an oxy-ferryl intermediate, $[Fe^{IV}=O]^{2+}$ and a protein free radical, the tyrosyl residue (Tyr_{103}) of the Mb polypeptide (Catalano *et al.*, 1989; Ortiz de Montellano, 1990; Ramirez *et al.*, 2003). Mb exists predominantly in the oxy-ferrous form *in situ*. The electrons are derived from the haem iron in the oxidation by H_2O_2, this produces the oxy-ferryl intermediate with no concomitant formation of the protein radical, hence no Mb-H is formed. The formation of ferryl myoglobin is strongly associated with inducing apoptosis in endothelial cells (D'Agnillo & Alayash, 2002) and may have contributed to the depressed perfusate flow (**Chapter 4**) following CO-induced oxidative stress (**Chapter 5**). This ferryl species is capable of inducing lipid peroxidation in rabbit cardiac myocytes (Turner *et al.*, 1991). Oxidation of substrates by the ferryl oxygen requires the substrate and haem group are in close proximity of the haem pocket in Mb (Boffi *et al.*, 1997; Hamberg, 1997). The altered protein chemistry may enhance the proteolysis of these modified proteins *in vivo* (Osawa & Pohl, 1989) and minimise the potential oxidization of cellular components, also making them less suitable as potential biomarkers here. Any *in vivo* modification of the haem group in Mb may result in an altered flux of intracellular oxygen diffusion and metabolism (Glabe *et al.*, 1998) and may contribute to tissue dysfunction following CO poisoning.

The lack of Mb-H in treated heart homogenate (relative to control hearts) is complicated by the redox cycling of myoglobin. Mb undergoes peroxidatic redox cycling *in situ*, driven by electrons from ascorbate and reduced glutathione (GSH) (Galaris *et al.*, 1989a). In the latter case, there is a possibility that the Tyr_{103} radical could be formed, as the peroxidatic cycle involves metMb. This lack of H_2O_2-induced Mb-H formation shown here *in situ* suggests that the occupancy of this form must be low or it decays rapidly (under the experimental conditions).

The early colour change observed relative to peak LDH release (5min versus 15min, respectively) in treated hearts, suggests that haem-iron had been oxidised to the ferryl oxidation state (Fe^{IV}). This greenish coloured species has been observed and identified by other workers and can be confidently attributed to the observed colour change described here (Arduini *et al.*, 1990; Galaris *et al.*, 1989a). The observed qualitative loss of ferryl iron could be attributed to a reductive mechanism resulting in ferrous (Fe^{II}) or ferric (Fe^{III}) iron. However, if the loss of Fe^{IV} was accounted for by its reduction, the green-brown colour formed in the tissue would have reverted back to the pretreatment colour. The 'bleaching' or loss of green-brown colour observed after 60min of H_2O_2-perfusion suggests loss of the ferryl species from the tissue. Therefore, we assayed for iron in the perfusate, to conclude whether the iron had been reduced or lost through tissue damage. Increasing levels of iron were found by *ca.* 15min of H_2O_2-perfusion relative to the controls (**Figure 7.2**).

The period of initial iron release in the perfusate coincided with the release of LDH in all treated hearts. The use of LDH to assess tissue viability suggests the likely disappearance of the ferryl-species is due to the loss of cellular integrity, therefore, resulting in the loss of cellular/tissue constituents (i.e. myoglobin in **Figure 7.4**). The role of metal transition ions in generating ROS is established with reference to iron in radical generating systems (Gutteridge, 1986; Sullivan, 1989). The addition of peroxynitrite, an oxidant, to caeruloplasmin releases copper (Halliwell & Gutteridge, 1999). Similarly, the production of an oxidising species (H_2O_2) may be responsible for the release of iron from cellular iron binding sites such as ferritin and haemoproteins. Conversely, the released iron may have primarily disrupted cell integrity by Fenton-mediated lipid peroxidation of the cell membrane(s), which would also result in a loss of tissue viability. The lag-time observed before the loss in viability and iron release is consistent with the depletion of intracellular antioxidants such as GSH or ascorbate (Vaage *et al.*, 1997). The release of iron into the perfusate of H_2O_2-treated hearts (relative to controls) could also account for the tissue bleaching observed (7.4.1).

The possible sources of cellular iron, such as haemoglobin, can be ruled out as most of the blood was washed before starting the experiment. The Ferrozine assay was also tested to examine if the reagent could displace haem iron from Mb. The Ferrozine reagent did not remove haem iron (results not shown), therefore, the Mb found in treated heart perfusate could not account for the released iron. However, if haem was degraded in Mb following oxidant treatment, then the possibility of haem as the source of iron cannot be ruled out (Turner *et al.*, 1991). The released iron could also derive from the degradation of the Mb-H (protein-haem) adduct (Osawa & Williams, 1996). We assessed the degradation of haem under physiological conditions by examining the absorbance spectra of the haem moiety. Our findings of haem degradation do not agree with those reported by Vuletich & Osawa (1998) who showed no degradation of haem under similar conditions. These workers reported that haem degradation occurred in Mb when H_2O_2 levels were used in 100-fold excess to Mb, however, this was determined using different conditions compared to our method. Our results showed a significant loss of the haem signal to occur when a three-fold excess of H_2O_2 was added to the Mb solution (**Figure 7.5**). This finding agrees with the reaction of a molar excess of oxidant producing iron release through haem degradation (Nagababu *et al.*, 1998). It is very likely that the iron in the perfusate of treated hearts was derived from haem iron of Mb in the presence of hydrogen peroxide (Harel *et al.*, 1988). Another likely source is intracellular ferritin (Gutteridge, 1986; Reddy *et al.*, 1989). The source of iron remains to be determined here, however, it is likely that the iron released by oxidant(s) could participate in producing further oxidative stress (Gutteridge, 1986). These findings along with the CO-induced oxidative stress (**Chapter 5**) may implicate haemoproteins in causing further damage to heart tissue(s) after CO-induced oxidative stress has ceased (Hidalgo *et al.*, 1990). The lowered intracellular pH (acidosis) found during CO poisoning *in vivo* could exacerbate tissue damage from the released iron through its increased reactivity favoured by its improved solubility (Schafer & Buettner, 2000). In fact, an acidic pH favours the formation of ferryl-Mb under oxidizing conditions (Reeder & Wilson, 2001), and this may suggest that myoglobin-induced oxidative stress could have contributed to the CO-induced oxidative stress (shown in **Chapter 5**). The presence of iron from haemoproteins (i.e. Mb) may account for the

oxidative stress found in the isolated hearts exposed to CO and could explain the absence of oxidative stress in isolated mitochondria exposed to CO and hyperoxia (Robb *et al.*, 1999; **Chapter 6**). Iron increases the activity of xanthine oxidase and this may play a role in CO-induced oxidative stress (Sullivan, 1989).

It is shown that the release of myoglobin from ruptured cardiac myocytes is an early indicator of myocardial infarction (Bourne *et al.*, 1997; Ebisuno *et al.*, 1986). The observed loss of heart myoglobin following oxidative stress could occur after CO poisoning *in vivo* and may be responsible for producing rhabdomyolysis resulting in renal failure (Cooper *et al.*, 2002). Myoglobin is directly implicated in regulating myocardial metabolism (Doeller & Wittenberg, 1991; Glabe *et al.*, 1998) and this may be inhibited by CO binding as a ligand. The human cases of myonecrosis described following CO poisoning (Finley *et al.*, 1977; Herman *et al.*, 1988; Shapiro *et al.*, 1989) could suggest that haemoproteins may themselves be cytotoxic under conditions of oxidative stress (Chen & Tappel, 1995; Eaton *et al.*, 2002; Mason *et al.*, 2002; Witting *et al.*, 2001).

The abundance of myoglobin in heart tissue may be protective during acute hypoxia, however, CO-hypoxia inhibits its facilitative function (Glabe *et al.*, 1998) and may even be a prooxidant under these conditions. Myoglobin may also have a protective cellular role by binding oxidant radicals produced in mitochondria (Hidalgo *et al.*, 1990). The reduced cardiac level of Mb in people with heart disease (McGrath, 2000) could exacerbate cardiac damage following CO poisoning in this group of people; furthermore, CO could be responsible for the depressed myoglobin levels in hearts prior to producing heart disease. The role of myoglobin in toxicology remains to be further investigated in light of its redox recycling, and pro- and anti-oxidant properties (Galaris *et al.*, 1989b).

The role of nitric oxide (NO) in endothelial functioning and its role as a 'stress' messenger under oxidative stress is established (Joe *et al.*, 1998; Sherman *et al.*, 1997). Any NO released into the perfusate was determined (using the Griess assay) by measuring its immediate breakdown product: nitrite (NO_2^-). We found no difference in perfusate nitrite

between either group of perfused hearts. Other studies in the literature have shown elevated NO levels and inducible nitric oxide synthase (iNOS) message and protein after 24 hours in culture models or after chronic exposure in animals (Igarashi *et al.*, 1998; Jones *et al.*, 1999; Kumura *et al.*, 1996). Our results rule out the role of NO in immediate oxidative damage.

7.6 Conclusions

The *in vitro* modification of myoglobin by oxidative stress was investigated as a possible biomarker of oxidative stress. Our findings showed that rat hearts perfused with hydrogen peroxide (H_2O_2) caused the release of LDH and free iron and produced a loss of tissue function. The H_2O_2-induced release of iron may undergo redox recycling in the cell by tissue antioxidants such as ascorbate, and could initiate oxidative stress in this manner. The release of iron may cause oxidative damage in other tissues by mediating the production of ROS via Fenton chemistry. The [H_2O_2] used was sufficient to lead to gross organ failure, however, it was envisaged this would modify Mb (under these conditions) to the peroxidatic Mb-H species. Our results showed no peroxidatic activity in perfusate or ventricle homogenate under these conditions, therefore, suggesting that Mb-H may not be used as a cardiac-specific marker of oxidative stress from biopsy or blood samples. Whether CO-induced oxidative stress oxidizes myoglobin *in vivo* remains to be determined.

7.7 Further Work

A diagnostic role of Mb-H can be ruled out from this study (above), however, the observation of peroxidatic activity from low molecular weight protein(s) has raised the possibility of microperoxidases being potential markers. It was found that the homogenate from H_2O_2 perfused hearts showed peroxidatic bands in the low molecular weight (MW) region after 90 minutes of H_2O_2 perfusion. Treated Mb showed a loss of this low MW peroxidatic material, therefore, this low-weight unidentified compound(s) could be microperoxidases. This is of interest as this type of peroxidase has not been found *in vivo* (personal communication with Professor Michael Wilson, University Of Essex).

The microperoxidases are short peptides fragments derived from haemoprotein polypeptide. These peptides have a haem group covalently attached for their peroxidatic activity (**Figure 7.8**). We will address the use of these microperoxidases as potential novel markers of oxidative stress by examining their possible degradation/modification under oxidative conditions (Nagababu *et al.*, 1998).

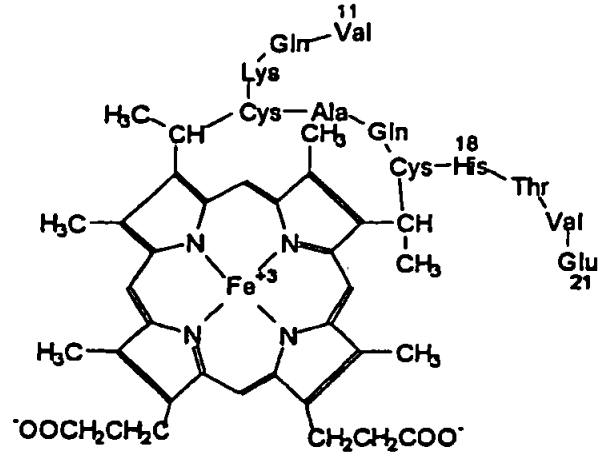

Figure 7.8 Structure of MP11 (microperoxidase-11), a ferrihaem undecapeptide derived from cytochrome *c*. The figure is taken from Spector *et al.* (2000).

Briefly, the proposed work involves characterizing the unknown low MW peroxidatic material. The samples for analysis were derived from rat heart ventricles prior to being perfused with hydrogen peroxide for 15 minutes. This is envisaged by using low MW markers on the polyacrylamide gel, in conjunction with a more cross-linked gel to limit the loss of these likely peptides, and to maintain the markers for molecular weight identification. The above was performed by our group using a 10% acrylamide and failed to retain the low MW markers, hence, using a more cross-linked gel is proposed to retain the markers to aid characterization. The peroxidatic activity would be detected (as performed here) to evaluate its potential use as a novel marker of cardiac oxidative stress.

Chapter Eight:

General Discussion & Conclusions

8.1 General Discussion

This chapter will discuss the presented findings by extrapolating to an *in vivo* scenario with specific emphasis on the likely mechanism(s) responsible for producing CO-associated cardiac morbidities.

8.1.1 The Cardiotoxicity Of Carbon Monoxide

8.1.1.1 In vivo myocardial hypoxia

The cardiotoxicity of carbon monoxide (CO) is established at the clinical level, however, the mechanism(s) of its toxicity are unclear and the direct effects within the body are also not fully known. Binding of CO to haemoglobin *in vivo* and the subsequent production of tissue hypoxia is well documented (Killick, 1940) and considered to be responsible for some of the symptoms of CO poisoning (**Chapter 1**). Our hypothesis postulated that CO exposure *in vivo* produces cardiac morbidities via an ischaemia/reperfusion (I/R)-like injury (**1.7**). Using isolated rat hearts as a suitable model (**Chapter 3**) allowed the hypothesis to be addressed and presented some changes similar to those found occurring in I/R injury. The novel findings (presented in **Chapters 4 and 5**) were evident under normoxic conditions and rule out hypoxia (with the levels of CO used here) in producing the described changes.

Lactic acidosis of the blood is associated with CO poisoning *in vivo* (**Chapter 5**). This increase in lactate production would appear to be protective under systemic hypoxia (following severe acute inhalation of CO) by increasing the release of oxygen from myoglobin (Giardina *et al.*, 1996). This could provide oxygen for the tissues, however, it could increase the availability of haem sites for CO to bind to (in myoglobin) and potentiate the CO-induced hypoxia *in vivo* (Jaffe, 1997). This deoxygenation of myoglobin may limit the availability of oxygen to the mitochondria (Wittenberg & Wittenberg, 1985).

In vivo hypoxia stimulates the production of NO (**Chapter 1**) and this may also arise during CO poisoning (Ischiropoulos *et al.*, 1996; and *8.1.1.2*). NO can bind readily to haemoproteins such as cytochrome *c* oxidase (Complex IV; *8.1.1.4*) and impair its function in cell respiration (Sarti *et al.*, 2003). This could account for the decrease in the activity of Complex IV shown by CO (Somogyi *et al.*, 1981). Although CO can only bind to the reduced haem centre of cytochrome *c* oxidase. NO can also nitrate Tyr residues in various proteins and interfere with many biochemical pathways within the myocardium (Mallozzi *et al.*, 2001). The inhibition of mitochondrial respiration by NO could contribute to the apoptosis (*8.1.1.2*) that is found after CO poisoning (Piantadosi *et al.*, 1997).

8.1.1.2 Oxidative stress

It is reasonable to conclude that CO may elicit its cardiotoxicity by producing oxidative stress based on the findings (in **Chapter 5**). Furthermore, the postulated hypothesis which may be more realistic in an *in vivo* setting could undergo I/R injury and exacerbate any CO-induced oxidative stress. However, the source of reactive oxygen species (ROS) production remains unclear as ROS are implicated in organ injury following I/R injury (Sussman & Bulkley, 1990) and CO poisoning (Zhang & Piantadosi, 1992). Isolated mitochondria showed no production of ROS here (**Chapter 6**), therefore, other sources of cellular oxidation are briefly discussed. Oxidative stress was inferred from the depletion of intracellular reduced glutathione in heart ventricle tissue exposed to CO (**Chapter 5**).

Oxidative stress may have arisen by an increase in the production of ROS induced by CO and/or a depletion of antioxidants by CO (**Chapter 5**). The source(s) of the oxidative stress do not lie solely in the ventricle tissue (that is comprised mostly of cardiac myocytes), as endothelial tissue is shown to be a source of ROS production, particularly the superoxide radical (Serrano Jr. *et al.*, 1996). Oxidative stress by the production of ROS are also partly responsible for the oxidant-mediated cytotoxicity through altered calcium channels (Az-ma *et al.*, 1999). The importance of glutathione in cells (described in

Chapter 5) and its thiol chemistry involved in regulating cellular redox state (Schafer & Buettner, 2001) and conferring cytoprotection may be associated with apoptosis. A molecule termed SAG (sensitive to apoptosis gene) has 12 cysteine residues and is thought to play a vital antioxidant role in the heart (Kim *et al.*, 2003). Any depletion of this protein (as shown for glutathione) could lead to myocardial apoptosis that may contribute to eventual myocardial failure. The depletion of GSH is shown to reduce the activities of myocardial glutathione peroxidase (GPX), and glutathione-*S*-transferase (Leeuwenburgh *et al.*, 1996) and increase the activity of superoxide dismutase (SOD). The activity of GPX is decreased in severe cases of atherosclerosis in the coronary artery (Ramos *et al.*, 1997) and could suggest that the CO-induced oxidative stress may lead to atherosclerosis (*8.1.1.5*). Apoptosis is triggered by oxidative stress (Chandra *et al.*, 2000) and found in the reperfused ischaemic myocardium (Maulik *et al.*, 1998). Following this, oxidative stress shown here and the likely validity of the postulated hypothesis *in vivo* suggests that apoptosis could play a major role in determining myocardial outcome following CO poisoning. Apoptosis is found to contribute in neuronal cell death in the brains of Sprague-Dawley male rats exposed to 0.25% CO for 30min (Piantadosi *et al.*, 1997). Lipid peroxidation (LPO) cannot be ruled out following the CO-induced oxidative stress. Tissues with limited antioxidant reserves show a generation of CO associated with LPO (Vreman *et al.*, 1998) and this could exacerbate the initial oxidative insult (by CO) leading to a vicious circle of cytotoxic events. This lethal cascade of events could propagate down to the mitochondrial level and alter the bioenergetics of the myocardium leading to heart failure (*8.1.1.5*). Recent findings report an anti-apoptotic property of CO at low levels (Zhang *et al.*, 2003) through its endogenous production by haem oxygenase (**Chapter 1**). The CO-induced oxidative stress may elicit such an anti-apoptotic effect by regulating the oxidant effects of hydrogen peroxide, as hydrogen peroxide is found to regulate apoptosis by inhibiting pro-apoptotic caspases (Borutaite & Brown, 2001).

Xanthine oxidase (XO) produces superoxide radicals following the hypoxia-induced conversion of xanthine dehydrogenase (XDH) to the oxidase (Russell *et al.*, 1989). The role of XO is established in I/R injury (Sussman & Bulkley, 1990), however, it is not

considered responsible for producing ROS as no hypoxic conditions were found under the experimental conditions used (**Chapter 5**). Furthermore, the presence of oxidative stress in the ventricles could not arise from XO, as XO is only found in endothelial cells and not cardiac myocytes (Gunther *et al.*, 1999; Kehrer *et al.*, 1987). Therefore, we conclude that XO may not be responsible for the oxidative stress from the lack of hypoxic conditions in this study. However, hypoxia is shown not to convert XDH to XO in rat hearts (Kehrer *et al.*, 1987), although, they did report a loss of myoglobin from isolated perfused hearts during reperfusion (*8.1.1.3*). XO is not considered to contribute significantly in humans during CO poisoning as it is found in low levels in human hearts (Morales *et al.*, 2002). Other evidence shows that peroxynitrite is produced by XDH under aerobic conditions (Godber *et al.*, 2000) may be responsible for the oxidative stress observed here in the presence of inorganic nitrite (or nitric oxide) and a reductant. CO-hypoxia *in vivo* and its concomitant production of nitric oxide (Ischiropoulos *et al.*, 1996; Kumura *et al.*, 1996) could participate in such a reaction with XDH to produce oxidative stress that may produce brain and heart failure associated with CO poisoning (**Chapter 1**).

8.1.1.3 Myoglobin as an oxidant

The abundance of myoglobin (Mb) in heart tissue is postulated to have a protective role in scavenging nitric oxide (NO) *in vivo* by Ascenzi *et al.* (2001). This could become impaired during CO poisoning and may account for oxidative stress through the production of reactive nitrogen species (RNS; described in **Chapter 5**). However, the production of NO following CO exposure is shown in rat brain tissues (Ischiropoulos *et al.*, 1996) and may not arise in heart tissue (**Chapter 3**) and rule out Mb as a possible NO scavenger in heart tissue. Although the absence of NO production in heart tissue following CO exposure could indicate effective scavenging of NO by Mb. Glutathione may reduce nitric oxide to nitrite, such that it inhibits Mb to affect myocardial function (Glabe *et al.*, 1998). Furthermore, nitrosation of cysteine residues by peroxynitrite (formed from NO and superoxide) could account for the depletion of reduced glutathione after CO exposure.

The *S*-nitrosated haemoglobin has a higher affinity for oxygen than native Hb (Patel *et al.*, 1999) and may contribute in CO-induced hypoxia *in vivo* and oxidative stress. The inhibition of Mb by oxidation of its haem iron and/or via CO-binding has negative chronotropic properties by impairing oxygen delivery to mitochondria (Glabe *et al.*, 1997; White & Wittenberg, 1997) that could decrease the heart rate (shown in **Chapter 4**). Of particular interest is that the [Mb] has a good correlation with the level of cytochrome *c* oxidase (Cole, 1983), another potential target of CO (**Chapter 1**) that shall be discussed later (in *8.1.1.4*).

Typical conditions found during oxidative stress could oxidise the haem iron of Mb to the ferric form and so prevent the binding of oxygen to the predominant cellular form, i.e. ferrous haem (**Chapter 7**). The ferryl form of Mb is found during ischaemia in isolated rat hearts (Arduini *et al.*, 1990) and may occur during CO poisoning *in vivo*. This oxidised ferryl form of myoglobin may function as an intracellular oxidant (discussed in **Chapter 7**) and such myoglobin-catalyzed oxidative reactions may initiate and/or exacerbate vascular tissue injury responsible for producing the depressed coronary flow (in **Chapter 4**; Jourd'heuil *et al.*, 1998). *In vivo* CO-induced hypoxia can produce lactic acidosis and these conditions favour the formation of the oxidising ferryl species of Mb (Reeder & Wilson, 2001). The oxidative form of Mb (ferryl) has not been found in heart tissue *in vivo* and this could be attributed to the enhanced proteolysis of this modified protein. Oxidative stress can alter intracellular calcium balance (**Chapter 4** and *8.1.1.2*) that could impair haem binding in Mb and/or haemoglobin (Pappa & Cass, 1993) to reduce the oxygen carrying function of these proteins. This may infer that aerobic metabolism may remain nonfunctional until the calcium balance is restored following CO poisoning *in vivo*. Mb may have a significant role in the cardiotoxicity of CO *in vivo* (following acidification) by augmenting the production of superoxide and hydrogen peroxide that occurs under normal conditions of metmyoglobin formation (Gunther *et al.*, 1999). The localisation of Mb along the sarcoplasmic reticulum and the mitochondria in myocytes may contribute to site-specific tissue damage contributing to stunning and arrhythmogenesis (**Chapter 4**).

The depletion of glutathione (GSH) following CO exposure (**Chapter 5**) may have arisen from the oxidative (ferryl) Mb species conjugating with intracellular proteins (Østdal *et al.*, 1999) such as GSH. The relative scavenging reactivities of the Trp_{14} peroxyl radical in Mb (produced from the reaction of Mb with hydrogen peroxide as oxidant) is ascorbic acid > urate > TroloxC > GSH (Irwin *et al.*, 1999). These scavenging properties support the protective role of antioxidants in CO poisoning (*8.1.1.6*) and highlight the role of ascorbic acid and TroloxC (in **Chapter 5**). The findings from Irwin *et al.* (1999) also suggested that ascorbate may be the primary antioxidant in preventing the depletion in GSH. It is possible that ascorbate reduced the oxidised ferryl species of Mb (Galaris et al., 1989) prior to it producing any oxidative damage within the cells, and/or directly scavenged the peroxyl radical before it can covalently modify Mb to its peroxidatic form (**Chapter 7**). GSH can reduce the ferryl form of Mb, however, it cannot reduce metmyoglobin (Galaris *et al.*, 1989) and interfere in the peroxidatic activity of modified Mb (Vuletich & Osawa, 1998). Metmyoglobin can bind NO to yield NO-adducts that could produce nitrosoglutathione in the presence of GSH (Reichenbach *et al.*, 2001) and possibly deplete GSH in this manner. However, this is unlikely *in vivo* as there is no Mb in brain tissue where NO production is reported to occur following CO exposure (above); neither is it anticipated in heart tissue as the production of NO in heart tissue were not found following CO exposure (**Chapters 3 and 7**). However, a neural equivalent of Mb termed neuroglobin has been identified (Ascenzi *et al.*, 2001), and this may have a role in brain tissue damage following CO poisoning similar to the proposed oxidative damage by Mb in heart tissue.

Although the above has described the oxidising nature of Mb, it must be stressed that other haem centres do undergo similar oxidative changes to yield oxidising species. Haemoglobin can also undergo similar oxidation to produce ferrylhaemoglobin that is thought responsible for oxidative lesions (Herold & Rehmann, 2003). When the irreversible covalent modification of haem to the globin (protein) occurrs, its peroxidatic activity cannot be dampened by CO binding to its reduced haem centre (Newton *et al.*, 1965). This is physiologically important as it implies that oxidative stress by these

haemoproteins cannot be regulated unless the haem centre is degraded or the metal ions at the haem centre is selectively sequestered (8.1.2).

8.1.1.4 Free radical theory of ageing and mitochondria

Mitochondrial respiratory function is gradually uncoupled and the activities of the respiratory enzymes decrease with age (Wei *et al.*, 1998). Mitochondrial DNA (mtDNA) is susceptible to oxidative damage by reactive oxygen species (ROS) and free radicals (Kadenbach *et al.*, 1995). Impaired respiratory components increase the electron leakage and ROS production by an one-electron reduction of molecular oxygen and this contributes to the oxidative stress and concomitant oxidative damage to the mitochondria. Heart tissue has an age-related decrease in its tolerance to oxidative stress (Abete *et al.*, 1999). The activity of catalase decreases with age in rat hearts (Boucher *et al.*, 1998). This mitochondrial production of free radicals and its associated oxidative stress with ageing encompass the 'free radical theory of ageing' (Cadenas & Davies, 2000). Human hearts show an age-associated damage in their mtDNA (Hayakawa *et al.*, 1992). The fragility of mtDNA (due to the lack of histones for binding) and its oxidising environment is thought to contribute to the failing heart through apoptosis (Ozawa, 1998). This loss of mitochondrial function is thought to be responsible for many pathologic conditions including those related to heart failure (Ramachandran *et al.*, 2002).

An increase in ROS production by deenergised mitochondria opens the mitochondrial permeability transition (MPT) pore in cardiac myocytes (Zorov *et al.*, 2000). This pore opening phenomenon is dependent upon the cellular ROS-scavenging mechanisms, particularly glutathione. This could suggest that an opening of the MPT pore following CO exposure may lead to loss of the mitochondrial transmembrane potential ($\Delta\psi_m$) by oxidative stress (**Chapter 5**) and produce more ROS (Zorov *et al.*, 2000) and/or apoptosis (*8.1.1.2*). However, no ROS production were found in isolated mitochondria following CO exposure under the experimental conditions used (in **Chapter 6**), also ruling out this

occurrence. Hydrogen peroxide is reported not to affect mitochondrial respiration in dog grastrocnemius-plantaris muscle (Cole, 1983). Although other factors could have contributed to the lack of ROS production by mitochondria exposed to CO *in vitro* (**Chapter 6**). Heart mitochondria are swollen and show fragmented cristae following CO poisoning *in vitro* (Somogyi *et al.*, 1981). The results suggest that mitochondria may not be directly responsible for producing oxidative damage and could be a target of other cellular damage leading to mitochondrial failure during CO poisoning.

The possible production of NO following CO exposure (*8.1.1.2*) and/or cytokines (*8.1.1.5*) may inhibit mitochondrial activity (Brown, 2001; Oddis & Finkel, 1995). NO can modulate other factors involved in the apoptotic cascade and directly influence mitochondrial function. The compromised function of mitochondria is involved in a number of vascular pathologies and could arise following CO-induced oxidative stress. Damage to mtDNA is found in ischaemic heart disease, cardiomyopathy, atherosclerosis, and hypertension (Ramachandran *et al.*, 2002). Cytochrome *c* oxidase may also have a protective role within mitochondria to scavenge NO (as described for myoglobin in *8.1.1.3*; Sarti *et al.*, 2003).

The fragility of the mtDNA does not appear to be the sole victim by attack from oxidising species. The membranes of the mitochondrion are also susceptible to lipid peroxidation (LPO) with age. Over time the cardiolipin content decreases *ca.* 40% in aged rats (Paradies *et al.*, 1999) and this may potentiate the intrinsic lethality of LPO. Exposure to CO may exacerbate the decline in cardiolipin content through oxidative stress. The decrease in cardiolipin content contributes to the depressed activity of cytochrome *c* oxidase with age, therefore, this suggests that CO could potentiate ageing within the heart tissue (above and **Chapter 6**). Apoptosis contributes to brain cell death in CO poisoning (Piantadosi *et al.*, 1997). The loss of mitochondrial membrane integrity could release cytochrome *c* and stimulate the pro-apoptotic pathway (Borutaite *et al.*, 2001). Cardiolipin is also closely associated with cytochrome *c* and their loss is thought to initiate apoptosis (McMillin & Dowhan, 2002). CO inhalation significantly lowers the content of cytochrome *c*

in heart ventricles (Penney *et al.*, 1983) and supports a possible loss of cytochrome *c* by oxidative stress through LPO. Whether apoptosis following the loss of cytochrome *c* occurred under our conditions remains to be determined; however, its loss would inevitably produce a shortfall in the activity of cytochrome *c* oxidase that may contribute to the lowered activity of Complex IV found after CO poisoning (Pankow & Ponsold, 1984).

There are two types of mitochondria found in rat heart tissue: interfibrillar mitochondria (IFM) found between myofibrils; and subsarcolemmal mitochondria (SSM) that reside under the plasma membrane. Evidence suggests that the IFM are more susceptible to oxidative stress than SSM (Fannin *et al.*, 1999) and may explain the loss of contractile function associated with ageing that could be potentiated following CO poisoning.

8.1.1.5 Pathogenesis of heart disease

Oxidative stress is one of the principle insults in promoting cardiac myocyte death (Clerk *et al.*, 2003). Cardiac myocyte death through apoptosis or necrosis may contribute to many cardiac pathologies (Latif *et al.*, 2000). DNA damage is frequently found in cells exposed to oxidative stress. Following this, coronary artery disease (CAD) is characterised with DNA damage (Botto *et al.*, 2001) and decreased activities of GPX (Ramos *et al.*, 1997) that are associated with the severity of atherosclerotic disease. DNA damage was assessed by Botto *et al.* (2001) by determining the levels of DNA adducts in cardiac tissues. Increased levels of DNA adducts are found in the hearts of both smokers and rats exposed to mainstream or environmental cigarette smoke (Izzotti *et al.*, 2001). The presence of CO in cigarette smoke may be responsible for DNA damage (either nuclear or mitochondrial; *8.1.1.4*) by oxidative stress and lead to heart failure in such a manner (above). Human hearts are more sensitive to atherosclerosis relative to rats, therefore, an equivalent depletion of glutathione (shown in **Chapter 5**) and possibly other myocardial antioxidants in the human heart could produce severe symptoms or potentiate the rate of myocardial damage or failure.

Further oxidative stress through the action of neutrophils may arise following the myocardial damage after CO poisoning. The recruitment of neutrophils and macrophages can compromise the microcirculation and prolong tissue injury (Massey *et al.*, 1995). ROS produced by neutrophils activate collagenases and gelatinases and lead to irreversible tissue damage (Weiss, 1989). Neutrophils are activated in response to ischaemia/reperfusion (I/R) injury to cardiac myocytes (Hansen & Stawski, 1994; Kukielka *et al.*, 1993). The recruitment of neutrophils to cells requires the expression of intercellular adhesion molecule-1 (ICAM-1) on the myocytes and an enhanced expression of CD-18 by neutrophils for intercellular adhesion (Entman *et al.*, 1992). The expression of ICAM-1 is expressed in the ischaemic myocardium in a time-dependent manner. Any oxidative myocardial injury following CO poisoning *in vivo* may be further exacerbated by neutrophil recruitment and activation. The likely reoxygenation (or reperfusion phase) following severe acute CO poisoning could potentiate the degree of neutrophil recruitment and exacerbate myocardial tissue injury after CO exposure has ceased.

Cytokines are important mediators of cardiovascular disease (Kukielka *et al.*, 1995). Tumour necrosis factor alpha (TNFα) will be discussed here, although, other cytokines such as the interleukins (ILs) show a similar pattern of expression and function. The expression of these proinflammatory cytokines is responsible for the pathogenesis of many cardiovascular diseases (Maekawa *et al.*, 2002; Meldrum *et al.*, 1998; Nozaki *et al.*, 1998). Briefly, TNFα (and ILs) are upregulated early relative to ICAM-1 during ischaemia and may mediate the expression of ICAM-1. These cytokines also stimulate the expression of low-density lipoprotein receptors (Mehta & Miller, 1999; and below). Coincidentally, I/R injury stimulates the expression of other genes with κB-response elements including inducible NOS (Chandrasekar *et al.*, 2000). This could potentiate myocardial injury after CO poisoning from cytokines/neutrophils contributing to further oxidative damage. Mitochondria could be involved in myocardial failure as cytokines can

directly inhibit mitochondrial function in cardiomyocytes (Zell *et al.*, 1997) and may lead to ROS production (*8.1.1.4*).

Cytokines mediate the induction of calcium-independent NOS in the heart (Schulz *et al.*, 1995) and this modulation of myocardial NO could have a role in CO-associated cardiac morbidities. Nitric oxide is thought to contribute in the depression of heart function through inhibition of cardiac mitochondrial respiration (Ramachandran *et al.*, 2002; Ramesh *et al.*, 1999; Xie *et al.*, 1996). This could explain the negative inotropic response shown by TNF (Yokoyama *et al.*, 1993; and *8.1.1.4*). NO is implicated in decreasing intracellular cAMP following β-adrenergic stimulation in cardiac myocytes (Joe *et al.*, 1998). Heart failure is also characterised by down-regulation of the β_1-adrenergic receptor message (Bristow *et al.*, 1993). The cascade of events described above highlight the likely progression of cardiac morbidity following CO poisoning. Furthermore, the above events may be potentiated further by CO from haem oxygenase (HO-1) induced by cytokines (Guo *et al.*, 2001), thereby producing a downward cycle of events for the heart.

The production of ROS is essential for activation of p38 mitogen-activated protein kinase (p38-MAPK) in rat hepatocytes (Herrera *et al.*, 2001) and could suggest this occurs in heart tissue following CO-induced oxidative stress. The failing heart has a higher activity of p38-MAPK compared to normal hearts (Cook *et al.*, 1999). This could suggest that protein kinases may have a role in heart failure following CO poisoning. p38-MAPK is shown to decrease the expression of low-density lipoprotein (LDL) receptors and consequently increase the plasma levels of LDL (Mehta & Miller, 1999). Oxidising myoglobin (*8.1.1.3*) from ruptured myocytes could oxidise this LDL (oxLDL) to a cytotoxic species (Hogg *et al.*, 1994; Morel *et al.*, 1983) that may lead to the initiation and/or progression of heart disease (Ramirez *et al.*, 2003; Reeder & Wilson, 2001) associated with CO poisoning. Oxidised LDL plays a major role in the formation of atherosclerotic plaques (Miller *et al.*, 1996; Sharman *et al.*, 2002). The increase in levels of oxLDL produces autoantibodies that is a risk factor for the initiation or progression of cardiovascular disease (Van Tits *et al.*, 2001).

8.1.1.6 Other contributing factors

Any factors involved in changing the state(s) involved in precipitating oxidative stress are briefly described here. Differences in **age** could have a role in patient outcome following CO poisoning as aged rats are more susceptible to apoptosis within the myocardium following I/R compared to young hearts (Liu *et al.*, 2002). This may be explained from the reduced antioxidant capacity of the myocardium that is found to occur with age (Abete *et al.*, 1999; Boucher *et al.*, 1998) and render the myocardium more prone to apoptotic death after oxidative stress. **Sex** may play a role as females have a higher antioxidant capacity in tissues and mitochondria (Borrás *et al.*, 2003), however, no studies have been found (to date) examining the outcome of female versus male victims of CO exposure. Male mice have a higher turnover of glutathione (Ma *et al.*, 1998) that may contribute to iron- and myoglobin- catalysed free radical reactions (*8.1.1.3*). **Diet** may alter the cellular antioxidant capacity that could influence patient outcome after CO poisoning. A diet high in haem iron is associated with an increased risk of MI (Klipstein-Grobusch *et al.*, 1999) presumably from its prooxidant properties that may be potentiated during CO poisoning. Diets high in polyunsaturated fatty acids render heart tissue more susceptible to oxidative damage (O'Farrell & Jackson, 1997). Cholesterol-rich diet can increase the activities of glutathione peroxidase (GPX) and catalase (CAT) in rat liver, whereas significant decreases in these enzymatic antioxidants are found in rabbit livers (Mahfouz & Kummerow, 2000) indicating possible outcomes of CO poisoning due to differences in **animal species**.

Circannual changes in heart antioxidants have been found (Belló-Klein *et al.*, 2000). Rat hearts have maximal activities of GPX and CAT in the summer season. Whether this occurs in humans remains to be determined, however, it may associate with the increased number of cases in accidental CO poisoning found over winter (attributed to faulty heating boilers). **Lifestyle** factors such as the consumption of alcohol (Husain & Somani, 1997)

and smoking cigarettes (Baskaran *et al.*, 1999) could exacerbate CO poisoning due to their prooxidant effects. The vapour phase of cigarette smoke contains CO (**Chapter 1**). The lower dietary-intake of antioxidants by smokers (Waldren *et al.*, 2001) could account for the higher oxidative stress found in smokers that may be potentiated after CO poisoning.

Other health disorders could potentiate CO poisoning and contribute significantly in myocardial failure. Diabetic patients have increased oxidative stress (Fitzl *et al.*, 2001; Stefek *et al.*, 2000) that could contribute to CO-induced oxidative stress. Post-chemotherapy treatment involving the administration of adriamycin may potentiate the cardiotoxicity of CO as this drug is cardiotoxic (Kumar *et al.*, 1999; Luo *et al.*, 1997). A history of **antibiotic(s)** may influence patient outcome following CO poisoning as some findings indicate that some bacteria may cause heart disease (Bradley, 2002; Nicholson, 1999). Finally, genomic screening has discovered several **polymorphisms** that may influence patient outcome according to their **race**. A polymorphism for the gene encoding the subunits of glutamate-cysteine ligase limits the biosynthesis of GSH in individuals of African descent (Willis *et al.*, 2003). This suggests that race could account for some of the differences in patient outcome after CO poisoning and may rule out some socio-economic factors.

8.1.2 Therapeutic Intervention

Myocardial ischaemic **preconditioning** is thought to adapt the heart to become resistant to any subsequent ischaemic insult (Nakano *et al.*, 2000). Although CO-preconditioning has not been evaluated or developed, this type of vaccine method may be beneficial to those at high risk such as fire-fighters. p38 mitogen-activated protein kinases (p38-MAPK) may be involved in this preconditioning mechanism (below), therefore, a fine balance would have to be determined to prevent any toxicity (*8.1.1.5*). Other therapeutic intervention for victims of severe CO poisoning could include minimising some (or all) of

the likely pathways involved in myocardial morbidity following CO exposure (described in 8.1.1).

The likely involvement of intercellular adhesion molecule-1 (ICAM-1) following CO poisoning (*8.1.1.5*) may minimise or prevent CO-induced myocardial failure. Expression of ICAM-1 could be inhibited by an inhibitor of the complement pathway (Buerke *et al.*, 1998) prior to reoxygenation (either normobaric or hyperbaric). **Hyperbaric oxygen** also inhibits the expression of ICAM-1 (induced by hypoxia) in endothelial cells through a mechanism involving nitric oxide (Buras *et al.*, 2000). Hyperbaric oxygen could also increase the antioxidant capacity by inducing SOD (Evelson & González-Flecha, 2000), however, its prooxidant properties (**Chapter 1**) may have to be evaluated for use during the treatment of CO poisoning. P-selectin (or granule membrane protein-140) is involved in recruiting neutrophils to endothelial cells (Weyrich *et al.*, 1993). Similarly as ICAM-1, the inhibition of P-selectin expression prior to myocardial reperfusion by HBO also protects the heart (Thom, 1993).

Carbon monoxide enhances the migration of human neutrophils (Andersson *et al.*, 2002; VanUffelen *et al.*, 1996) and may directly activate neutrophils and target them to the heart due to the sink conditions of the myocardium through its abundance of myoglobin (*8.1.1.3*). Such an indirect mechanism is plausible and remains to be determined. If proven, **CO scavenging drugs** may be a potential development to minimise CO-associated cardiac morbidities. Other inhibitors of neutrophil recruitment are also potential targets for therapeutic intervention (Entman *et al.*, 1991). This could include **anti-TNF-α** (Ferrari, 1999; *8.1.1.5*) and/or **anti-ICAM-1 treatment** (Ma *et al.*, 1992) following CO poisoning particularly prior to any (post-CO) HBO treatment. Furthermore, neutrophil recruitment via the activation of cytokines and expression of adhesion molecules precedes by activation of p38-MAPK (Gao *et al.*, 2002). The **inhibition of p38-MAPK** may also be a potential therapy after CO poisoning, although, the extent of its inhibition could otherwise be pro-apoptotic as it found to mediate the anti-apoptotic properties of CO (Brouard *et al.*, 2000).

Myocyte death is responsible for many cardiac pathologies, therefore, limiting the death pathways of **mitochondria** may offer therapeutic strategies in cardiology. In the isolated perfused heart model where myocardial reoxygenation injury (after CO exposure) may be produced by mechanisms other than neutrophils, the inhibition of p38-MAPK may attenuate myocardial injury via apoptosis (Mehta & Miller, 1999; and *8.1.1.4*). The possible role of mitochondria in myocardial failure (Oddis & Finkel, 1995) after CO poisoning also warrants further investigation. Decreasing the degree of mitochondrial damage may reduce the amount of myocardial injury (Sakamoto *et al.*, 1998). This could be achieved by administering antioxidant drugs that specifically target the mitochondria. Dietary changes including a higher intake of antioxidants in various combinations (Haramaki *et al.*, 1998) may alleviate the rate of myocardial failure after CO poisoning. The use of antioxidants with oxygen therapy needs cautious development in CO poisoning. **Hypothermia** may reduce the production of ROS/oxidative species after CO poisoning (Kudo *et al.*, 2001).

8.2 Conclusions

The work has shown many effects of carbon monoxide (CO) using the *in vitro* isolated perfused rat heart model. The physiological effects of CO (in **Chapter 4**) suggested that CO has direct effects in the heart that may arise not due to hypoxia (**Chapter 5**). The degree of CO-induced oxidative stress in the myocardium may be exacerbated *in vivo* due to the hypoxic conditions that can arise during severe CO poisoning. The discussion (**8.1**) has raised the paradoxical effect of CO (Omaye, 2002; Otterbein, 2002) and suggests that further work at the molecular level is required.

Appendices

Appendix A - Determination of Glutathione (GSH) & Its Disulphide (GSSG)

The glutathione pool is measured with an enzymatic recycling assay using glutathione reductase (GR) modified from the method of Tietze (1969). This assay sequentially reduces glutathione by GR using NADPH. The resulting reduced GSH is oxidized by 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB; Ellman's reagent). The rate of 2-nitro-5-thiobenzoic acid formation is monitored at 412nm. The principle of the assay has been summarized in Figure A.1.

To determine the redox state of the tissue the reduced glutathione (GSH) and oxidized glutathione (GSSG) levels must be quantified. GSH was derivatized with 2-vinylpyridine (2-VP) prior to assaying, so that GSH would not be oxidized by DTNB and interfere with the determination of GSSG, i.e. contribute to the GSH derived from the GSSG pool via GR. If derivatisation is *not* performed (using 2-VP), the assay will yield the *total pool* of glutathione, i.e. GSH plus GSSG.

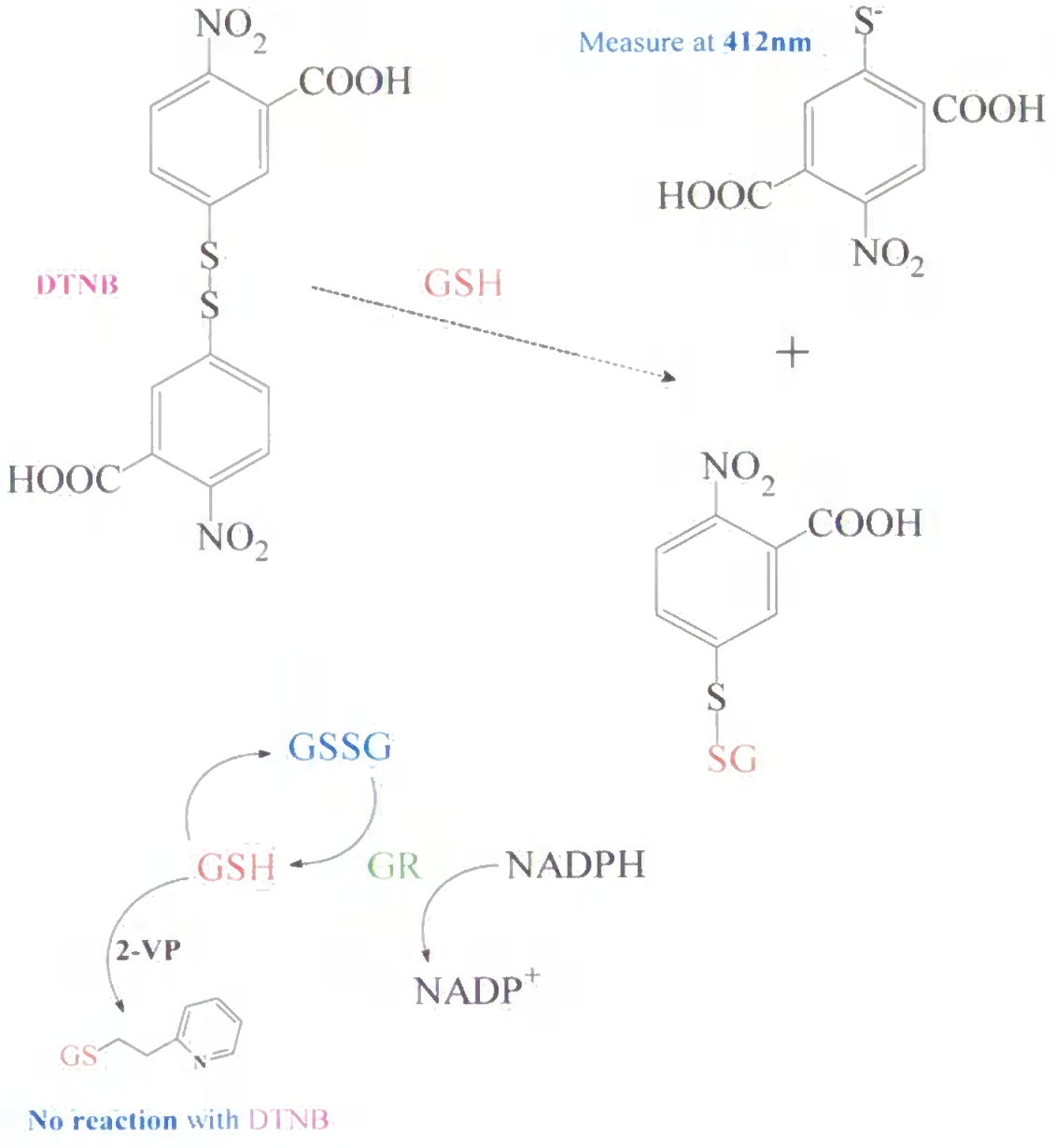

Figure A.1 Schematic of the glutathione assay used to determine the reduced and oxidized glutathione, GSH and GSSG, respectively. 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) oxidizes GSH producing the glutathione conjugate and the *p*-nitrothiobenzoate anion is measured at 412nm. GSSG is reduced by glutathione reductase (GR) and the resulting GSH is determined with DTNB. Prior to GSSG determination, the reduced GSH is derivatised with 2-vinylpyridine (2-VP) to prevent it being oxidized by DTNB, thereby allowing quantification of both GSH and GSSG.

Our assay were modified to accurately determine the levels of GSH and GSSG. Firstly, the tissue homogenates were deproteinised using perchloric acid ($HClO_4$) to limit the degree of oxidation of GSH (Bernt & Bergmeyer, 1974). Other workers have also used sulphosalicyclic acid to remove protein (Baker *et al.*, 1990; Griffith, 1980; Hoshida *et al.* 1996), however, we used perchloric acid as it would be compatible with the determination of other analytes, e.g. pyruvate. The derivatisation step was performed to block the GSH and prevent it from contributing to the GSH derived from the reduction of GSSG (via GR). Various reagents can be used for derivatisation (Abete *et al.*, 1999; Cavins & Friedman, 1970; Ellman, 1958; Katritzky *et al.*, 1984; Winters *et al.*; 1995; Wu *et al.*, 1971). 2-vinylpyridine (2-VP) were chosen to derivatize GSH, as it does not inhibit GR like *N*-ethylmaleimide, therefore we eliminate the clean-up step (Winters *et al.*; 1995). Although excess use of 2-VP can underestimate the GSSG content as it would block the oxidation of GSH (derived from the GSSG pool) by DTNB. Derivatisation of GSH with 2-VP requires neutral pH, therefore, the deproteinised extract was neutralized with KOH (in Tris buffer) as performed by Humphrey *et al.* (1987). The titration data is not included here. Note, that using triethanolamine (to neutralize the pH) is shown to interfere with the glutathione assay (Griffith, 1980). The *p*-nitrothiobenzoate anion which results (after the oxidation of DTNB) is highly coloured and measured at 412nm (ε_m= 13 600 at 412nm) (Ellman, 1959).

Alternative methods for determining glutathione include the use of reverse-phase HPLC (Jue & Hale, 1993; Morrison *et al.*, 1985). These assays can also be performed in microplates and sensitivity is increased further by using fluorescent plate readers. DTNB can be replaced with *o*-phthalaldehyde when using the fluorimetric assay (personal communication with Dr. Matthew Whiteman, Nanyang University, Singapore).

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Publications

Patel *et al.* (2000)

Patel, A. P.; Moody, A. J.; Handy, R. D. & Sneyd, J. R. (2000) Myoglobin As A Marker Of Myocardial Damage. *Toxicol. Lett.* **116** (Suppl. 1), 78-79.

We have investigated myoglobin (Mb) as a marker of cardiac damage due to its abundance in heart tissue. Oxidative stress was induced by perfusing isolated rat hearts with H2O2. Tissue viability was determined by measuring LDH activity in the perfusate and was maximal at 30 minutes in all H2O2-treated hearts relative to the controls. Treated hearts released Mb into the perfusate, whereas no release was detected in control hearts. Covalently bound haem to protein (globin) results in modified Mb (Mb-H) which shows increased peroxidatic activity. Findings showed no difference in peroxidatic activity in myoglobin from treated heart ventricles relative to controls. Mb released into the perfusate also showed no peroxidase activity.

Treated hearts showed an increase in perfusate free iron levels with respect to the controls. The release of iron implies further oxidative stress by the mediation of Fenton and Haber-Weiss generation of hydroxyl radicals. The role of various antioxidants: NAC, DMTU, and ascorbate were investigated by assessing the degradation of Mb. Our results showed no antioxidant activity with NAC or DMTU. Ascorbate showed minor antioxidant activity during the incubation, however, the haem was fully degraded by the end of the study.

These findings suggest that blood/plasma Mb can be used as a marker to show cardiac damage. Absence of Mb-H peroxidase activity suggests that Mb is not a suitable biomarker of oxidative stress. The work also highlights the problems in choosing suitable antioxidant regimes to minimise damage caused by trauma such as in hyperbaric oxygen treatment or surgery.

Patel *et al.* (2001)

Patel, A. P.; Moody, A. J.; Handy, R. D. & Sneyd, J. R. (2001) Carbon monoxide toxicity in the heart: evidence for ischaemia/reperfusion like injury. *Biochem. Soc. Trans.* **30** (1), A34-A34.

We hypothesise that carbon monoxide (CO) induced cardiac dysfunction is associated with ROS production in an ischaemia-reperfusion (I/R)-like injury. Isolated rat hearts were used to investigate the effects of CO on cardiac function.Hearts were exposed to CO (30min) under normoxic conditions followed by a 90min CO-free period. In hearts treated with 0.01 and 0.05% CO heart rate (HR) decreased to ca. 85±10% (mean±SE) compared to controls (0% CO). The 0.01% CO group showed signs of contractile recovery during the post-CO period, whereas the 0.05% CO group showed no recovery. Coronary flow (CF) decreased signifi-cantly ($P<0.05$) during the post-CO period, to 93±0.7% and 87±3% of controls with 0.01 and 0.05% CO, respectively. Activities of LDH, CK and troponin I were elevated in perfusate from treated hearts. To investigate the likely role of ROS hearts were perfused in the presence of two antioxidants (TroloxC and ascorbate). These eliminated the decrease in CF following exposure to 0.05% CO (30min) relative to controls. Whereas the decline in HR was augmented with antioxidant treatment to 57±14%. These findings suggest some oxidative role in regulating contractile function that cannot be attributed solely to cytochrome c oxidase inhibition since normoxic conditions were used. ROS may be produced during the (hypoxic) CO period and are responsible for a decline in coronary function following CO exposure.

Patel *et al.* (2003)

Patel, A. P.; Moody, A. J.; Handy, R. D. & Sneyd, J. R. (2003) Carbon monoxide exposure in rat heart: glutathione depletion is prevented by antioxidants. *Biochem. Biophys. Res. Comm.* **302** (2), 392-396.

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Biochemical and Biophysical Research Communications 302 (2003) 392–396

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Carbon monoxide exposure in rat heart: glutathione depletion is prevented by antioxidants

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Received 13 January 2003

Abstract

Rat hearts were perfused for 15 min with buffer equilibrated with 0.01% or 0.05% CO. The buffer was equilibrated with 21% O_2 throughout. The ventricular glutathione content decreased by 76% and 84%, 90 min post-exposure to 0.01% and 0.05% CO, respectively, compared with 0% CO controls (0.45 ± 0.01 μmol/g wet tissue; ±SEM, $n = 3$). Both reduced and oxidised glutathione contributed to this decline. When ascorbate and Trolox C were included during exposure to 0.05% CO the glutathione pool was partly protected; here the glutathione decrease was 46%. In most hearts additional creatine kinase activity in the perfusate indicated minor tissue injury occurring immediately after the start and/or about 10 min after the end of exposure to 0.01% CO or 0.05% CO. Ventricle lactate levels were unaffected by exposure to 0.01% CO. This evidence supports a role for oxidative stress in CO cardiotoxicity.

Keywords: Carbon monoxide; Oxidative stress; Rat heart; Glutathione; Antioxidants

Since cardiac and nervous tissues are highly oxygen dependent they are vulnerable to CO toxicity via CO-induced tissue hypoxia [1]. CO can induce tissue hypoxia by competitively reducing the oxygen carrying capacity of haemoglobin (Hb) and by attenuating the dissociation of oxygen bound to Hb at low partial pressure. However, as discussed in detail elsewhere, this does not explain all of the pathophysiology of CO toxicity [1].

Oxidative stress has been implicated in the cellular mechanisms of CO toxicity in brain tissue reviewed in [1]. Thom [2] found evidence for oxidative stress post-CO exposure in the form of enhanced lipid peroxidation, while Zhang and Piantadosi [3] found a decrease in the mitochondrial reduced glutathione (GSH) to oxidised glutathione (GSSG) ratio. These results are consistent with the idea that exposure of brain to CO leads to tissue hypoxia. Hence, on removal of the CO exposure, the situation is analogous to that seen in reperfusion after ischaemia, where the elevated reactive oxygen species (ROS) production, at least partly of mitochondrial origin, leads to tissue damage [4].

While a role for oxidative stress in CO toxicity in the brain seems certain there is a lack of equivalent evidence for this in the heart. It is already established that glutathione levels decline during reperfusion of isolated rat hearts following ischaemia [5]. Hence, in this study we have examined the effect of CO on the ventricular glutathione in perfused isolated rat hearts. CO caused a depletion of both reduced and oxidised glutathione, which could partly be protected against by including antioxidants in the perfusion buffer, thereby providing clear evidence of an involvement of oxidative stress in CO cardiotoxicity. However, the concentrations of CO used appear to have been insufficient to have caused tissue hypoxia during the exposure, raising the question of the source of the oxidative stress.

Materials and methods

Adult male Sprague–Dawley rats (262 ± 30 g body weight; means ± SD, $n = 35$) were used. Animals were maintained with free

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doi:10.1016/S0006-291X(03)00190-6

access to food and water before collection of the heart; experiments were conducted in accordance with ethical approval. Certified CO gas mixtures (0–0.05% CO in 21% oxygen, 5% carbon dioxide and balanced with nitrogen) were supplied by BOC (Guildford, UK). Trolox C, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid, came from Fluka (Poole, UK). All other reagents were obtained from Sigma (Poole, UK).

Hearts (1.48 ± 0.15 g; means ± SD, $n = 35$) were rapidly excised following anaesthesia (with intraperitonial sodium pentobarbitone) and perfused (at 36.9 °C) in a non-recirculating Langendorff apparatus at 6 ml/min/g wet weight of heart as described previously [6]. Perfusion buffer (118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.8 mM $CaCl_2$, 20 mM $NaHCO_3$, and 11 mM glucose) was constantly gassed with 21% oxygen, 5% carbon dioxide (balanced with nitrogen) to pH 7.4.

Hearts were perfused for 15 min to wash out contaminating blood. CO exposure was then started by perfusion with buffer that was saturated with 0%, 0.01%, or 0.05% CO (0, 100, or 500 ppm, respectively), for 30 min, followed by a 90 min CO-free period. Note that the buffer was saturated with 21% oxygen throughout. In range-finding experiments (unpublished) CO concentrations of 0.2% led to heart failure. The heart ventricles were then blotted and weighed before snap freezing for biochemical analysis (see below). In some perfusions hearts were collected immediately after the 30 min of perfusion with CO. In others the antioxidants Trolox C and sodium ascorbate were included in the perfusion buffer (at 0.2 and 1 mM, respectively) during the initial equilibration period and during the exposure to CO, but not during the 90 min of perfusion after the CO exposure. In these a stock solution of ascorbate was prepared and added to the perfusion buffer immediately before the start of the perfusion. Note that the presence of ascorbate required greater buffering capacity in the perfusion buffer, so this was supplemented with 10 mM Hepes.

In all experiments, in order to assess tissue injury, the activities of creatine kinase and lactate dehydrogenase in the perfusate were determined using commercial kits (Sigma kits 47 and 228, respectively).

In all experiments, for the determination of glutathione, lactate, and protein, the frozen rat heart ventricles (stored at −80 °C) were homogenised as described previously; however, tetrahydrobiopterin was omitted from the homogenisation buffer [7]. The 10% (w/v) homogenates (1 g of wet weight tissue per 10 ml homogenisation buffer) were deproteinised immediately on ice (15 min) by adding 8% (w/v) perchloric acid (2 volumes) to the homogenate (1 volume), with occasional gentle inversion, followed by centrifugation (10 min at 2000*g*). The perchloric acid extract (supernatant) was snap-frozen for later analysis of glutathione content using the method of Griffith [8]. The glutathione analysis of each heart extract was carried out in triplicate. The lactate contents of the homogenates were determined using a commercial kit (Sigma kit 826).

Unless otherwise stated Student's *t* test was used for statistical comparison of means.

Results

Fig. 1A shows the effects of CO exposure on the glutathione content of rat heart ventricles. There were significant decreases in the total glutathione pool of 76% ($P < 1 \times 10^{-11}$) and 84% ($P < 1 \times 10^{-7}$) for hearts treated with 0.01% and 0.05% CO, respectively, compared with controls perfused in the same manner but without exposure to CO. Both the reduced (GSH) and the oxidised (GSSG) forms contributed to the decline in total glutathione.

To investigate the possible role of reactive oxygen species (ROS) in the CO-induced depletion of glutathione, we performed perfusions with 0.05% CO in the presence of a combination of two antioxidants (Trolox C and ascorbate). Fig. 1B shows that at the end of the perfusions both the control groups (with or without antioxidants) had similar levels of GSH. When antioxidants were present there was a lower level of GSSG (decrease of 31% ± 15%, 95% confidence interval); a

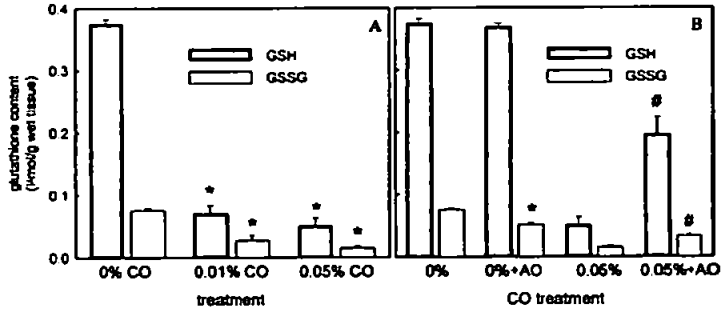

Fig. 1. Depletion of the glutathione content of rat heart ventricle tissue induced by carbon monoxide (CO) in the presence of oxygen and the protective effect of antioxidants. (A) Isolated rat hearts were perfused with buffer equilibrated with 0%, 0.01%, or 0.05% CO (as indicated) for 30 min followed by a 90 min CO-free period; buffer was saturated with 21% oxygen throughout. Reduced glutathione (GSH) and oxidised glutathione disulfide (GSSG) contents of the ventricle tissue were determined as described in the Materials and methods. The data shown are means ± SEM, $n = 3$ (0% CO), $n = 10$ (0.01% CO) and $n = 5$ (0.05% CO). Asterisks indicate significant differences at the 95% confidence level ($P < 0.05$) relative to the 0% CO control. The hatched symbol (#) indicates a significant difference at the 90% confidence level ($P < 0.1$) relative to the 0.01% CO treated hearts. (B) Isolated rat hearts were exposed to 0% or 0.05% CO, with or without antioxidants (AO) as indicated. The antioxidants (0.2 mM Trolox C and 1.0 mM ascorbate) were administered before and during the CO exposure, followed by a 90 min CO-free period with no antioxidants present. Oxygen was present at 21% throughout. The data shown are means ± SEM ($n = 3$, 0% CO; $n = 10$, 0.01% CO; $n = 5$, 0.05% CO; $n = 3$, 0% CO + AO; and $n = 5$, 0.05% CO + AO). The asterisk indicates a significant difference at the 95% confidence level ($P < 0.05$) relative to the 0% CO control. The hatched symbols (#) indicate significant differences at the 95% confidence level ($P < 0.05$) relative to the 0.05% CO-treated hearts.

slightly lowered total glutathione pool (decrease of 6.7% ± 6.0%, 95% confidence interval); and an increased GSH:GSSG ratio (median value of 7.1 with antioxidants compared to 4.9 without; $P = 0.08$, comparison of medians using Mann–Whitney W test). Fig. 1B shows that the combination of antioxidants used partially protected the glutathione pool from the depletion as a result of CO exposure. The decrease was only 46% with the antioxidants present compared to 84% without (relative to the appropriate control in each case).

The CO-induced glutathione depletion that we have observed is not explained by non-specific leak of intracellular material from damaged ventricle into the perfusate. In all the perfusions described here we also monitored the activity of creatine kinase (CK) and lactate dehydrogenase (LDH) in the perfusate as indicators of tissue injury. Fig. 2 shows the cumulative CK activity in the perfusate for control hearts and for those exposed to 0.01% CO. Note that the level of CK in the perfusate was also monitored in the 15 min equilibration period (see Materials and methods) before the start of the exposure ($t = 0$). This starting level was small, but varied (cumulative CK activity, $t = 0$, was 3.60 ± 1.65 IU/g wet tissue, means ± SD, $n = 13$), so the time courses of CK accumulation in perfusate are expressed as the percentage increase above the cumulative CK activity at time zero. The mean cumulative CK activities at $t = 120$ min were 18.2 ± 2.2 IU/g wet tissue (±SEM, $n = 3$) for

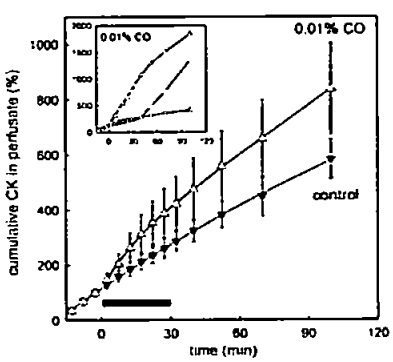

Fig. 2. Time courses of creatine kinase (CK) accumulation in the perfusate from rat hearts exposed to CO in the presence of oxygen. The isolated rat hearts exposed to 0 (control) or 0.01% CO for 30 min ($n = 3$ and 10, respectively) followed by a 90 min CO-free period. The black bar denotes the period of CO exposure. The CK activity in perfusate fractions was measured as described in Materials and methods. The data shown are means (± SEM) of cumulative CK activities expressed as percentages relative to the CK activity accumulated in the perfusate in the 15 min equilibration period before the CO exposure (see Materials and methods). Note that the times indicated represent the mid-point of the period over which the perfusate fraction was collected. The *inset* shows the time courses for three individual hearts exposed to 0.01% CO.

control hearts and 31.4 ± 6.3 IU/g wet tissue (±SEM, $n = 10$) for hearts exposed to CO. These values are not significantly different ($P = 0.29$) and there is no correlation between cumulative CK activity in the perfusate and ventricular glutathione content.

For individual control hearts there was a uniform exponential decrease in CK activity in the perfusate with time; hence, the mean cumulative CK data shown in Fig. 2 for the control hearts can be fitted well with a single exponential rise to a maximum. However, this is not the case for the mean cumulative CK data for the hearts exposed to 0.01% CO. Moreover, the mean data obscure a variety of responses that individual hearts showed to the CO perfusion. This is illustrated in the inset in Fig. 2. Individual hearts showed (a) no CO-induced tissue injury (3 out of 10), (b) CO-induced injury, i.e., an increase in the rate of accumulation of CK, at the beginning of the exposure to CO (2 out of 10), (c) injury shortly after the end of the exposure to CO (3 out of 10), or (d) injury both at the beginning and shortly after the end of the exposure to CO (2 out of 10). In all cases the cumulative LDH activity in the perfusate showed the same pattern of behaviour as that shown by cumulative CK activity (data not shown).

Since there was evidence for some tissue injury at the beginning and shortly after the end of perfusions with CO, it was of interest to find out when the CO-induced depletion of glutathione occurred in relation to this. Further information was gained by measuring the glutathione levels in ventricles from hearts where the perfusion was stopped immediately after the equilibration period, before exposure (i.e., at $t = 0$) or immediately after the CO exposure (i.e., at $t = 30$ min). The results from these are summarised in Table 1. The CO-induced depletion seems to have occurred post-exposure. In the controls there was a significant decrease (26%; $P = 0.004$) in the total glutathione content of the ventricles over 120 min of perfusion; most of this seems to have occurred within the first 30 min. This was mirrored by a 3.9-fold increase ($P = 0.0004$) in GSSG content. Although there was also depletion of total glutathione during the 30 min exposure to 0.01% CO, this was no greater than that seen in the control.

Table 1 also shows a comparison of the lactate content of ventricle tissue from hearts exposed to 0% and 0.01% CO; there is no significant difference in the lactate content either immediately post-exposure or 90 min post-exposure, implying at the lack of CO-induced tissue hypoxia.

Discussion

In this study the perfused isolated rat heart model was used to evaluate whether oxidative stress during or after CO exposure may contribute to the cardiotoxicity

Table 1
Glutathione and lactate contents of rat heart ventricles following exposure to 0.01% CO

	Control (%)	$t = 0$ i.e., pre-exposure	$t = 30$ min i.e., immediately post-exposure	$t = 120$ min i.e., 90 min post-exposure
GSH + GSSG (μmol/g wet tissue)	0.01 CO	0.61 ± 0.07 ($n = 3$)	0.46 ± 0.10# ($n = 3$) 0.44 ± 0.07# ($n = 3$)	0.45 ± 0.01*** ($n = 3$) 0.10 ± 0.02*** ($n = 10$)
GSH (μmol/g wet tissue)	0.01 CO	0.59 ± 0.07 ($n = 3$)	0.41 ± 0.08# ($n = 3$) 0.43 ± 0.07# ($n = 3$)	0.38 ± 0.01** ($n = 3$) 0.07 ± 0.01*** ($n = 10$)
GSSG (μmol/g wet tissue)	0.01 CO	0.020 ± 0.004 ($n = 3$)	0.053 ± 0.019# ($n = 3$) 0.007 ± 0.003* ($n = 3$)	0.075 ± 0.003** ($n = 3$) 0.027 ± 0.008 ($n = 10$)
Lactate (μmol/g wet tissue)	0.01 CO	43.1 ± 3.2 ($n = 3$)	38.8 ± 1.1 ($n = 3$) 39.3 ± 1.6 ($n = 3$)	18.8 ± 1.0 ($n = 3$) 17.7 ± 2.0 ($n = 10$)

Note. Isolated rat hearts were exposed to 0% or 0.01% CO for 30 min followed by a 90 min CO-free period, with 21% oxygen present throughout. In some cases perfusion was stopped immediately after the initial equilibration period before exposure to CO ($t = 0$) and in others it was stopped immediately after the CO exposure ($t = 30$ min, immediately post-exposure). The data shown are means ± SEM. The hatched symbols (#), single asterisks (*), double asterisks (**), and triple asterisks (***) indicate significant differences at the 80%, 90%, 95%, and 99% confidence levels between values and the appropriate value at $t = 0$.

of CO. To do this the ventricle glutathione pool, a major component in cardiac antioxidant defence [5], was monitored. Our key finding is that CO induces a dose-dependent depletion of both oxidised (GSSG) and reduced (GSH) glutathione (Fig. 1A), most of which appears to occur post-exposure (Table 1). The decline in glutathione levels suggests that oxidative stress occurred as a result of the CO exposure. Oxidative stress arises from excess production of oxidants (such as reactive oxygen species: ROS), or a compromise in antioxidant defences, or both [4].

To determine if ROS were responsible for the depleted glutathione levels following CO exposure, heart perfusions were conducted in the presence of two water-soluble antioxidants. The use of the vitamin E analog Trolox C and ascorbate is well-established [4]. These antioxidants preserved glutathione levels in hearts exposed to 0.05% CO (Fig. 1B). Although the glutathione pool was not entirely protected this finding strongly suggests that additional ROS were produced as a result of CO exposure. The evidence (Table 1) suggests that most, if not all, of the decline in glutathione levels occurred after the CO exposure. Hence, the level of protection of the glutathione pool afforded by the antioxidants could perhaps have been improved if they had been present in the perfusion buffer throughout, rather than before and during the CO exposure.

Exposure to 0.01% CO did not result in a statistically significant increase in mean cumulative creatine kinase (Fig. 2) or lactate dehydrogenase (not shown) activities in the perfusate at any point in the perfusions, although with most individual hearts some tissue injury was seen immediately after the start and/or shortly after the end of the perfusion with 0.01% CO. Hence, the loss of glutathione appears to arise from its function either as a single antioxidant or as part of the network of intracellular antioxidants [5] rather than as the result of non-specific leakage via widespread tissue damage. It is likely, therefore, that the glutathione depletion occurs by oxidation of GSH followed by loss of GSSG into the perfusate [9].

The depletion of glutathione after the CO exposure is reminiscent of the loss of intracellular antioxidants during reperfusion after ischaemia [5], where a key event is thought to be excess ROS production by mitochondria during the reperfusion [10]. The binding of carbon monoxide to myoglobin (Mb), which is abundant in the myocardium, can limit the supply of O_2 for use by the electron transfer chain (ETC) and hence cause hypoxia [1]. However, at the concentrations of CO and O_2 used here levels of carboxymyoglobin (MbCO) should be negligible. Using NMR signals Glabe et al. [11] found an intracellular partition coefficient between CO and O_2 of 36. On this basis $MbCO:MbO_2$ ratios (equal to $PCO/PO_2 \times 36$) of 0.017 and 0.086 can be calculated at the concentrations of CO used here (0.01% and 0.05%, respectively). Glabe et al. found only evidence, in the form of an increase in lactate production, of inhibition of the mitochondrial ETC when $MbCO:MbO_2$ ratios were in excess of about 1.5. Consistent with this, we found no evidence for elevated lactate levels in ventricle tissue immediately after perfusion with 0.01% CO (Table 1).

Zhang and Piantadosi [3] have observed oxidative stress associated with mitochondria after exposure of rat brain to CO. However, they used a much higher concentration of CO (1%) than that used here, sufficient, on the basis of their previous work, to cause inhibition of cytochrome-*c* oxidase. Hence, their experimental conditions are somewhat analogous to those found during ischaemia/reperfusion, so the decrease in the mitochondrial GSSG:GSH ratio that they observed after CO exposure is perhaps not surprising. However, this raises the question of why in our work there is evidence for a substantial decline in total glutathione as a result of CO exposure, when there is apparently no CO-induced hypoxia during the exposure.

An interesting observation made by Zhang and Piantadosi [3] is that there were two episodes of CO-

induced oxidative stress as indicated by decreases in the mitochondrial GSH:GSSG ratio, one seen just after the CO exposure and another, more severe episode, 120 min after the end of the CO exposure. Somewhat similar behaviour was seen in our experiments: when present, tissue injury was seen just after the start of the CO exposure and/or lagging after the end of the CO exposure (Fig. 2, inset). The lag of at least 10 min after the end of CO exposure, before the appearance of tissue injury, is consistent with the slow in vivo dissociation of CO from the myocardium; a rate constant of 1.16×10^{-4} s^{-1} has been reported for this dissociation [11]. This suggests that the CO:O_2 ratio is an important factor here, i.e., that the CO-induced ROS production that leads to depletion of the glutathione pool (and sometimes to tissue injury) may occur only at low CO:O_2 ratios, seen transiently just after the start of the perfusion, as the CO in the perfusion buffer equilibrates with the heart (1–1.5 min), and again after the end of the perfusion as the CO dissociates.

The cellular component(s) with which the CO is interacting to induce the ROS production is unknown. One possibility is that cytochrome-*c* oxidase is involved. Significant inhibition of cytochrome-*c* oxidase by CO in our experiments can be ruled out because the CO:O_2 ratios are too low [1]. However, cytochrome-*c* oxidase can also catalyse the oxidation of CO by oxygen to CO_2, i.e., it has CO oxygenase activity [12,13], and this activity may involve the release of hydrogen peroxide from the enzyme to complete the catalytic cycle [13,14].

Another candidate for the site of CO-induced ROS production is xanthine oxidase. Thom [15] has already established that the conversion of xanthine dehydrogenase to xanthine oxidase, and the consequent production of ROS via xanthine oxidase activity, can account for the lipid peroxidation seen after exposure of rat brain to CO [2]. This is consistent with the idea that CO toxicity "is a type of post-ischaemic reperfusion phenomenon" [15], since a role for xanthine dehydrogenase to oxidase conversion has been implicated in reperfusion (reoxygenation) injury after ischaemia [4]. However, in our experiments there is no CO-induced hypoxia, so there appears to be no chain of events that could lead to the dehydrogenase to oxidase conversion. Nevertheless, given the fact that xanthine dehydrogenase is part of the same family of molybdenum-cofactor-containing enzymes that includes bacterial CO dehydrogenases [16], there still remains the possibility that the CO-induced ROS production comes from a direct interaction between CO, O_2, and xanthine dehydrogenase.

In conclusion, while it is clear that oxidative stress has a role in CO cardiotoxicity it is questionable whether this always arises as a result of reoxygenation following CO-induced tissue hypoxia. The indications here, and elsewhere with rat brain [3], that oxidative injury/stress occurs during exposure to CO as well as post-exposure suggest a more direct mechanism of CO-induced ROS production that remains to be elucidated.

Acknowledgments

The authors thank the following bodies for their financial support: the Diving Diseases Research Centre (DDRC), Plymouth, UK; the British Antarctic Survey (BAS), and the British Journal of Anaesthesia (BJA).

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