STUDY OF LIVER ISCHAEMIA-REPERFUSION INJURY AND ITS MODULATION BY N-ACETYLCYSTEINE

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This thesis is submitted to the University of London in partial fulfilment of the requirements for the degree of Doctor of Philosophy. Except for where indicated, it presents entirely my own work and describes the results of my own research.

This thesis is dedicated to my wife Anna and our children Constantinos, Katerina and Filia for their loving support and understanding, especially during the periods of my absence.

ABSTRACT

Liver ischaemia-reperfusion (I/R) injury occurs in a number of clinical settings, including liver surgery, transplantation and haemorrhagic shock with subsequent fluid resuscitation. It is well recognised as a significant cause of morbidity and mortality and characterised by oxidative stress accompanied with depletion of antioxidants. Its pathophysiology is complex.

This thesis investigates the effect of lobar liver I/R injury on liver microcirculation, hepatic oxygenation, cellular energetics and nitric oxide ($NO^.$) metabolism. It evaluates also the hypothesis that the antioxidant N-acetylcysteine (NAC) ameliorates liver I/R injury.

Initially an experimental rabbit model was established where both phases (early and late) of liver I/R injury could be studied. New Zealand white rabbits underwent 60 min of lobar ischaemia followed by 7 h of reperfusion. It was found that cannulation of the femoral artery, for monitoring of the arterial blood pressure, induced remote liver injury, which could be avoided by use of the ear artery.

Lobar liver I/R caused significant decrease in hepatic microcirculation, liver tissue oxygenation and cellular energetics. It also caused significant alterations in $NO^.$ metabolism during the late phase (increase in plasma nitrites and plasma S-nitrosothiols, decrease in liver tissue nitrotyrosine) and significant oxidative stress.

Intravenous administration of NAC (150 mg/Kg/h over the 15 min before reperfusion and maintenance at 10 mg /Kg/h during the 7 h reperfusion period) had a clear protective effect during the late phase of reperfusion injury in rabbits with normal and steatotic liver. This effect was associated with improved cellular energy metabolism (maintenance of cytochrome oxidase activity, increased acetates and decreased lactates

in bile) and altered NO˙ metabolism (reduced plasma nitrites, S-nitrosothiols and reactive nitrogen species).

In conclusion, this thesis has shown that lobar hepatic I/R induces significant alterations on cellular energetics and NO˙ metabolism. It provides also significant new information about the timing and the possible mechanism of the protective effect of NAC. It could form the basis for the performance of large scale prospective randomised clinical trials where the effect of thiols in clinical settings of liver I/R injury will be investigated.

ACKNOWLEDGEMENTS

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THESIS DESCRIPTION

Chapter 1 is an overview of the clinical significance of hepatic ischaemia-reperfusion (I/R) injury, the pathophysiology of liver I/R and the role of reactive oxygen and nitrogen species in I/R injury.

Chapter 2 reviews the role of antioxidant therapy on liver I/R with special emphasis on the role of thiols.

In chapter 3, materials and methods used in this study are described.

In chapter 4 the establishment and refinement of an experimental rabbit model of lobar liver I/R is described. This model allowed the study of both the early and late phases of liver I/R injury. The cannulation of the ear artery for continuous monitoring of the arterial blood pressure, instead of the femoral artery, was found to avoid remote liver injury.

Chapter 5 describes the application of proton nuclear magnetic resonance (^{1}HNMR) spectroscopy in analyzing the changes in bile composition during liver I/R.

In chapter 6, the effect of N-acetylcysteine (NAC) administration on liver function following hepatic I/R injury in normal liver is investigated and discussed.

In chapter 7 the effect of NAC administration on liver function during hepatic I/R injury in steatotic liver has been studied.

Chapter 8 investigates the possible mechanism of the protective effect of NAC in liver warm I/R injury. It has been demonstrated that NAC administration preserves cellular energetics and modulates nitric oxide metabolism.

Chapter 9 is a general discussion of the thesis and the directed future research.

References and an appendix listing the presentations and publications arising out of the work described appear at the end of the thesis.

LIST OF ABBREVIATIONS

ALT Alanine aminotransferase

ARDS Adult respiratory distress syndrome

AST Aspartate aminotransferase

AP-1 Activator protein-1

ATP Adenosine triphosphate

BW Body weight

c GMP Cyclic guanosine monophosphate

CK Creatine kinase

CO Carbon monoxide

Cyt Ox Cytochrome oxidase

Cu_A Copper-copper dimer

DHLA Dihydrolipoate

DHR Dihydrorhodamine

EC Endothelial cells

EPR Electron paramagnetic resonance

eNOS endothelial NOS

GGA Geranylgeranylacetone

GSH Glutathione

GPX Glutathione peroxidase

GSSG Oxidised glutathione

H & E Haematoxylin and eosin

Hb Deoxyhaemoglobin

HbO_2 Oxyhaemoglobin

HM Hepatic microcirculation

^1H NMR Proton Nuclear Magnetic Resonance

H_2O_2 Hydrogen peroxide

HOCl Hypochlorous acid

HO-1 Haem oxygenase 1

ICG Indocyanine green

iNOS inducible NOS

IL-1 Inteleukin 1

IL-6 Interleukin 6

I/R Ischaemia-reperfusion

ISLAND Infarct Size Limitation: Acute N-acetylcysteine Defense

KC Kupffer cells

LDF Laser Doppler flowmeter

LDH Lactate dehydrogenase

LV Left ventricular

LW Liver weight

MABP Mean arterial blood pressure

NAC N-acetylcysteine

NADPH nicotinamide adenine dinucleotide phosphate

NAFLD Non alcoholic fatty liver disease

NEM N-ethylmalemide

NIRS Near infrared spectroscopy

NT Nitrotyrosine

$NO^{.}$ Nitric oxide

NOS Nitric oxide synthase

NO_2^{-} Nitrite

NO_3^{-} Nitrate

N_2O Nitrus oxide

O_2 Oxygen

$\cdot OH$ Hydroxyl radical

$O_2^{\cdot-}$ Superoxide anion

$ONOO^-$ Peroxynitrite

PC Phosphatidylcholine

PMN Polymorphonuclear

PR Pulse rate

Prx Peroxiredoxin

PVBF Portal vein blood flow

Redox Reduction-oxidation

RH Rhodamine

ROS Reactive oxygen species

RNS Reactive nitrogen species

$RSSR^{\cdot-}$ Disulfide radical anion

$RS\cdot$ Thiyl radical

RSNOs S-nitrosothiols

SaO_2 Oxygen saturation

SD Standard deviation

-SH Sulfydryl

SOD Superoxide dismutase

THBF Total hepatic blood flow

TNF-α Tumour Necrosis Factor alpha

Trx Thioredoxin

T Tyrosine

TSP Trimethysilypropionate

XOR Xanthine oxidoreductase

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CHAPTER 1

INTRODUCTION

1.1 Clinical significance of liver ischaemia - reperfusion (I/R) injury

Liver I/R injury is well recognized as a significant cause of morbidity and mortality in two principal settings. Firstly, it occurs in major liver resections (Liu *et al.* 1996) and transplantation (Caldwell-Kenkel *et al.* 1991; Deschenes *et al.* 1998; Silva *et al.* 2006) where anoxic or ischaemic liver injury takes place. Secondly, it happens as a consequence of systemic hypoxia or with conditions which cause low blood flow to the liver resulting in insufficient perfusion. The latter occurs in haemorrhagic, cardiogenic or septic shock with subsequent fluid resuscitation (Yamakawa *et al.* 2000), in cardiovascular surgery with extracorporeal circulation (Okano *et al.* 2002), in laparoscopic surgery (Glantzounis *et al.* 2001; Glantzounis *et al.* 2005b) and in abdominal compartment syndromes (Rezende-Neto *et al.* 2003).

Severe warm liver I/R can lead to liver or even to multiple organ failure (Helling 2006). The extent of hepatic injury caused by I/R depends primarily on the condition of the liver prior to the ischaemic insults and its duration (Isozaki *et al.* 1995). In liver surgery new techniques have been applied to increase the resectability rate of liver tumours such as down staging chemotherapy and portal vein embolisation. However, these techniques can make the liver more susceptible to ischaemic insults (Pocard *et al.* 2001). Furthermore, the commonest primary liver cancer, hepatocellular carcinoma usually develops on the background of liver cirrhosis, which increases the risk of liver failure during any subsequent surgery (Kaibori *et al.* 2003).

In the field of liver transplantation I/R injury is closely related to the development of primary graft non-function (occurs in < 5 % of grafts) and primary graft dysfunction

(occurs in 10-30 % of grafts) (Clavien *et al.* 1992). Both conditions are associated with high rates of morbidity and mortality. I/R injury also increases the incidence of subsequent graft rejection (Fellstrom *et al.* 1998) and of non-anastomotic biliary strictures of transplanted livers (Lemasters *et al.* 1995) .

Another field where I/R injury affects outcome is hepatic resection or transplantation with steatotic livers. It is reported that 25 % of the western population has some degree of hepatic steatosis (Selzner *et al.* 2001) which is the result of the abnormal accumulation of triacylglycerol within the cytoplasm of hepatocytes, attributed to the effects of alcohol excess, obesity, diabetes or drugs. Furthermore, hepatic steatosis is associated with an impaired microcirculation (Seifalian *et al.* 1998; Ijaz *et al.* 2003), increased postoperative morbidity and mortality and poor graft function (Selzner and Clavien 2001).

It is obvious that liver I/R occurs in many diverse clinical settings and has a major impact on clinical outcome. Reoxygenation of the ischaemic liver causes the generation of numerous reactive oxygen (ROS) and nitrogen species (RNS). Although ROS and RNS in low concentrations play an important role as mediators in normal cellular metabolism and signal transduction (Czaja 2002; Haddad 2002) in higher concentrations they can be damaging. In light of this a complex defence network, the antioxidant system, developed in mammals, to prevent or reduce the injury caused by high concentrations of ROS or RNS. Free radical scavenging or administration of agents that enhance the endogenous antioxidant system could reduce post ischaemic tissue injury and so be useful in clinical settings against hepatic I/R damage.

1.2 Pathophysiology of liver I/R

Ischaemic injury

When oxygen supply to cells becomes insufficient as a direct result of reduced blood flow or hypoxia the mitochondrial respiratory chain function alters and the reduction-oxidation (redox) state of the mitochondrial enzymes becomes reduced. This results in the inhibition of the oxidative phosphorylation process with a subsequent reduction in ATP (Adenosine Triphosphate) synthesis (Gonzalez-Flecha *et al.* 1993). Reduction of cellular ATP causes disturbances in membrane ion translocation by inhibition of the ATP-dependent sodium (Na^+) / potassium (K^+) ATPase, resulting in sodium influx and intracellular sodium accumulation with corresponding cell swelling and death (Blum *et al.* 1991).

Intracellular calcium accumulation is also strongly implicated in the development of ischaemic injury and is thought to be a crucial step in the transition to irreversible damage (Dhar *et al.* 1996). The increased cytosolic calcium level causes activation of cell membrane phospholipases resulting in phospholipids degradation and cell membrane disruption (Farber 1981). Prior to cell death hepatocytes and other cells develop a state characterized by mitochondrial permeability transition (Kim *et al.* 2003), lysosomal disruption, bleb formation and growth, cell swelling and leakage of small molecular mass solutes (Nishimura and Lemasters 2001; Zahrebelski *et al.* 1995). Calcium also activates xanthine oxidoreductase (XOR) which has a role in oxygen free radical production following reperfusion (Ishii *et al.* 1990; Glantzounis *et al.* 2005c). Although the basic mechanisms of ischaemic injury after warm and cold liver ischaemia

share several similarities, there are also significant differences. In liver transplantation the liver undergoes cold ischaemic storage followed by rewarming ischaemia and reperfusion (Selzner *et al.* 2003). Cold ischaemia is associated with reduced oxidative phosphorylation, lower cellular ATP levels and increased glycolysis (Churchill *et al.* 1994), while warm ischaemia leads to greater oxidative stress and mitochondrial dysfunction (Baumann *et al.* 1989; Mochida *et al.* 1994). The main site of injury in cold ischaemia are non-parenchymal cells (Kupffer, sinusoidal endothelial cells (EC), Ito cells and biliary epithelium) whereas in warm ischaemia are hepatocytes (Ikeda *et al.* 1992).

Reperfusion injury

Although ischaemia causes significant injury to tissue and cells; the injury during reperfusion is more severe. A complex network of hepatic and extra hepatic mechanisms is involved in the pathophysiology of hepatic I/R. Experimental evidence shows that there are two distinct phases of liver reperfusion injury. The early phase covers the first two hours after reperfusion. During this phase the main event is the activation of Kupffer cells (Jaeschke and Farhood 1991). Complement activation and the recruitment and activation of $CD4^+$ T lymphocytes are factors that enable the activation of the Kupffer cells (Jaeschke 2003; Fondevila *et al.* 2003).

Kupffer cell activation leads to structural changes, formation of vascular ROS and production of cytokines such as tumour necrosis factor (TNF-α) and interleukin 1 (IL-1) (Lentsch *et al.* 2000; Liu *et al.* 2001). These ROS and cytokines have a direct cytotoxic effect on EC and hepatocytes; can induce changes in cell membrane receptors in hepatocytes and release of cytokines. TNF-α acts as the central mediator in the hepatic response to I/R. The production of TNF-α induces the expression of adhesion molecules

on vascular EC and stimulates the production and release of neutrophil-attracting chemokines. The final result is the recruitment of neutrophils. These activated neutrophils release ROS and proteases that are responsible for the induced oxidative stress during the late phase of reperfusion injury (Jaeschke 2000; Weiss 1989) which is much more severe compared to the early phase.

Recent evidence suggests that the T-lymphocytes can also be important mediators in short and long term liver I/R injury. Their role seems to be a multifactorial one. There is evidence that systemic immunosuppression attenuates hepatocellular injury following I/R (Matsuda *et al.* 1998; Shen *et al.* 2002; Suzuki *et al.* 1993). The adherence of CD4$^+$ T-lymphocytes in hepatic sinusoids occurs during the early phase of reperfusion and is mediated by TNF-α and IL-1 (Clavien *et al.* 1993). These T-lymphocytes can increase Kupffer cell activation and can act as cellular mediators in polymorphonuclear (PMN) cell recruitment through the release of substances such as granulocyte colony stimulating factor and interferon γ (Zwacka *et al.* 1997).

The basic pathophysiological mechanisms in hepatic I/R injury are summarized in figure 1.
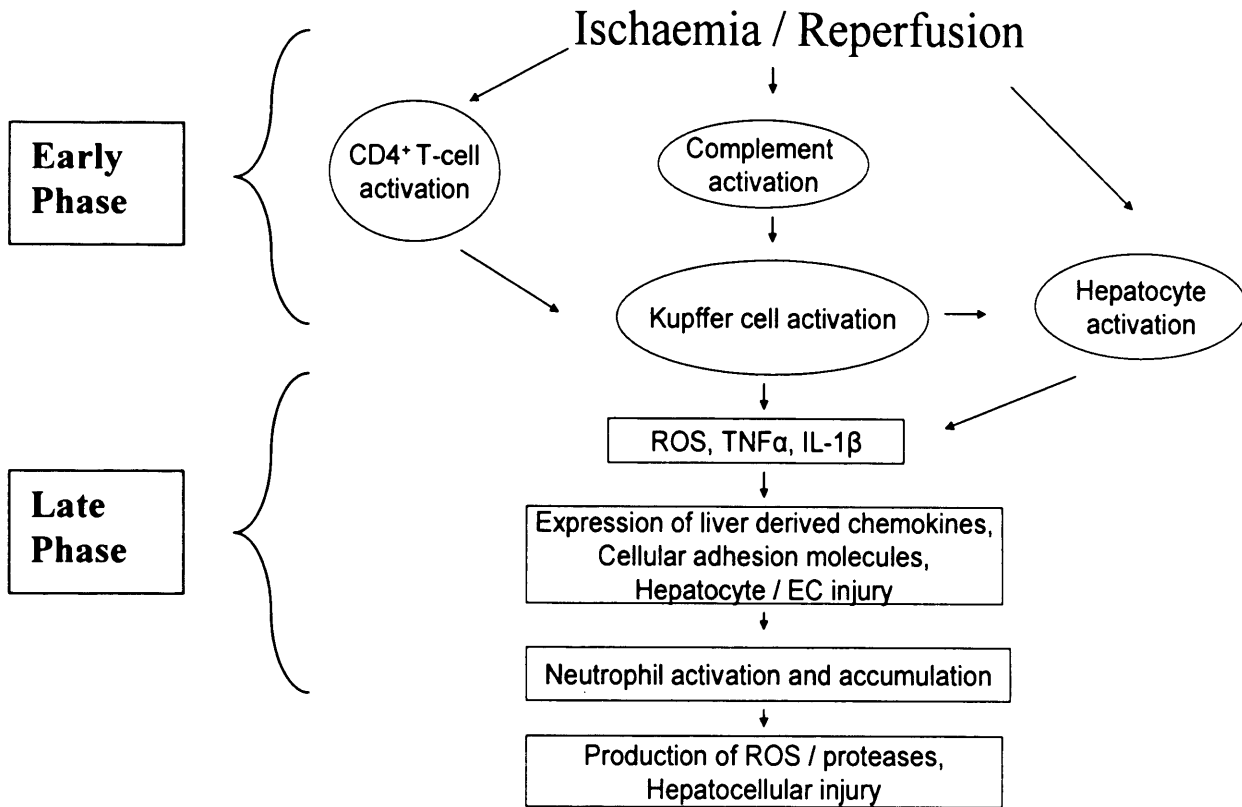
Figure 1.1. Schematic presentation of the pathophysiology of liver ischaemia / reperfusion injury. Keys: ROS, reactive oxygen species; TNFα, tumour necrosis factor α; IL-1β, inteleukin 1β; EC, endothelial cells.

1.3 Reactive oxygen species and I/R injury

A "radical" is defined as any atom or biomolecule that contains unpaired electrons (Halliwell B and Gutteridge JMC 1999). These unpaired electrons influence the chemical reactivity making the radical more reactive than the corresponding non-radical. Although oxygen (O_2) is the most important biological molecule for sustaining life, it is also the main source for free radical formation due to its high availability.

The biologically relevant radicals are the superoxide anion (O_2^-), the hydroxyl radical (^-OH) and nitric oxide (NO^-). Under normal conditions around 1-3 % of the oxygen metabolized in the mitochondria is converted to the radical O_2^- (Nohl *et al.* 2003). Some

other species are intermediate in the metabolism of O_2 or $NO^{\cdot}$ but are not radicals as they do not contain unpaired electrons. These intermediate species along with the radical species are called ROS and RNS respectively. The most representative examples of non radical ROS are hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl). The most representative of non radical RNS is peroxynitrite ($ONOO^{-}$) (Ischiropoulos et al. 1992). Peroxynitrite is formed when there is simultaneous production of $NO^{\cdot}$ with superoxide anion (eq. 1):

$$NO^{\cdot} + O_2^{-} \longrightarrow ONOO^{-} \qquad (1)$$
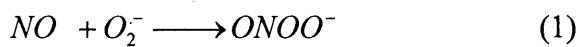
Tissue toxicity from superoxide generation is based on its direct reactivity with numerous types of biological molecules (typically lipids, DNA, RNA, catecholamines and steroids) and from its dismutation to form H_2O_2 (Cuzzocrea et al. 2001). Trace amounts of metals ions (principally iron or copper) react with H_2O_2 in what is known as the Fenton reaction to produce the toxic radical $^{\cdot}OH$ (Sutton and Winterbourn 1989). This radical can cleave covalent bonds in proteins and carbohydrates and destroy cell membranes.

Liver injury induced by I/R is caused, at least partially, by ROS and RNS. There is evidence that during hepatic I/R there is generation and release of ROS and RNS with concomitant consumption of endogenous antioxidants and apoptotic or necrotic cell death (Bilzer et al. 1999; Giakoustidis et al. 2002; Jaeschke 2000; Rauen et al. 1999; Rudiger and Clavien 2002).

Although the exact sources of ROS generation in liver I/R are still under investigation, the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, the xanthine /XOR system and the mitochondria have been suggested to play key roles (Fan et al. 1999).

Although XOR was regarded as the principal source of post ischaemic oxidant stress in the liver, recent evidence suggests that XOR plays a minor role as compared to the mitochondria (Jaeschke 2002). Mitochondria are the site of the production of large amounts of superoxide, under conditions of oxidative stress. It is this stress that finally leads to the formation of membrane permeability transition pores and the breakdown of the mitochondrial membrane potential that can cause cellular death (Nieminen *et al.* 1997).

ROS and RNS can have an important role in signal transduction pathways coordinating the body inflammatory response after liver I/R injury (Zwacka *et al.* 1998a; Jaeschke 2000; Harada *et al.* 2003; Jaeschke 2003). They are involved as mediators in the production of substances regulating liver blood flow (Paxian *et al.* 2001) and regeneration (Nakatani *et al.* 1997; Pannen 2002). Transgenic mice with over expression of antioxidant enzymes have decreased cellular ploidy during liver regeneration suggesting that ROS are involved in cell cycle control (Nakatani *et al.* 1997). ROS also induce stress genes such as haem oxygenase-1 (HO-1) (Bauer and Bauer 2002). The induction of HO-1 leads to formation of the antioxidant biliverdin, the vasodilator carbon monoxide (CO) and iron. The multiple roles of ROS in liver I/R are summarised in table 1.

Table 1.1 Role of reactive oxygen species (ROS) in liver ischaemia-reperfusion (I/R) injury.

Function	References
Enhance pro-inflammatory gene expression (TNF-a, IL-1, IL-8, cellular adhesion molecules)	Lentch, 2000
Induce expression of the transcription factors NF-kB and activator protein-1	Zwacka, 1998 Harada, 2003
Direct cellular damage through protein oxidation and degradation, lipid peroxidation and DNA damage	Jaeschke, 2000
Direct induction and regulation of apoptotic and necrotic cell death	Rauen, 1999 Rudiger, 2002
Inactivation of antiproteases	Weiss, 1989 Jaescke, 2000
Induction of protective stress genes in hepatocytes	Bauer, 2002
Formation of mediators involved in regulating sinusoidal blood flow & liver regeneration	Nakatani, 1997 Paxian, 2001

Keys: TNF-α, tumor necrosis factor-α; IL-1, interleukin-1; IL-8, interleukin-8; NF-kB, nuclear factor-kappa B.

1.4. Role of nitric oxide in liver I/R injury

NO is a radical synthesized via the oxidation of L-arginine by NO synthetase (NOS) (Moncada and Higgs 1993). There are two major isoforms of NOS in the liver, endothelial NOS (eNOS) and inducible NOS (iNOS). eNOS is expressed constitutively and its activity is dependent on Ca^{2+} and calmodulin (Vasquez-Vivar et al. 1998). iNOS is synthesised by EC, hepatocytes and Kupffer cells and its activity is Ca^{2+} independent. NO is a lipophilic biomolecule that diffuses to adjacent cells and enters the cytosol, where it activates soluble guanylyl cyclase by binding to the iron in the haem centre resulting in an intracellular increase of cyclic guanosine monophosphate (cGMP) levels (Schmidt et al. 1993). Many of the biological actions of NO are mediated through the guanyl cyclase/cyclic GMP system.

Under physiologic conditions only constitutive eNOS is present in the liver and the low level of NO produced regulates hepatic perfusion, prevents platelet adhesion, thrombosis, PMN cell accumulation and secretion of inflammatory mediators (Gauthier et al. 1994; Mittal et al. 1994) . NO also induces vasodilatation at the level of the sinusoid and at presinusoid sites (McCuskey 2000; Ming et al. 1999), this in order to keep a balance with vasoconstrictors such as endothelin (Pannen 2002). Induction of iNOS may have either toxic or protective effects. The effects are dependent on the type of insult, the level and duration of iNOS expression and the simultaneous production of superoxide anion (Cuzzocrea et al. 2001).

In liver I/R iNOS mRNA expression starts 1 h post reperfusion with increased iNOS activity at 5 h post reperfusion (Hur et al. 1999). The literature concerning the effect of

iNOS in liver I/R injury is still ambivalent. Some studies suggest that iNOS expression has detrimental effects (Kimura *et al.* 2003; Meguro *et al.* 2002; Serracino-Inglott *et al.* 2001) to liver function whilst others suggest that it is beneficial (Hsu *et al.* 2002; Wang *et al.* 1998) or has no effect at all (Hines *et al.* 2002; Rivera-Chavez *et al.* 2001). One study with mice deficient in iNOS showed a moderate reduction in reperfusion injury (Lee *et al.* 2001). The toxic effects of NO˙ are linked with the production of peroxynitrite which is the product of O_2^- and NO˙. Peroxynitrite can cause cell injury through multiple pathways: initiation of lipid peroxidation, direct inhibition of mitochondrial respiratory chain enzymes, inhibition of membrane Na^+ / K^+ ATP-ase activity, or oxidative protein modification such as formation of nitrotyrosine (Doulias *et al.* 2001; Hon *et al.* 2002; Szabo 2003). However, whether NO˙ will act as a cytoprotective or cytotoxic agent depends on a number of factors such as NO˙-superoxide radical ratios, hepatic stores of reduced glutathione and the duration of ischaemia.

1.5 Therapeutic strategies for amelioration of liver I/R injury

Understanding the mechanisms of liver I/R injury allows therapeutic strategies to be developed. The current strategies are either mechanical (e.g. ischaemic preconditioning or remote preconditioning) or pharmacological. Ischaemic preconditioning is the application of short periods of ischaemia and reperfusion to an organ prior to prolonged ischaemia whereas in remote preconditioning cycles of brief ischaemia are applied to a remote organ (e.g. lower limb) prior to prolonged ischaemia of the target organ (e.g. liver, heart). Preliminary studies with both techniques have shown a reduction in liver I/R injury although further studies and refinement of technique are required (Banga *et al.* 2005; Kharbanda *et al.* 2002; Koti *et al.* 2003). Mechanical methods of reducing I/R

injury are worthwhile but are limited in their application whereas pharmacological modulation may have universal application.

One of the main group of pharmacological agents which can enhance the antioxidant defence system is thiols. Thiols are molecules containing sulfydryl (-SH) groups and have an important role in maintaining the body's redox balance and defence against oxidants (Sen 1998; Wlodek 2002). N-acetylcysteine is a synthetic thiol used currently as the drug of choice in the management of fulminant liver failure due to paracetamol overdose (Chyka *et al.* 2000; Cotgreave 1997). It has been used in different experimental settings linked to oxidative stress showing mainly beneficial effects. However, its application in small clinical trials has shown inconsistent results (Aitio 2006). Its role in warm liver I/R injury is still ambivalent (Chavez-Cartaya *et al.* 1999; Koeppel *et al.* 1996). Progress in the use of protective strategies such as antioxidant therapy in liver I/R requires well designed experimental and clinical studies, in order to gain a better understanding of the basic mechanisms of I/R injury, of the mechanism of protective action and timing of any beneficial effects.

1.6 Aim and objectives of this thesis

1. To investigate further the pathophysiology of warm liver I/R injury

2. To investigate the effect of the administration of the antioxidant NAC on liver function and the mechanism of action. NAC was chosen as a classical representative of thiols, which had already been tested in experimental studies of liver I/R, producing conflicting results.

The next chapter provides an overview of the endogenous antioxidant system and reviews the experimental and clinical evidence on the role of antioxidant therapy on liver I/R, with special emphasis on the role of thiols.

CHAPTER 2

THE ANTIOXIDANT SYSTEM AND THE ROLE OF ANTIOXIDANT THERAPY IN LIVER I/R INJURY WITH EMPHASIS ON THIOLS

2.1 The antioxidant system

The body has developed major antioxidant defence mechanisms to protect it from damage due to free radicals. An antioxidant is any substance that when present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of the substrate (Gutteridge 1995). The endogenous antioxidants mainly are small molecular weight substances that are able to prevent initiation of oxidative damage or to limit its propagation and enzymes that convert and detoxify ROS and RNS. Cellular redox balance is in normal circumstances under tight control. However, when ROS and RNS are produced at levels that cannot be counteracted by endogenous antioxidant systems, an imbalance takes place that is called oxidative stress (Sies H 1991). This condition can lead to the damage of lipids, proteins, carbohydrates and nucleic acids. Hepatocytes tend to be resistant to injury by ROS and RNS since they contain high intracellular concentrations of glutathione (GSH), superoxide dismutase (SOD), catalase and lipid soluble antioxidants.

The antioxidant system is very important for the living species and has allowed them to use oxygen (O_2) for energy production, without being exposed to the deleterious effects of O_2. The composition of antioxidant defences differs from tissue to tissue and from cell to cell in a given tissue. Also, different organs contain different concentrations of antioxidants and for this reason there is variability in organ resistance to I/R. However, there is evidence that the antioxidants operate as a balanced and coordinated system and each relies on the action of the others (Marczin *et al.* 2003; Vertuani *et al.* 2004).

Antioxidants are a heterogenous family of molecules. Several classifications have been used in the past taking into account the origin (natural or synthetic), nature (enzymatic or non-enzymatic), properties (hydrophilic or lipophilic), mechanism (catalytical removal of ROS, metal chelation, scavenging of ROS) and site of action (intracellular; membrane and extracellular). This review is presented according to the site of action of the antioxidants.

Intracellular antioxidant defences include: the superoxide dismutase; catalase; glutathione peroxidase and reductase enzymes, the tripeptide glutathione, the polypeptide thioredoxine, the enzyme haem oxygenase (HO) and peroxidases of the peroxiredoxin family.

SOD catalyses the dismutation of superoxide to hydrogen peroxide and oxygen (eq. 1):

$$2O_2^- + 2H^+ \xrightarrow{\quad SOD \quad} H_2O_2 + O_2 \qquad (1)$$
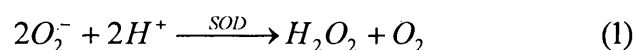
Three forms of SOD exist with different subcellular localizations. Those containing copper and zinc (Cu/ZnSOD) are located in the cytosol (Sherman *et al.* 1983), manganese (MnSOD) in the mitochondria (Ho and Crapo 1988) and the extracellular form usually located on the outside of plasma membrane interacting with matrix components. The product of reaction 1, H_2O_2, is a weak oxidant and is relatively stable. However, unlike superoxide, H_2O_2 can rapidly diffuse across cell membranes and in the presence of transition metal ions it can be converted to toxic hydroxyl radicals via Fenton chemistry (eq. 2):

$$Fe^{+2} + H_2O_2 \longrightarrow Fe^{+3} + OH + OH^- \qquad (2)$$
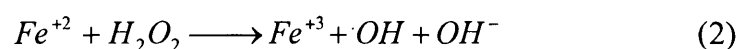
Three main systems can break down H_2O_2. One of them is the haemoprotein catalase. Catalase is present in all major body organs being especially concentrated in liver. It catalyzes the breakdown of hydrogen peroxide to oxygen and water (eq. 3):

$$2H_2O_2 \longrightarrow O_2 + 2H_2O \qquad (3)$$
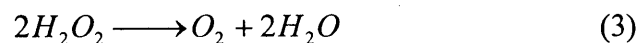
The second system consists of the glutathione peroxidases. This group includes four different isoforms such as cellular, gastrointestinal, extracellular and phospholipids (Dufaure et al. 1996). They have a major role in removing hydrogen peroxide generated by superoxide dismutase with the oxidation of GSH to its oxidized form glutathione disulphide (GSSG) (eq. 4):

$$2GSH + H_2O_2 \longrightarrow GSSG + 2H_2O (4)$$
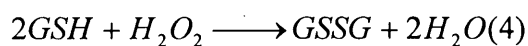
Glutathione reductase is also an important enzyme in this system. It expresses its action through the regeneration of GSH from GSSG using NADPH (Meister 1988).

The third system consists of peroxidases of the peroxiredoxin (Prx) family that reduce hydrogen peroxide and alkyl hydroperoxides to water and alcohol respectively using reducing equivalents. These equivalents are derived specifically from thiol-containing donor molecules, such as thioredoxin. They are located in the cytoplasm (Prx I and II) and in mitochondria (Prx III) (Kang et al. 1998; Seo et al. 2000).

HO is an enzyme found in the endoplasmic reticulum that catalyses the breakdown of haem to biliverdin with the release of iron ions and CO. Three isoforms of HO have been characterized: HO-1 that is highly inducible in conditions of inflammation and oxidative stress and HO-2 and HO-3 that are constitutively expressed (Guo et al. 2001).

Induction of HO-1 protects the cell against oxidative injury by controlling intracellular levels of free haem (a prooxidant), producing biliverdin (an antioxidant) and improving microcirculation via CO release (Bauer and Bauer 2002). Recent evidence suggests that HO-1 expressed early after intermittent porta hepatis clamping and possibly contributes to the early protective effects of ischaemic preconditioning (Patel *et al.* 2004)

Major extracellular antioxidant defences include the metal-binding proteins (Betteridge 2000). Free metals iron and copper can promote free radical damage, accelerating lipid peroxidation and catalyzing hydroxyl radical formation. The body is protected against these potentially adverse effects by metal- binding proteins which ensure that these metals are maintained in a nonreactive state (Halliwell and Gutteridge 1990). Transferrin and lactoferrin bind iron while caeruloplasmin and albumin bind copper. Haemoglobin and myoglobin are normally intracellular proteins. When these proteins are exposed to a large amount of oxidative stress such as H_2O_2, they are degraded releasing both haem and iron ions that can then stimulate lipid peroxidation. Haemoglobin binding proteins known as haptoglobins and haem binding proteins such as haemopexin decrease the effectiveness of these substances in stimulating lipid peroxidation (Gutteridge 1989).

In addition to the major protective role of the metal-binding proteins, various low-molecular-weight molecules that are synthesised in vivo have antioxidant properties (Ames *et al.* 1981; Frei *et al.* 1988; Layton *et al.* 1996). The most important of these substances are bilirubin, melatonin, lipoic acid, coenzyme Q, uric acid and the melamins. Recent evidence suggests that uric acid has an important role in the endogenous antioxidant system (Glantzounis *et al.* 2005c; Reyes and Leary 2003) and

that exogenous administration of uric acid could have beneficial effects in situations associated with oxidative stress (Chamorro *et al.* 2004; Waring *et al.* 2003).

A large number of dietary constituents exert antioxidant effects in vivo. The most important are hydrophilic ascorbic acid (vitamin C) and lipophilic α-tocopherol (the most active form of vitamin E) that are important components of the human antioxidant system. Ascorbate is required in vivo as a cofactor for many enzymes, of which the best known are proline hydroxylase and lysine hydroxylase both involved in the biosynthesis of collagen. Its main chemical property is its ability to act as a reducing agent. It can scavenge most radicals such as O_2^-, ·OH, peroxyl, thiyl, oxysulphur radicals and peroxynitrite (Halliwell and Gutteridge 1990; Birlouez-Aragon and Tessier 2003). The lipophilic α-tocopherol is a highly effective antioxidant when incorporated in the lipid core of cell membranes. It has the ability to scavenge intermediate peroxyl radicals and therefore interrupt the chain reactions of lipid peroxidation (Traber 1994).

Carotenoids are a group of coloured pigments that are widespread in plant tissues. They serve as a precursor of vitamin A and are the principal dietary source of vitamin A in humans. They exert their antioxidant action as free-radical scavengers (Liebler and McClure 1996). Another group of antioxidants are the plant phenols. Plants contain a huge range of phenols including tocopherols, tocotrienols, flavonoids, anthocyanidins and phenylpropanoids. They inhibit peroxidation by acting as chain-breaking peroxyl-radical scavengers. In addition, they scavenge ROS and RNS such as ·OH, ONOO⁻ and HOCl and they act as metal chelators (Rice-Evans *et al.* 1996; Shahidi and Wanasundara 1992).

There is synergism among the different antioxidants and they are linked to each other in a systematic relationship as part of the antioxidant network (Vertuani *et al.* 2004). Recent studies in myocardial I/R injury suggest that the hydrophilic agents (ascorbic acid, GSH) are consumed first followed by the lipophilic species (Vitamin E) (Haramaki *et al.* 1998; Marczin *et al.* 2003) . It has also been shown that ascorbate can recycle α-tocopherol (Vertuani *et al.* 2004).

Figure 2.1 summarizes the main antioxidant mechanisms.
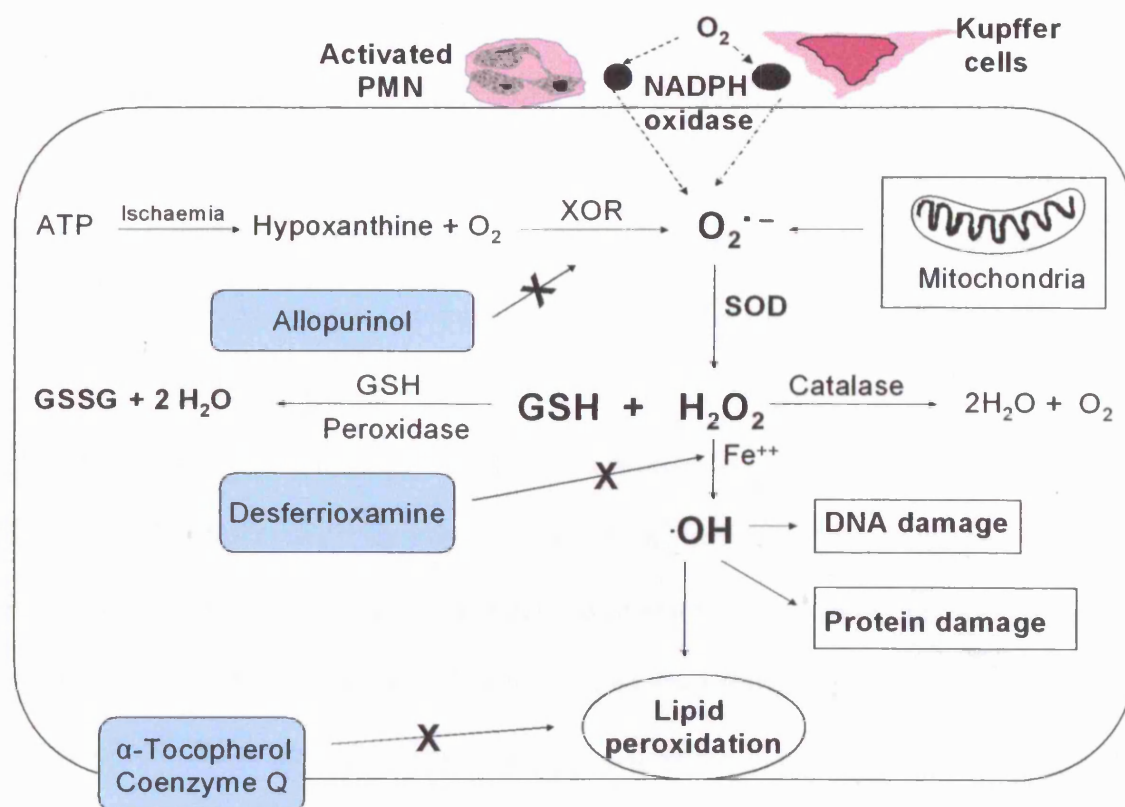
Figure 2.1. Basic mechanisms of the endogenous antioxidant system. Xanthine oxidoreductase (XOR) and mitochondria are the main intracellular sources of production of reactive oxygen species (ROS). Kupffer cells (KC) and activated neutrophils (act PMN) are the main extracellular sources for production of ROS during the early and late phase of reperfusion respectively. The main intracellular antioxidant enzymes are superoxide dismutase (SOD), catalase and glutathione (GSH) peroxidase. A-tocopherol and coenzyme Q are the main antioxidants in cellular membranes.

2.2 Antioxidant therapy

There is a large number of antioxidant agents which have been shown experimentally or clinically to be of benefit in the treatment of liver I/R injury. Table 2.1 summarises the effects of these agents in liver I/R injury.

2.2.1 Membrane and extracellular antioxidants

α-tocopherol is the most important inhibitor of the free radical chain reaction of lipid peroxidation. It acts as a direct free radical scavenger, increases GSH levels and has other non-antioxidant properties such as the inhibition of protein kinase C (Brigelius-Flohe *et al.* 2002; Calfee-Mason *et al.* 2002; Masaki *et al.* 2002; Ricciarelli *et al.* 2002). A significant reduction in liver α-tocopherol levels was observed during the first hour of reperfusion in a rat model of liver ischaemia (Marubayashi *et al.* 1984). Pretreatment with high and very high doses of α-tocopherol (30 and 300 mg/kg of body weight i.m, respectively) improved ATP levels, prevented the increase in lipid peroxidation products such as thiobarbituric acid reactive substances and attenuated the loss of hepatic glutathione during the early phase of reperfusion after warm ischaemia in rats (Giakoustidis *et al.* 2002). High doses of α-tocopherol (1000 U/kg) also increased the survival of rats with steatotic liver which underwent warm liver ischaemia (Soltys *et al.* 2001). A-tocopherol has also shown beneficial effects in cold I/R injury (Gondolesi *et al.* 2002). Trolox C, the hydrophilic analog of α-tocopherol has shown beneficial effects in experimental liver I/R injury (Eum *et al.* 2002). The combination of α-tocopherol and pentoxyphilline, a drug that affects microcirculation as used in the treatment of peripheral vascular disease, reduces experimental warm liver I/R injury (Vardareli *et al.* 1998).

Ascorbic acid in low doses (<100 mg/kg) has also shown protective effects in hepatic function in an experimental study of liver I/R injury. However high doses of ascorbic acid (1000 mg/kg) aggravated the injury. This harmful effect could be due to the increased reduction of ferric iron to the ferrous form under high ascorbic acid concentration (Seo and Lee 2002). A prospective randomized clinical study in patients undergoing liver resection used an antioxidant multivitamin infusion containing 10 mg α- tocopherol acetate and one g ascorbate administered prior to reperfusion. In the treated group less plasma lipid peroxidation occured and less acute liver damage as assessed by measurement of the prothrombin time and transaminase levels. The treated group also had fewer postoperative complications (postoperative liver failure and infections) (Cerwenka *et al.* 1999).

Melatonin is a hormone produced by the pineal gland that helps regulate circadian rhythms and exhibits antioxidant activity. In an experimental study with rats that underwent 60 minutes of total liver ischaemia and 2 h reperfusion, melatonin administration preserved functional and energetic status, reduced TNF-α production and inhibited expression of iNOS (Rodriguez-Reynoso *et al.* 2001). One recent clinical study in patients undergoing liver resections suggested that the protective effect of melatonin may be through the enhancement of neutrophil apoptosis (Chen *et al.* 2003).

Pretreatment with coenzyme Q prevented the post ischaemic loss of hepatic α-tocopherol and glutathione (Marubayashi *et al.* 1984) and also attenuated the increase in lipid peroxidation and the decrease in mitochondrial respiration in rats submitted to partial hepatic ischaemia (Genova *et al.* 1999). Also the administration of idebenone that is an analog of coenzyme Q-10, in isolated perfused pig livers reduced significantly

the neutrophil mediated reperfusion injury (Schutz *et al.* 1997). The combination of coenzyme Q and pentoxyphylline had a protective effect in warm I/R injury by maintaining GSH levels and by inhibiting lipid peroxidation (Portakal and Inal-Erden 1999).

As mentioned before free iron in its ferrous state can catalyze the formation of hydroxyl radical from hydrogen peroxide (Fenton reaction). In this way it can initiate lipid peroxidation. Desferrioxamine is an iron chelator used for the treatment of iron loading diseases such as Thalasaemia. Pretreatment with desferioxamine in vivo protected in experimental settings with warm and cold hepatic ischaemia (Omar *et al.* 1989; Bailey and Reinke 2000; Park *et al.* 2003).

There is a number of experimental studies using biomolecules principally trimetazidine (Tsimoyiannis *et al.* 1993) or trimetazidine analogs (Elimadi *et al.* 2001) that have shown beneficial effects after warm liver ischaemia. The mode of action has been postulated to be through the chelation of metals mainly Copper (Tselepis *et al.* 2001). In addition, plant phenols (Su *et al.* 2003; Tsuda *et al.* 2002) or herbal medicines (Nagai *et al.* 1992; Chiu *et al.* 1999; Singh *et al.* 2000; Topp *et al.* 2001; Zhong *et al.* 2002; Sanchez *et al.* 2003) have shown beneficial effects (Table 2.1).

From the above it is clear that substantial experimental evidence exists about the protective effects of extracellular antioxidants in hepatic I/R injury. However, the majority of these antioxidants have not as yet been evaluated in clinical trials.

2.2. 2 Intracellular antioxidants

Thiols

Sulphydryl groups exert their antioxidant action through the oxidation of the thiol (-SH) group of cysteine. Furthermore, they have a central role as mediators to the majority of redox-sensitive cell signaling mechanisms (Moran *et al.* 2001; Vertuani *et al.* 2004) . Due to their significance the role of thiols in I/R injury is presented separately at the end of this chapter.

Superoxide dismutase

The rational behind using SOD is to accelerate the detoxification of the superoxide anion, thus preventing the generation of the highly reactive OH radical. In vivo experimental studies in hepatic I/R have reported protective effects with SOD pre-treatment (Atalla *et al.* 1985; Nauta *et al.* 1990). However, some other studies failed to show a protective effect with SOD administration (Lambotte *et al.* 1988; Minor *et al.* 1992). In order to detoxify effectively intracellular ROS the SOD molecule has to enter the cells intact or superoxide has to leave the cell to be metabolized extracellularly. There is no evidence in the liver that superoxide moves through cell membranes. The main problems with SOD administration are the short half-elimination life (about 6 min) and the lack of uptake of the intact protein into cells (Jaeschke 1991). Inadequate delivery to target sites could be the cause for the mixed results reported to date in the literature.

In order to improve the intracellular availability of SOD new derivatives have been developed. These include the conjugation of SOD with carbohydrate structures which facilitate the uptake into liver nonpenchymal cells. The techniques that have been developed are: mannosylation, succinylation (Yabe *et al.* 1999b) and pegylation

(Veronese *et al.* 2002). Kupffer and sinusoidal endothelial cells have receptors that recognize and internalize ligands containing mannose, succinylated and pegylated proteins. Targeted SOD derivatives showed beneficial effects in preventing experimental warm liver I/R injury (Fujita *et al.* 1992; Nguyen *et al.* 1999).

Catalase

The results in the literature are ambivalent concerning the role of catalase in liver I/R injury (Nordstrom *et al.* 1988; Tanaka *et al.* 1990). The main problems are the same as with SOD, the short half-elimination life in plasma and the difficulty of protein uptake into cells. In order to bypass these difficulties catalase targeted derivatives have been developed from conjugation with carbohydrates. The initial results appear promising (Yabe *et al.* 1999a), and the administration of a combination of both enzymes (catalase and SOD) has been tried and the results were found to be effective (Yabe *et al.* 2001). Among the different combinations Man-SOD and Suc-catalase have shown the greatest efficacy in preventing liver injury. This combination reduced significantly ICAM-1 expression and neutrophil infiltration.

Allopurinol

Allopurinol is a XOR inhibitor. Low doses (5-10 mg/kg) are sufficient to inhibit hepatic activities of xanthine oxidase and xanthine dehydrogenase almost completely, but these doses were not protective of liver I/R (Jaeschke 1991). On the other hand, an experimental study where allopurinol was administered ip, before ischaemia, at high doses (50 mg/kg) showed a clear protective role in rats subjected to liver I/R injury (Jeon *et al.* 2001). There is experimental evidence that allopurinol has a protective role in acetaminophen-induced toxicity (Knight *et al.* 2001) The most likely mechanism for this protective effect is the prevention of mitochondrial oxidative stress. An alternative mechanism is the action as a free radical scavenger and most probably as a scavenger of

peroxynitrite (Knight *et al.* 2001). Table 2.1 summarises substances with extracellular

and intracellular antioxidant action that have shown beneficial effects in liver I/R injury.

2.2. 3 Antioxidants that modulate NO˙ metabolism

NO˙ can have either beneficial or detrimental effects in liver I/R injury. Recent evidence

has shown that during the reperfusion period eNOS is down regulated despite iNOS

expression in both hepatocytes and inflammatory cells during the late phase (Serracino-

Inglott *et al.* 2003). The expression of iNOS is associated with evidence of ONOO⁻

formation, although the exact role of peroxynitrite in liver I/R is not clear (Jaeschke

2003). It has been postulated that when the endogenous amount of SOD or GSH is not

enough to inhibit ONOO⁻ formation cellular damage can occur. Pharmacological

intervention to block ONOO⁻ formation could have a protective role against the toxic

effects of massive ONOO⁻ production (Cuzzocrea *et al.* 2001). This intervention could

act either at the level of the reactant (NO and O_2^-) or the product (ONOO⁻). Blocking

the action of O_2^- can be achieved by the use of SOD or its derivatives. There are reports

in the literature that showed beneficial effects with use of selective inhibitors of iNOS

in both warm and cold liver I/R injury (Isobe *et al.* 1999; Kimura *et al.* 2003).

Strategies that aim at decreasing the intrinsic life time of ONOO⁻ could be either

competitive stoichiometric trapping of ONOO⁻ or catalysis of ONOO⁻ decomposition to

benign products (e.g. isomerization to nitrate). Such ONOO⁻ decompositors have been

developed and have as their base iron porphyrin complexes (Cuzzocrea *et al.* 2001). The

search for other redox-active complexes that will accomplish catalysis of the

peroxynitrite isomerization to nitrate still continues.

Table 2.1 List of antioxidant agents with beneficial effects in liver ischaemia / reperfusion injury.

Antioxidant	Category	Species	Injury Type	Mode of Adm.	Dose	Main protective effect	Authors (first)	
A-Tocopherol	Vitamin-diet	Rat	WI	im	30 and 300 mg/kg	Survival, Histology	Giacoustidis,	2002
A-Tocopherol	Vitamin-diet	Rat	CI/WI	iv	50 IU/Kg	Histology	Gondolesi,	2002
A-Tocopherol / Ascorbate	Vitamins-diet	Clinical	WI	iv	2 mg / 1000 mg	Better PT ↓postop.complications	Cerwenka,	1999
Ascorbate	Vitamin-diet	Rat	WI	iv	30 and 100 mg/kg	↓ lipid peroxidation	Seo,	2002
Coenzyme Q / Pentoxyfylline	In vivo LMM agent	Rat	WI	In/ip	10 mg/kg / 50 mg/kg	↓ lipid peroxidation ↑ GSH levels	Portakal,	1999
Idebenone	Coenzyme Q deriv	Pig	CI				Schutz,	1997
Lipoic acid	In vivo LMM agent	Rat	CI	iv	500 μM	Histology	Muller,	2003
Deferrioxamine	Iron chelator	Dog	CI/WI	iv	20 mg / kg	↓ AST activity	Park,	2003
Trimetazidine	Metal chelator	Rat	WI	iv /ip	2.5 mg / kg	Histology	Tsimoyiannis,	1993
Quercetin	Plant phenol	Rat	WI	po	0.13 mmol / kg	↓ ALT, AST	Su,	2003
Cyanidin	Plant phenol	Rat	WI	po	0.9 mmol / kg	↓ lipid peroxidation	Tsuda,	2002
Green Tea Extracts	Plant extracts (Catechines)	Rat	WI	po	0.1 %	Histology	Zhong,	2002
Magnifera indica	Plant extracts	Rat	WI	po	250 mg / kg	↓ AST, ALT ↓ lipid peroxidation	Sanchez,	2003
Glutathione (GSH)	In vivo LMM agent	Rat	WI	iv	100 μM/h/kg	↓ ALT ↑ survival	Schauer-a,	2004
Glutathione	In vivo LMM agent	Rat	CI/WI	iv	100 μM/h/kg	↓ ALT, ↑ bile flow	Schauer-b,	2004
N-acetylcysteine	Thiol compound GSH precursor	Rabbit	WI	iv	150 mg / kg	↓ ALT ↑ microcirculation	Glantzounis,	2004
N-acetylcysteine/ Melatonin	Thiol compound	Rat	WI	ip	150 mg/kg / 10 mg / kg	↓ AST, ALT ↓ lipid peroxidation	Sener,	2003
N-acetylcysteine	Thiol compound	Clinical	CI/ WI	iv	150 mg / kg	↓ICAM, ↓ α-GST	Weigand,	2001
Bucillamine	Thiol compound	Rat	CI/ WI	Ipr iv	15 mg / kg 10 mg /kg	Survival	Amersi,	2002
SOD derivatives	Intracellular enzyme	Rat	WI	iv	5000 IU / Kg	↓ lipid peroxidation	Nguyen,	1999
CAT derivatives	Intracellular enzyme	Rat	WI	iv	0.1 mg / kg	↓ ALT, AST	Yabe,	1999
Allopurinol	XO Inhibitor	Rat	WI	ip	50 mg / kg	↓ AST	Jeon,	2001
Aminoguanidine	iNOS inhibitor	Pig	CI /WI	iv	10 mg / kg	Survival, histology	Kimura,	2003

Keys: Admin, administration; WI, warm ischaemia; im, intramuscular; CI, cold ischaemia; iv, intravenous; PT, prothrombin time; LMM, low molecular molecule; deriv, derivatives; In, Intragastric; ip, intraperitoneal; AST, aspartate transaminase ; ALT, alanine transaminase; ICAM-1, intercellular adhesion molecule-1; α-GST:α-glutathione S-transferase; ipr, intraportally; XO, xanthine oxidase

2.2. 4 Antioxidant gene therapy

Gene therapy has recently been applied as a therapeutic strategy against I/R injury. Two categories of vector systems have been used, viral and non viral. The advantages of virus based systems include higher infection efficiencies and the ability to encode multiple large genes (Engelhardt 1999). The disadvantages include immunogenicity and vector production issues. For these reasons non-viral vector systems using lysosomes and DNA-protein complexes have been developed.

In liver I/R injury predominantly to date viral vectors typically recombinant virus have been used. High doses of mitochondrial SOD administered via viral vector in rats prevented liver I/R injury via inactivation of the transcription factors nuclear factor – kappa B (NF-kB) and activator protein-1 (AP-1)(Zwacka et al. 1998b). Experimental studies in a rat liver transplantation model with normal and steatotic livers have shown that cytosolic and mitochondrial SOD markedly improved survival whereas extracellular SOD was not protective (Lehmann et al. 2000;Lehmann et al. 2003). In another experimental study (Wheeler et al. 2001) overexpression of the three SOD isoforms were all shown to protect against an increase in serum transminases and hepatocellular necrosis following I/R injury. They also reduced significantly the production of lipid derived free radicals. The extracellular form was protective when administrated in high doses.

A recent in vitro study has shown that the induction of human genes expressing the peroxidase peroxiredoxin, can protect murine cells effectively from oxidative stress (Shau et al. 2000). Gene therapy therefore has potential for amelioration of the effects of liver I/R. Although its value to cadaveric liver transplantation may be limited by the

emergency nature of the procedure, it could have application to elective liver surgery such as liver resection for tumours or living donor liver transplantation. However, the future success of gene therapies requires better understanding of the pathophysiology of I/R. Although ROS can cause cell injury during I/R they may also have a significant role in the normal cell growth and proliferation. The targets should be ROS that are responsible for cell injury, in addition the subcellular compartments that produce detrimental ROS should be identified. A recombinant adenoviral vector has been used to inhibit NF-kB activation in a model of partial hepatectomy (Iimuro et al. 1998), resulting in massive apoptosis and also delaying the regeneration process. This study showed that NB-kB is important in preventing apoptosis and also in enabling liver regeneration.

2.2.5 THIOLS

Thiol compounds contain sulphydryl (-SH) groups and exert their protective effect against I/R injury by reacting with ROS and RNS. They undergo one-electron oxidation with the formation of thiyl radicals (RS) and exert their antioxidant properties through scavenging of free radicals, chelation of metal ions and the thiol/disulfide component of redox buffer (Laragione et al. 2003). There is significant in vitro and in vivo evidence showing the consumption of thiols in conditions associated with oxidative stress (Bilzer et al. 1999; Haramaki et al. 1998; Leichtweis and Ji 2001; Schauer et al. 2004a; Sirsjo et al. 1996).

Thiols are central to cellular antioxidant defence in mammals. Glutathione and thioredoxin are thiols which act as substrates for the enzymes glutathione peroxidases and peroxyredoxines respectively. These molecules are the main part of the endogenous

antioxidant system responsible for removal of the H_2O_2 generated under conditions of oxidative stress (Halliwell and Gutteridge 1999).

Thiols also play an important role in most redox-sensitive signalling mechanisms. Recent evidence suggests that thiols can act as redox sensitive switches providing a common trigger for a variety of ROS and RNS mediated signalling events. Under normal conditions they use ROS as a mediator to regulate their protein activity (Leichert and Jakob 2004; Linke and Jakob 2003; Moran *et al.* 2001). A typical example is the reversible redox state of cysteine, an amino acid that plays an important role in the structure, function and regulation of proteins. The active element is the thiol (-SH) group of cysteine which when performing its antioxidant activity is oxidised to cystine or cysteine disulfide (Leichert and Jakob 2004).

The endogenous thiols thioredoxin and glutathione are also of central importance in redox signalling (Droge *et al.* 1994; Nakamura *et al.* 1997). The oxidation and enzymatically catalysed reduction of methionine is a novel molecular mechanism for cellular regulation. Methionine sulfoxide reductase is an enzyme that reduces methionine sulphoxide to methionine in a thioredoxin-dependent manner. There is evidence that it plays an important role in the repair of age- or degenerative disease - related protein modification (Hoshi and Heinemann 2001).

However, antioxidant reactions of thiols yield thiyl radicals ($RS^.$) that can be toxic. Under normal conditions thiyl radicals can react with thiolate anion (RS^-) producing disulfide radical anions ($RSSR^{.-}$) as an intermediate and then disulfides and superoxide anion ($O_2^{.-}$) which can have also toxic effects. Winterbourn proposed in 1993 'the

radical sink hypothesis' which suggests a coordinated antioxidant action between GSH and SOD. According to this hypothesis SOD will catalyze the dismutation of superoxide anion terminating the biologically generated free radical (Winterbourn 1993; Winterbourn and Metodiewa 1994). More recent evidence suggests that vitamin C plays a very important role in the detoxification of thiyl radicals and that reaction of thiyl radicals with ascorbate with the formation of the low activity ascorbyl radical is the most probable mechanism associated with detoxification of thiyl radicals (Sturgeon *et al.* 1998). Figure 2.2 summarises the reactions between thiols, oxidants and antioxidants.

$$RSH + R^{\cdot} \longrightarrow RH + RS^{\cdot}$$

$$RS^{\cdot} + RS^{-} \longrightarrow RSSR^{\cdot -}$$

$$RSSR^{\cdot -} + O_2 \longrightarrow RSSR + O_2^{\cdot -}$$

$$2\,O_2^{\cdot -} + 2H^{+} \xrightarrow{\text{SOD}} O_2 + H_2O_2$$

$$RS^{\cdot} + Asc\text{-} \longrightarrow RSH + Asc^{\cdot}$$
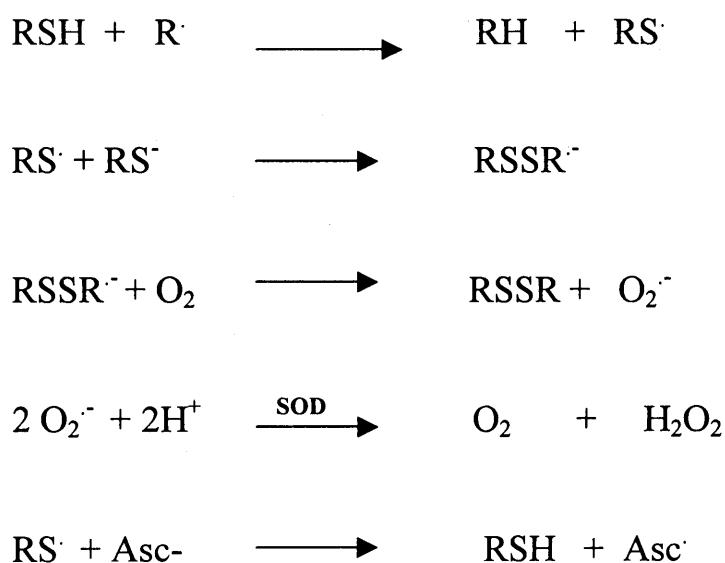
Figure 2.2: Reactions of thiols with oxidants yield thiyl radicals first, then disulfide radical anion and finally disulfides and superoxide anion, which is believed to be inactivated in the reaction catalysed by the enzyme superoxide dismutase. An alternative pathway that probably plays a more important role in the detoxification of thiyl radicals is the reaction with ascorbate (vitamin C). *Keys:* RSH: thiol, R$^{\cdot}$: free radical, RS$^{\cdot}$: thiyl radical, RSSR^{-} : disulfide radical anion, O$_2^{\cdot -}$: superoxide anion, SOD: superoxide dismutase, H$_2$O$_2$: hydrogen peroxide, Asc- : ascorbate, Asc$^{\cdot}$: ascorbic radical.

Another recent study showed that the copper, zinc superoxide dismutase isoenzyme (Cu, Zn-SOD) catalyses autoxidation of thiols such as cysteine and cysteamine, generating substantial amounts of hydrogen peroxide which may contribute to the toxicity of thiols (Winterbourn *et al.* 2002). This data shows that the metabolic pathways of thiol reactions with oxidants are not yet fully elucidated and that a coordinated action is required between the major endogenous antioxidants for the toxic effects of thiyl radicals to be eliminated.

THIOREDOXIN

Thioredoxin is a polypeptide especially concentrated in the endoplasmic reticulum (Trx1) but it is also found in mitochondria (Trx2). It is used as an electron donor by the antioxidant enzymes peroxyredoxins for the reduction of hydroperoxides and peroxynitrite (Rhee *et al.* 2005). Thioredoxin contains two adjacent –SH groups in its reduced form, which are converted to a disulphide in the oxidized form of thioredoxin. Because of its dithiol/disulfide exchange activity, thioredoxin determines the oxidation state of many protein thiols. Therefore it plays important roles in antioxidant defence, redox regulation of protein function and signal transduction. While GSH has one cysteine, thioredoxin has two cysteins in its active site. Thioredoxin together with thioredoxin reductase and peroxyredoxins forms a complete redox system similar to GSH system. GSH and thioredoxin often have complementary if not overlapping roles in cytoprotection.

Antioxidant properties of thioredoxin include removal of hydrogen peroxide (Spector *et al.* 1988), free radical scavenging (Schallreuter and Wood 1986), and protection of cells against oxidative stress (Nakamura *et al.* 1994; Tanaka *et al.* 1997). In the current

literature, there is indirect evidence on the effect of thioredoxin on liver I/R. An experimental study from Japan showed that the agent geranylgeranylacetone (GGA), an acyclic polyisoprenoid, which is used as an antiulcer drug, showed protective effects in a rat model undergoing warm liver I/R (Fudaba *et al.* 1999). Subsequently another study showed that GGA induces expression of m-RNA and protein of thioredoxin suggesting cytoprotective effects through thioredoxin (Hirota *et al.* 2000).

There are more data on the efficacy of thioredoxin in the myocardium. A recent study demonstrated a reduction of thioredoxin-1, in the ischaemic reperfused myocardium. There was an increased induction of thioredoxin-1 expression when the heart was adapted to ischaemic stress by ischaemic preconditioning. Inhibition of thioredoxin-1 expression resulted in reduced postischaemic ventricular recovery and increased myocardial infarct size in the preconditioned heart. Corroborating these findings, transgenic mouse hearts overexpressing thioredoxin were resistant to I/R injury as compared with the hearts from wild-type mice (Das and Maulik 2003; Das 2004; Turoczi *et al.* 2003). Thus, it appears that thioredoxin plays a crucial role in cardioprotection induced by preconditioning. A recent study has shown that exogenously applied thioredoxin reduced myocardial I/R injury in mice likely through its antiapoptotic effects (Tao *et al.* 2004).

Thioredoxin has also been shown to have a protective effect in lung I/R injury (Nakamura *et al.* 2005). The role of human thioredoxin and L-cysteine in I/R lung injury has been studied in isolated rat lungs. Thioredoxin and L-cysteine added to the pulmonary artery flush solution before ischaemia significantly reduced the wet to dry lung weight ratio and the albumin concentration in the bronchoalveolar lavage fluid

(Wada *et al.* 1995). In another experimental study, rats underwent lung I/R and treated with thioredoxin had significant better survival rate compared with controls. Thioredoxin also increased arterial oxygen tension and reduced lipid peroxide, superoxide dismutase, and glutathione peroxide levels. Histology showed less thickening and oedema of the alveolar walls in the thioredoxin group than in controls (Fukuse *et al.* 1995). The thioredoxin administered during reperfusion significantly increased arterial oxygen tension after reperfusion (Okubo *et al.* 1997). Human thioredoxin also reduced reperfusion injury in a canine lung transplantation model (Yagi *et al.* 1994).

Thioredoxin has also been investigated in the kidney, in C57BL/6 and transgenic mice overexpressing thioredoxin during renal I/R (Kasuno *et al.* 2003). With I/R, endogenous murine thioredoxin was rapidly depleted from the cytosol in the cortical proximal tubules and detected in the urinary lumen. The urinary excretion of thioredoxin increased transiently after I/R. These transgenic mice with overexpression of thioredoxin were more resistant to I/R injury compared with wild-type mice, suggesting that exogenous application of thioredoxin may reduce renal I/R injury.

Thioredoxin can permeate the blood-brain barrier in cerebral ischaemia, which has an important biological role in the regulation of various intracellular molecules. Exogenous thioredoxin exerts distinct cytoprotective effects on cerebral I/R injury in mice by means of its redox-regulating activity (Hattori *et al.* 2004). Expression of thioredoxin in transgenic mice decreased ischaemic neuronal injury, after focal brain ischaemia, in comparison to wild-type mice. This shows that thioredoxin and the redox state modified

by thioredoxin play a crucial role in brain damage during I/R injury (Takagi *et al.* 1999).

The role of thioredoxin in redox signalling and amelioration of I/R injury in the ischaemic liver is a promising field for research.

LIPOIC ACID

Lipoic acid is a dithiol (8-carbon open or cyclic disulfide) that is found as a prosthetic group in α-keto acid dehydrogenase complexes of mitochondria and has an important role in cell metabolism (Packer 1998) . It is a cofactor in the multi-enzyme complexes that catalyse the oxidative decarboxylation of α-keto acids, such as pyruvate and α-ketoglutarate (Takaoka *et al.* 2002). It has antioxidant properties due to direct radical scavenging and metal chelating by both lipoic acid and its reduced form dihydrolipoate (DHLA). It also has regulatory action on signal transduction processes. Levels of free lipoic acid in tissues and body fluids are very low, so it is unlikely to have antioxidant effects in vivo. However, exogenous administration of lipoic acid could have beneficial effects in conditions associated with oxidative stress, since it has a wide range of antioxidant properties and also the ability to promote the synthesis of other endogenous antioxidants such as α- tocopherol and GSH (Packer *et al.* 1995). When administered to cells, lipoate is reduced rapidly to DHLA and released outside the cell. DHLA released from cells reduces extracellular cystine to cysteine, and thus promotes cellular cysteine uptake and through this GSH synthesis. It can also reduce GSSG to GSH.

The administration of α-lipoic acid has been shown to be beneficial in various organ oxidative stress injury. It has potential applications both in the prevention and treatment

of diabetic complications such as diabetic neuropathy (Packer *et al.* 1995) where

oxidative stress is regarded as main pathogenetic factor (Pacher *et al.* 2005). α-lipoic

acid (thioctic acid) is available in Germany and has been used extensively in clinical

trials for the treatment of diabetic neuropathy. A previous review of 15 clinical trials

(Ziegler *et al.* 1999) and subsequent meta-analysis (Ziegler *et al.* 2004) involving the

use of α-lipoic in the treatment of diabetic neuropathy showed promising improvement

in neuropathic symptoms and neuropathic deficits in patients with diabetic

polyneuropathy. A small case series suggests that α-lipoic acid may also ameliorate

chemotherapy-induced neuropathy caused by docetaxel and cisplatin (Gedlicka *et al.*

2003). In vitro and in vivo experimental models of I/R injury have shown a beneficial

role for α-lipoic acid administration in protection to heart (Coombes *et al.* 2000; Scheer

and Zimmer 1993), brain (Packer *et al.* 1997), kidney (Takaoka *et al.* 2002) and retina

(Chidlow *et al.* 2002). In liver I/R injury, pre-treatment with α-lipoic acid decreased the

indices of I/R injury both in isolated perfused livers and in vivo (Muller *et al.* 2003). In

the latter study (Muller *et al.* 2003) the authors observed that inhibition of the

phosphatidylinositol PI3-kinase/Akt pathway, blocked the protective effect of α-lipoic

acid on I/R injury. In another experimental study with rat hearts, phosphatidylinositol-3-

kinase activation upstream of protein kinase C has been proposed as a signalling

pathway in the mechanism of ischaemic preconditioning (Tong *et al.* 2000). Although

the cellular contents of α-lipoic acid were not measured in Muller's study (Muller *et al.*

2003) and the evidence for liver protection is indirect through pharmacological

manipulation, this study provides encouragement to further studies in order to explore

the potential role for α-lipoic acid as a therapeutic agent in liver I/R injury. Clinical

trials regarding pharmacological benefits of α-lipoic acid in liver I/R injury are lacking.

There are no reports of serious side effects with α-lipoic acid administration in animal

or human studies and this should encourage clinical trials with α-lipoic acid in liver I/R injury.

GLUTATHIONE

Glutathione (GSH) is an endogenous molecule present in all animal cells, in millimolar concentrations. It has multifaceted physiological functions (Smith *et al.* 1996; Wu *et al.* 2004) such as antioxidant defence, detoxification of xenobiotics, modulation of redox-regulated signal transduction, regulation of cell proliferation, synthesis of deoxy-ribonucleotides, regulation of the immune response and regulation of leukotriene and prostaglandin metabolism. The cysteine sulfhydryl (-SH) side chain of glutathione accounts for most of its physiological properties.

The antioxidant function of GSH occurs through two mechanisms of reaction with ROS: direct or spontaneous and glutathione peroxidase (GPX) catalysed. As a major by-product of such reactions oxidised glutathione (GSSG) is produced (reaction 4). GSSG, can then be again reduced to GSH with the participation of glutathione reductase and NADPH (reaction 5):

$$GSSG + NADPH + H + \longrightarrow 2GSH + NADP \qquad (5)$$
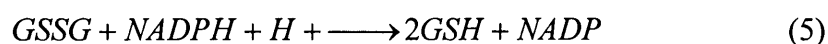
Depletion of reduced GSH indicating a decrease in the antioxidant capacity of the cell, has been associated with aging (Droge 2002; Thomas and Mallis 2001; Suh *et al.* 2005), inflammation (Villa *et al.* 2002), I/R injury (Leichtweis and Ji 2001) and neurodegenerative diseases (Drake *et al.* 2002; Bharath *et al.* 2002).

In liver I/R injury, two experimental studies with a rat model of liver transplant (Schauer *et al.* 2004b) and liver I/R (Schauer *et al.* 2004a), have used a continuous intravenous administration of GSH during reperfusion. This decreased sinusoidal endothelial cell and hepatocellular injury, improved postischemic bile flow and restored sinusoidal flow. The GSH-mediated protection of the liver grafts was associated with an increase of GSSG in plasma, suggesting detoxification of ROS by the intravenously administered GSH. Previous studies from the same group showed that continuous infusion of GSH during reperfusion of cold preserved livers (Bilzer *et al.* 1999) and after warm I/R (Bilzer *et al.* 2002) prevented Kupffer cell-related reperfusion injury. Continuous infusion of GSH has been used in another experimental study and protected the liver from endotoxin induced liver injury during I/R (Liu *et al.* 1994). These studies show a therapeutic potential for GSH in ROS- mediated reperfusion injury.

A similar finding with GSH administration was found with myocardial I/R. In an experimental study where pigs underwent coronary I/R the use of a glutathione monoethylester reduced reperfusion-induced myocardial damage (Guarnieri *et al.* 1993). The infusion with glutathione monoethylester decreased the depletion of tissue GSH and improved the GSH/GSSG ratio. Moreover, the drug decreased mitochondrial dysfunction at the level of pyruvate utilization and partially prevented the fall in ATP in reperfused tissue. GSH administration has also been shown to improve the recovery of myocardial function after I/R in perfused hearts. This protective effect could be through detoxification of peroxynitrite and the stimulation of soluble guanylate cyclase (Cheung *et al.* 2000). Addition of GSH in crystalloid cardioplegia detoxifies $ONOO^-$ and forms cardioprotective nitrosoglutathione, resulting in attenuated neutrophil adherence and

endothelial protection through the inhibition of neutrophil-mediated damage (Nakamura *et al.* 2000).

Overall, the evidence suggests that exogenous GSH administration is associated with reduction of I/R injury in experimental settings associated with extracellular oxidative stress. Also, GSH has low toxicity in humans (Aebi *et al.* 1991), low cost and appears to be an attractive agent for use in clinical settings. However, we should bear in mind that GSH is a large molecule that is not transported efficiently into cells and exogenous GSH administration may not exert significant antioxidant effects in situations associated mainly with intracellular oxidative stress(Amersi *et al.* 2002; De Flora *et al.* 2001; Jaeschke 1991).

N-ACETYLCYSTEINE

As mentioned above, exogenous GSH administration has limited cellular uptake due to its large molecular size and this may limit its value in situations associated with severe intracellular oxidative stress. Also a common limiting factor in GSH synthesis is the bio-availability of cysteine inside the cell. Cysteine per se is highly unstable in its reduced form. As a result, considerable research has focused on alternative strategies for cysteine delivery. In the N-acetylated form, i.e. N-acetylcysteine (NAC) the redox state of cysteine is markedly stabilized. After free NAC enters a cell, it is rapidly hydrolysed to release cysteine (Cotgreave 1997). NAC (figure 3) has a much smaller molecular size compared to GSH and was initially introduced for the treatment of congestive and obstructive lung diseases, primarily those associated with hypersecretion of mucus (e.g. chronic bronchitis and cystic fibrosis) (Boman *et al.* 1983). NAC is currently the drug

of choice for the management of fulminant liver failure due to paracetamol overdose (Cotgreave 1997). The diversity in the pharmacological uses of NAC is due to the multiple chemical properties of the cysteinyl thiol of the molecule. These include its nucleophilicity and redox reactions. The main mechanism of action of NAC is through the metabolism to cysteine in vivo and synthesis of GSH (Winniford *et al.* 1986). However, some effects of NAC are not mediated by an increase of GSH, as suggested by experiments with inhibitors of GSH synthesis or the D-stereoisomer of NAC (D-NAC) that cannot be converted to GSH. These effects include direct scavenging of ROS and RNS, inhibition of apoptosis (Yan *et al.* 1995), antiproliferative effects (Kim *et al.* 2001) and direct reduction of functional protein thiols at the cell surface (Laragione *et al.* 2003).
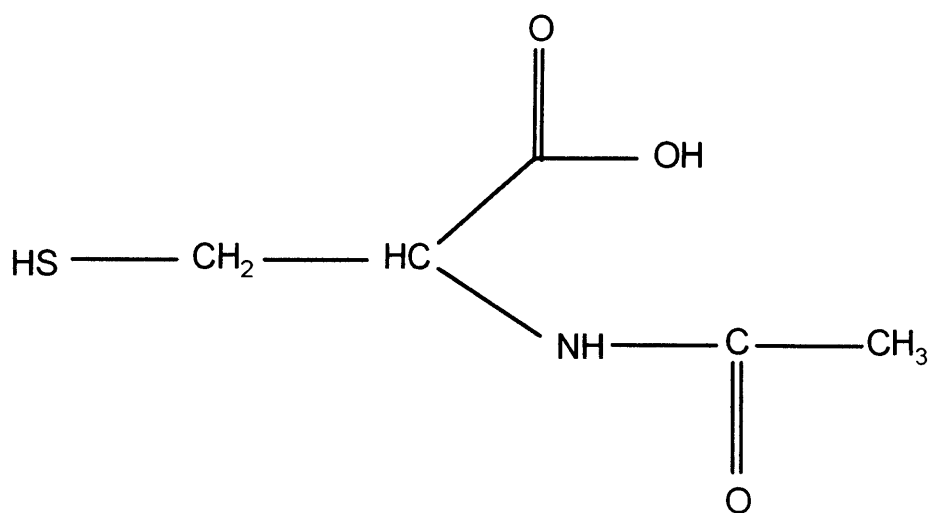
Figure 2.3: Chemical structure of N-acetylcysteine

The clinical benefit of NAC in liver I/R is still ambivalent (Chavez-Cartaya et al. 1999; Koeppel et al. 1996). This probably relates to differences in the method of NAC administration and the duration of follow up.

In the field of liver transplantation, there are six published small (9-30 patients in each group) clinical trials (5 randomised controlled trials and one open label pilot study) that assessed the effect of intravenous NAC administration and patient outcome. In the first study NAC improved oxygen delivery and consumption but had no effect in postoperative graft function, morbidity or mortality (Bromley et al. 1995). The second failed to show any clear protection on postoperative graft function (Steib et al. 1998). The third clinical study showed that NAC administration was associated with better liver function, less hepatocellular injury and lower incidence of primary graft dysfunction (Thies et al. 1998). The fourth study showed that NAC attenuated the increase in α-glutathione S-transferase, circulating ICAM-1 and VCAM-1 after liver transplantation, indicating cytoprotective effects (Weigand et al. 2001). In the fifth study NAC was used in combination with prostaglandin E_1 in pediatric liver transplant recipients. In this pilot study peak serum ALT was lower and median postoperative in-hospital stay was shorter in the treated group, while rejection was less severe (Bucuvalas et al. 2001). The most recent published study (Khan et al. 2005) is a small study (9 patients each group), where NAC was administered (intravenously and via the portal vein) only to the donors. NAC administration did not affect peak transaminases or episodes of acute rejection in the recipients.

It is obvious that there is a significant variability in the results of these clinical trials. This could be due to differences in methodology, time and length of administration and primary end points. No large scale randomised trial has been performed as yet.

Another interesting effect of NAC administration is the reversal of the effects of ischaemic preconditioning. Ischaemic preconditioning is the application of short periods of I/R prior to major ischemic insult and is associated with a protective effect in different I/R settings (Koti et al. 2002; Koti et al. 2003; Murry et al. 1986; Peralta et al. 1997). ROS are thought be essential for the preconditioning response against I/R injury (Jaeschke 2003; Sindram et al. 2002) suggesting a role for ROS in redox signalling. Two studies found that the inhibition of ROS by NAC was detrimental to liver protection from ischaemic preconditioning. Sindram et al (Sindram et al. 2002) found that pre-treatment with NAC reversed the beneficial effects of ischaemic preconditioning on sinusoidal endothelial cell detachment and apoptosis in rat livers subjected to cold preservation and reperfusion injury. Tejima et al (Tejima et al. 2004) found that scavenging of ROS with NAC treatment reversed the protective effects of ischaemic preconditioning in rat livers subjected to warm I/R injury. These findings suggest that small amounts of ROS are acting as signalling molecules for the body's response against I/R injury.

NAC has been investigated extensively in the treatment of I/R in other organs. Animal studies utilising NAC in myocardial I/R injury showed that NAC administration reduced the extent of infarction and limited oxidative stress with enhanced contractile recovery (Fischer et al. 2003; Sochman et al. 1990; Tang et al. 1991). A pilot clinical study (Arstall et al. 1995) showed that the combination of NAC, nitroglycerin and

streptokinase in the treatment of myocardial infarction, produced less oxidative stress, more rapid reperfusion and better preservation of left ventricular function. The ISLAND (Infarct Size Limitation: Acute N-acetylcysteine Defense) a randomized angiography- and echocardiography-controlled study (Arstall *et al.* 1995; Sochman *et al.* 1995; Sochman *et al.* 1996; Sochman 2002) showed that a combination of NAC and streptokinase reduced infarct size and improved left ventricular (LV) function, in comparison to patients with reperfusion induced by streptokinase alone or in comparison to patients with failed reperfusion.

In a small prospective randomised clinical trial (Andersen *et al.* 1995) the perioperative administration of N-acetylcysteine in patients undergoing coronary artery bypass grafting significantly reduced the postoperative neutrophil oxidative burst response, an indicator of oxidative stress. Subsequently Tossios et al (Tossios *et al.* 2003) studied the effect of NAC administration on oxidative stress markers, in LV biopsy specimens, taken from patients who underwent coronary artery bypass surgery. They found that in the NAC group the levels of 8-iso-prostaglandin-F_2a (a marker of lipid peroxidation) and of nitrotyrosine (a marker of nitrosative stress) were significantly lower compared with the placebo group, at the end of the cardiopulmonary bypass. The same group (Fischer *et al.* 2004) also studied the effect of NAC on myocardial apoptosis in the same patient group. They found that cardioplegic arrest initiates the apoptosis signal cascade in human LV cardiac myocytes (as expressed by caspases −3 and −7). NAC prevented the cardioplegia-induced apoptosis. Despite these promising small-scale trials, no large-scale trial has been conducted to confirm a clear benefit of NAC in reducing reperfusion injury in the treatment of acute myocardial infarction or surgically induced I/R during cardiac surgery (Marczin *et al.* 2003; Sochman 2002).

In a recent study NAC ameliorated lung reperfusion injury in dogs after deep hypothermia and total circulatory arrest (Cakir *et al.* 2004). Renal I/R injury is of clinical interest because of its role in renal failure and renal allograft rejection. Experimental studies have shown that NAC administered prior to ischaemia and immediately before the reperfusion period reverses I/R induced nephrotoxicity, as evidenced by decreases in blood urea nitrogen and creatinine and better histology (Sehirli *et al.* 2003). This protective effect is associated with less oxidative stress as indicated by less lipid peroxidation and maintenance of GSH levels. NAC has also been shown to ameliorate ischaemic acute renal failure in experimental models of kidney transplantation. Donor pretreatment with NAC preserved renal metabolism and improved outcomes of I/R injured kidney transplants (Fuller *et al.* 2004; Lin *et al.* 2004).

Administration of NAC before ischaemia attenuated cerebral I/R injury in a rat model of experimental cerebral ischaemia (Cuzzocrea *et al.* 2000b; Sekhon *et al.* 2003). Animals treated with NAC showed significant reductions in brain infarct volume and the neurological score as compared to the untreated animals. NAC treatment also maintained GSH levels and reduced apoptotic cell death in ischaemic brain. NAC treatment also blocked the I/R-induced expression of TNF-α and inducible nitric oxide synthase (Khan *et al.* 2004).

A newly designed amide form of NAC, N-acetylcysteinamide (AD4), in which the carboxylic group is neutralized, is more lipophilic and cell-permeating. This compound was shown to cross the blood-brain barrier, scavenge free radicals, chelate copper ions

and protect mice from experimental autoimmune encephalomyelitis, a condition used as a animal model of multiple sclerosis and characterized by significant oxidative stress (Offen *et al.* 2004). Also AD4 protected human red cells from oxidative stress more efficiently than NAC (Grinberg *et al.* 2005).

BUCILLAMINE

Bucillamine is a low molecular weight thiol, containing two sulphydryl groups. Along with its antioxidant properties it has anti-inflammatory properties and is used for the treatment of rheumatoid arthritis (Horwitz 2003). Bucillamine is fourfold more potent than NAC in *in vitro* studies (Whitekus *et al.* 2002) and in mice 20 mg /kg i.p of bucillamine had similar effects to a 16-fold greater dose of NAC (320 mg/kg i.p.).

Similar to other cysteine derivatives such as NAC, bucillamine has the ability to replenish intracellular GSH, prevent decrease in intracellular GSH and thus maintain the antioxidant line of defence against oxidative stress. Its effects in I/R injury have only been evaluated in experimental studies so far. Amersi and colleagues (Amersi *et al.* 2002) conducted the first study investigating the effect of bucillamine on oxidative stress and I/R injury in a rat model of liver transplantation. This was a well conceived and executed study, involving ex vivo perfusion of normal and fatty livers and an in vivo model of syngenic orthotopic liver transplants in rats. In these models bucillamine treatment resulted in significantly decreased I/R injury and increased survival after transplant, increased levels of GSH in the liver and decreased levels of oxidized glutathione in both liver and blood. These findings suggest that this agent may be a useful target in liver protection against I/R injury. In a phase I human study in normal

volunteers, bucillamine at doses up to 25 mg/kg/h i.v. for 3 h elicited no serious drug-related adverse effects (Horwitz 2003).

Regarding cardiac I/R injury Horwitz and Sherman (Horwitz and Sherman 2001) demonstrated in isolated rat cardiac myocytes that it is a potent antioxidant; bucillamine (125-500 μM) prevented lactate dehydrogenase (LDH) release in cardiac myocytes exposed to hydrogen peroxide or a xanthine/xanthine oxidase system. Further, in dogs subjected to 90 min of coronary artery occlusion and 48 h of reperfusion, bucillamine, administered during reperfusion decreased myocardial infarct size by 41% (Horwitz and Sherman 2001).

2.2.6 CONCLUSIONS

Liver I/R injury occur in a number of clinical settings in general surgery and is associated with increased morbidity and mortality. ROS and RNS play a major role in the pathophysiology of I/R injury. The antioxidant defence system is a complex system that includes intracellular enzymes, non-enzymatic substances that act as scavengers and dietary components. It normally controls the production of ROS and RNS. Oxidative stress occurs when there is significant imbalance between production and removal of ROS and RNS. This occurs principally when antioxidants are depleted or oxidants are overproduced.

Antioxidant therapy is a promising therapeutic strategy to ameliorate liver I/R injury. Drug therapy has distinct advantages when compared to surgical strategies such as ischaemic preconditioning and intermittent clamping, since it can be applied in conditions where the surgical strategies can not be applied (typically transplantation or

haemorrhagic shock) and at the same time avoid the detrimental effects that the surgical techniques possess. In recent years new strategies have been developed using for example SOD and CAT derivatives, thiol compounds, selective NOS inhibitors, peroxynitrite decompositors, and gene therapy. Although the results are still not entirely clear; there is accumulative evidence, mainly from in vitro and in vivo experimental studies, that the administration of antioxidant substances can reduce the post reperfusion liver injury.

However important parameters have to be taken in account before antioxidant therapy is applied: a) Free radical pathways are very complex and more studies are needed for an in depth understanding of the mechanisms involved. ROS and RNS at low concentrations have an important role as signal, trigger and messenger molecules in cellular growth and metabolism (Gutteridge and Halliwell 2000; Olthoff 2002). The understanding of the signal transduction mechanisms and the role of ROS and RNS in them enables us to develop therapeutic strategies that will then target detrimental oxidative stress but without affecting the pathways that are responsible for normal cell growth, repair and regeneration after liver I/R. b) Free radicals are very reactive with extremely short half lives. Timing of intervention is critical. c) Certain antioxidants can also exert prooxidant effects under specific conditions (Vertuani *et al.* 2004). d) Differences between species, duration of ischaemia, dose, timing and mode of drug administration, distribution problems and different end points may account for the inconsistency found in the results.

Regarding thiols antioxidant action is determined by both, efficient detoxification of free radicals and inactivation of the concurrently created thiyl radicals. Determining

which signalling pathways lead to alterations in thiol metabolism is critical for understanding the mechanisms of and developing therapies for exogenous oxidative stress.

In choosing a thiol for a specific action the global effects should be considered. It has been found that large increases in free thiols in the circulation are associated with toxic effects. This could be the results of thiyl radical mediated reactions. The changes also in the thiol redox gradient across cells could affect transport or cell signalling processes which are dependent on formation and rupture of disulphide linkages in membrane proteins.

In the next chapter the experimental model, along with the methodology used in this thesis is described.

2.3 Chapter summary

Oxidative stress is an important factor in many pathological conditions such as inflammation, cancer, ageing and organ response to ischaemia-reperfusion. Humans have developed a complex antioxidant system in order to eliminate or attenuate oxidative stress. Liver ischaemia-reperfusion injury occurs in a number of clinical settings, including liver surgery, transplantation and haemorrhagic shock with subsequent fluid resuscitation; leading to significant morbidity and mortality. It is characterized by significant oxidative stress and accompanied with depletion of endogenous antioxidants. The administration of antioxidant agents could provide protection from the harmful effects of I/R injury.

Thiol-containing compounds have an essential role as antioxidants and signalling molecules. They participate in many biochemical reactions due to their ability to be easily oxidised and then quickly regenerated. Main representatives are glutathione, lipoic acid and thioredoxin which are synthesised de novo in mammalian cells. N-acetylcysteine and Bucillamine are synthetic thiols which have been administered in experimental and clinical studies for treatment of conditions associated with oxidative stress.

This review-chapter had two aims: firstly, to describe the main pathways by which the antioxidants function. Secondly, to highlight the new developments that are ongoing in antioxidant therapy and to present the experimental and clinical evidence about the role of antioxidants in modulating hepatic ischaemia-reperfusion injury with particular emphasis on thiols.

CHAPTER 3

MATERIALS AND METHODS

3.1 Animal model of lobar liver I/R injury

The study was conducted under a project license granted by the Home Office in accordance with the Animals (Scientific Procedures) Act 1986. New Zealand white rabbits were used for the experiments. All animals were kept in temperature controlled environment with 12 h light-dark cycle. Animals were kept without food overnight prior to the experiments. Experimental research protocols were approved by the hospital ethics committee.

Anaesthesia was induced by intramuscular injection of 0.5 ml/kg Hypnorm™ (fentanyl citrate and fluanisone; Janssen Animal Health, High Wycombe, Buckinghamshire, UK). Following tracheostomy, anaesthesia was maintained with 0.5–3 % isoflurane through an anaesthetic circuit. Body temperature was maintained at 37–38.5°C by a warming blanket (Harvard Apparatus, Southmatick, Massachusetts, USA) and monitored with rectal temperature probe. Haemoglobin saturation and heart rate were recorded continuously by a pulse oximeter (Ohmeda® Biox 3740 pulse oximeter; Ohmeda, Louisville, Colorado, USA). A radio-opaque catheter 20 gauge (GA) was inserted into the right femoral artery or into the right ear marginal artery and connected to a pressure transducer for monitoring of mean arterial blood pressure. Ear marginal veins were cannulated with radio-opaque catheters (22 GA) for the administration of anaesthetics, fluids and medication.

Laparotomy was performed through a midline or transverse subcostal incision. The ligamentous attachments from the liver to the diaphragm were divided and the liver was exposed. Lobar ischaemia was induced by clamping the vascular pedicles of the median and left lobes of the liver, using an atraumatic microvascular clip. This method produces

a severe ischaemic insult without mesenteric venous hypertension (Koo *et al.* 1992). After 60 min of ischaemia, the vascular clip was removed and reperfusion was allowed for up to 7 h. At the end of the experiment the animals were killed by exsanguination.

Hepatic microcirculation was continuously measured via a probe placed on a fixed site on the median lobe of the liver and held in place by a retort holder during ischaemia and reperfusion period. Hepatic tissue oxygenation was continuously measured via optodes placed on the surface of the left liver lobe during ischaemia and reperfusion period.

Blood samples (1 ml each) were collected from the arterial line at different time points during the experiment for blood gases, serum transminases, CK, LDH, plasma nitrite, nitrates and nitrosothiols.

Liver tissue at baseline and at the end of the experiment was taken from the ischaemic liver lobe, placed immediately in liquid nitrogen and stored in -80 °C for nitrotyrosine measurement. Also liver tissue samples fixed in 10% formalin for histological study.

3.2 Hepatic microcirculation

3.2.1 Basic principles

Hepatic microcirculation (HM) was measured by a surface laser Doppler flowmeter (LDF) (DRT4, Moor Instruments Limited, Axminster, UK) in flux units. Measurement is easy to perform and provides a continuous signal without interference with tissue blood flow (Almond and Wheatley 1992; Shepherd *et al.* 1987; Wheatley *et al.* 1993).

Briefly, a monochromatic laser light from a 2mV-helium neon laser operating at 632 nm is guided to the tissue via optical fibres. The back-scattered light from the tissue is

transmitted through optical fibres to photodetectors. Only the photons which are scattered by moving red blood cells will have a Doppler frequency shift, whereas those from the static tissue matrix will not be Doppler shifted. Mixing of these components at the photo detector surface produces an electrical signal containing all of the Doppler frequency shift information. Further processing of the signal produces an output voltage that varies linearly with the product of total number of moving red blood cells in the measured volume of a few cubic millimetres multiplied by the mean velocity of these cells.

Linearity of the LDF signal from the liver with total organ perfusion has been demonstrated and the technique has been shown to be sensitive to rapid changes in organ blood flow (Almond and Wheatley 1992; Hewett and Murray 1993; Shepherd *et al.* 1987; Wheatley *et al.* 1993). The LDF measurements are expressed in arbitrary perfusion units (flux). Due to the problems associated with variation in signal across the surface of the liver, it is not possible to apply a conversion factor so that the LDF signal can be expressed in absolute flow units (Almond and Wheatley 1992; Hewett and Murray 1993; Wheatley *et al.* 1993).

The application and reproducibility of LDF measurement for assessment of liver microcirculation has been validated in both experimental animals (Hewett and Murray 1993; Wheatley *et al.* 1993) and human liver transplantation (Seifalian *et al.* 1997).

3.2.2 LDF application in the rabbit, data collection, analysis

The hepatic microcirculation in this study was assessed using a commercially available dual channel surface laser Doppler flowmeter (DRT4, Moor Instruments Ltd., Devon,

UK) (Figure 3.1). The LDF was calibrated before each study against a standard reference (Brownian motion of polystyrene micro spheres in water) provided by the manufacturer. The LDF probe was placed on a fixed site on the median lobe of the liver in order to avoid any error due to anatomical variation in the microcirculation. It was held in place by a probe holder in order to minimise any disturbance to blood flow by the LDF probe pressure on the tissue. Data were collected continuously. Data from the continuous measurement by LDF was collected via the near infrared spectroscopy (NIRS) program that can accept the input of 4 different clinical monitors. After conversion of the NIRS data to excel sheets, the LDF data at the relevant points in each experiment was calculated as a mean of 2-min data.
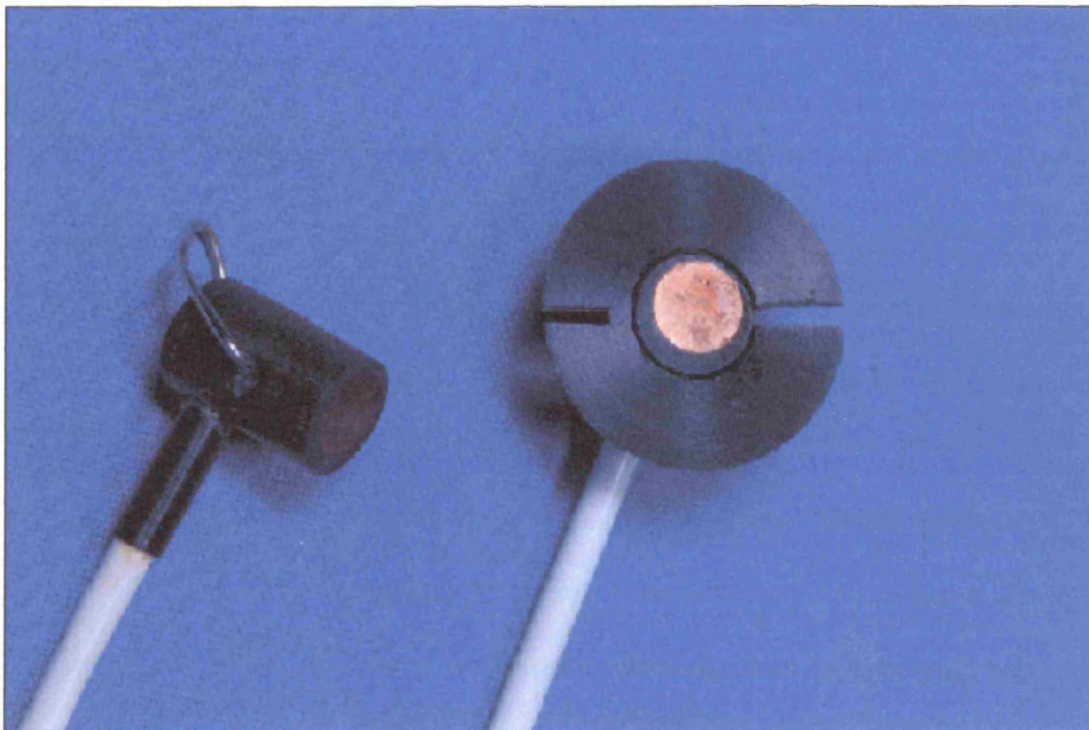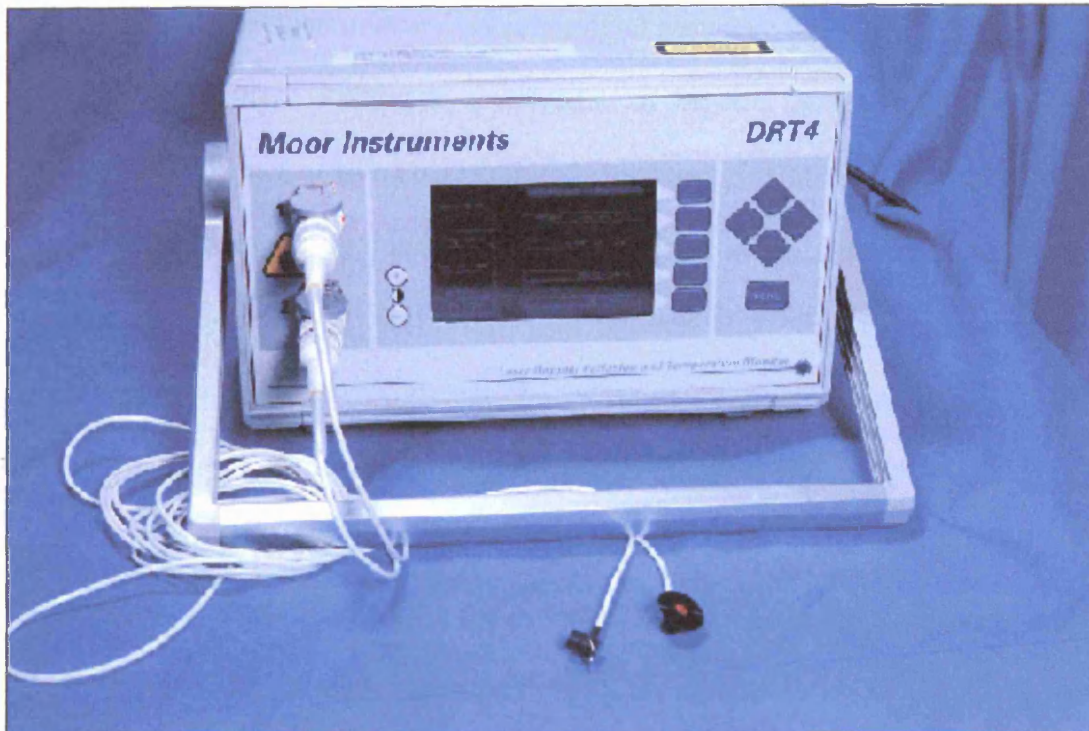
Figure 3.1. Dual channel surface laser Doppler flowmeter and its probes
(DRT4, Moor Instruments Ltd., Devon, UK).

3.3 Intracellular oxygenation / mitochondrial activity

Cytochrome oxidase activity as a reflection of hepatic intracellular oxygenation was continuously measured using near infrared spectroscopy (NIRS). In all tissues a number of colour-bearing compounds (chromophores), namely oxyhaemoglobin (HbO_2), deoxyhaemoglobin (Hb) and cytochrome oxidase (Cyt Ox) are present in variable concentrations. They have different absorption spectra in the near infrared (NIR) light and their absorption characteristics are oxygen dependent (Jobsis FF 1992).

Cytochrome oxidase (Cyt Ox) is the terminal complex of the mitochondrial respiratory chain (Figure 3.2). It takes electrons from cytochrome c and catalyses the reduction of oxygen to water with the concomitant synthesis of ATP through the oxidative phosphorylation process (Capaldi 1990; Edwards *et al.* 1991). In hepatocytes, approximately 90 % of the oxygen is consumed by mitochondrial cytochrome oxidase. Cyt Ox contains four redox-active metal sites: two haem iron (haem a and haem a_3) centres, the copper-copper dimer (Cu_A), and the binuclear haem-copper coupled centre (haem a_3/Cu_B). These four metal centres accept or donate electrons during the electron transfer through the respiratory chain, changing their redox state. The oxygen-binding site of the enzyme is the binuclear unit formed of the Cu_B and haem a_3. The donation of electrons from this unit to oxygen accounts for the majority of oxygen consumption in the tissues. The Cu_A and haem a centres donate electrons to the binuclear unit and therefore are not directly involved in oxygen reduction (Capaldi 1990; Nicholls and Ferguson 1982). In the absence of oxygen, electron transfer to oxygen cannot take place. Electrons accumulate on the haem and copper atoms and Cyt Ox becomes reduced. With oxygen availability the electrons are transferred rapidly from the metal centres to oxygen and Cyt Ox becomes oxidised. Many factors can affect the Cyt Ox

redox state in vivo, but the most significant factors are the oxygen concentration (Capaldi 1990; Cooper *et al.* 1994); nitric oxide in physiological circumstances (Cleeter *et al.* 1994; Cooper 2002) and oxidants such as ROS and RNS in oxidative stress situations (Shiva and Darley-Usmar 2003; Shiva *et al.* 2005).

All 4 centres exhibit different absorption characteristics depending on their redox state. The copper centres are optically sensitive in the NIR region in contrast with the haem centres that absorb visible light (Capaldi 1990; Cooper 2002; Edwards *et al.* 1991; Jobsis *et al.* 1976). Absorption of the NIR light by Cyt Ox occurs primarily at the Cu_A centre within Cyt Ox. The oxidised Cu $_A$ centre has a characteristic shape spectrum with a broad peak centred around 845 nm (Cooper *et al.* 1994; Jobsis FF 1992). The signal intensity decreases on reduction of this centre. The contribution of haem iron centres to absorption of NIR is less than 10 % of the total signal in the reduced − oxidised spectrum (Boelens and Wever 1980; Cooper *et al.* 1994).

The redox state of Cyt Ox Cu_A is dependent on cellular oxygen availability (Capaldi 1990; Cooper 2002; Edwards *et al.* 1991; Takashima *et al.* 1995). In the presence of oxygen electron transfer occurs and the enzyme becomes oxidised whereas lack of oxygen results in a decreased flow of electrons and Cyt Ox becomes reduced. The increase in the reduction state of Cyt Ox reflects severe cellular hypoxia.

Another synthetic chromophore that can be measured by NIRS is Indocyanine green (ICG). ICG is an anionic dye that has been used for many years to measure hepatic blood flow and as a test of liver function (Caeser *et al.* 1961; Grainger *et al.* 1983). It has a characteristic maximum absorption peak at 805 nm in the NIR light region

allowing its absorption coefficient to be incorporated in the NIRS algorithm to measure directly its concentration in the hepatic tissue (Shinohara *et al.* 1996).
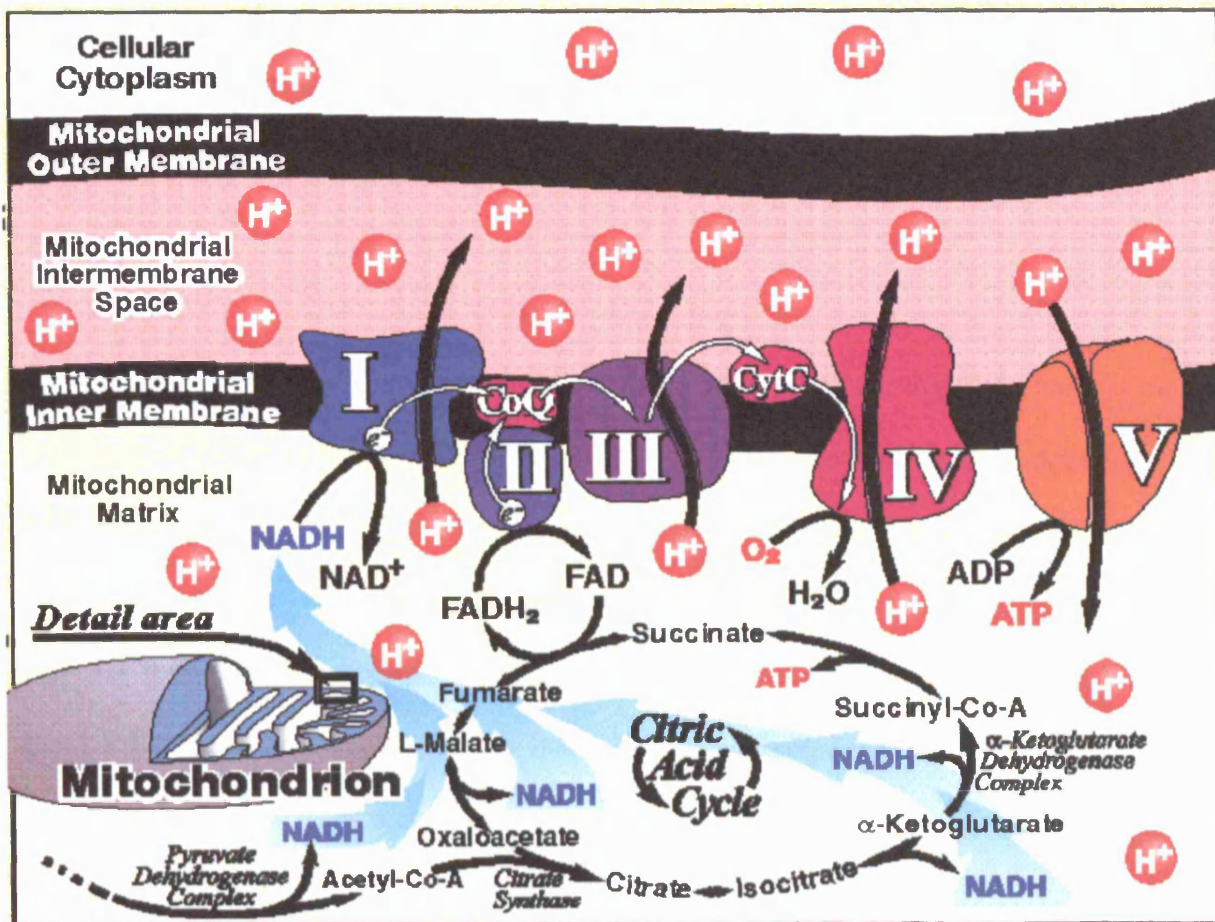
Figure 3.2. Schematic presentation of the mitochondrial respiratory chain

3.3.1 Basic principles of NIRS

Light interactions with the tissue involve the combination of reflectance, scattering and absorption which depends upon many factors including the light wavelength and the illuminated tissue type (Jobsis FF 1992). In the visible region of the spectrum (450-650 nm) light is strongly attenuated due to the intense absorption by haemoglobin and light scattering in tissues, which increases with decreasing the wavelength. Therefore, light

fails to penetrate more than 1 cm of tissue (Sterpetti *et al.* 1988; Jobsis FF 1992; Kitai *et al.* 1993). However, the NIR region of the electromagnetic spectrum (700-1000 nm) represents an optical window of relative transparency and a significant amount of radiation can be effectively transmitted through biological materials over distances of up to 8 cm (Jobsis FF 1992; Kitai *et al.* 1993; Sterpetti *et al.* 1988).

The technique of NIRS relies upon two main physical properties: (1) the relative transparency of biological tissue to light in the NIR region of the spectrum and (2) the existence of different tissue chromophores with characteristic absorption spectra in the NIR light spectrum (Elwell *et al.* 1994; Ojha *et al.* 1993; Wray *et al.* 1988). In tissue with homogeneous scattering the calculation of light attenuation and the relationship between the optical absorption and chromophore concentration may be described by a modified Beer - Lambert's law. The law modifications include (a) an additive term, G, due to scattering losses and (b) a multiplier, to account for the increased optical pathlength due to scattering. This law can be used to convert the obtained optical densities to concentration changes of Hb, HbO_2, and Cyt Ox in $\mu mole/L$ per optical pathlength (Ojha *et al.* 1993; Wray *et al.* 1988): A = α. c. d. B + G; where A is the attenuation of light (optical density), α is the absorption coefficient of the chromophore ($\mu mole^{-1}.cm^{-1}$), c is the concentration of the absorbing compound ($\mu mole/L$) and d is the geometrical distance between the points where light enters and leaves the tissue (cm). B the differential pathlength factor (DPF) which accounts for the increase in optical pathlength due to light scattering (which causes the optical pathlength to be greater than d) and G is a constant geometrical factor which accounts for loss of photons by scattering. As G cannot be quantified in vivo and is dependent upon the scattering coefficient of the tissue interrogated, it is not possible to measure the absolute

concentration of the chromophore in the tissue from measurement of the absolute attenuation. If α, B, and d are known and G assumed to remain constant during measurement, we can measure the change in the chromophore concentration (Δc) from measuring the change in attenuation (ΔA) from the following formula:

$$\Delta c = \Delta A \, / \, \alpha. \, d. \, B.$$

Since the absolute concentration of tissue chromophores are unknown and cannot be calculated due to the effect of light scattering within the tissue, all NIRS measurements are expressed as absolute concentration changes (μmole/L) from an arbitrary zero at the start of the measurement. The absorption coefficient of Cyt Ox was obtained in vivo from the brains of experimental animals whose blood had been replaced by a blood substitute (fluorocarbon) with exposure to 100% O_2 or N_2 to obtain the oxidised and reduced Cyt Ox spectra (De Blasi *et al.* 1997; Ojha *et al.* 1993; Williams *et al.* 1990; Wray *et al.* 1988)

For simultaneous computation of the changes in concentration of a number of chromophores from changes in attenuation at a number of wavelength, a mathematical operation (algorithm) can be used which incorporates the relevant absorption coefficient for each chromophore at each wavelength (Cooke *et al.* 1991; Cope 1991; Ojha *et al.* 1993; Wray *et al.* 1988).

3.3.2 Near infrared spectrophotometer

The NIR spectrometer used in this study is the NIRO 500 (Hamamatsu Photonics K.K., Hamamatsu, Japan) (Figure 3.3). This spectrometer is the commercial version of an

instrument developed by colleagues in the Department of Medical Physics and Bioengineering, University College London (Cope 1991). In the NIRO 500, the light source is monochromatic light generated from semiconductor laser diodes (LD). The light is produced at four wavelengths (774, 826, 849, and 906 nm). The choice of the wavelengths is based on 765 nm, the absorption maximum for Hb; 810 nm, the isobestic wavelength at which the extinction coefficients of HbO_2 and Hb are equal which can be used to calculate haemoglobin concentration independent of oxygen saturation; 845 nm, the absorption maximum for oxidised Cyt Ox; and 900 nm, a reference wavelength (Jobsis FF 1992; Ojha et al. 1993; Wray et al. 1988).

The light is produced by laser diodes and carried to the liver via a bundle of optical fibres in sequential pulses. The optical fibres are covered by a light proof protective sheath and its distal end terminated in a very small glass prism which reflects the light through 90° to direct it into the tissue (Elwell et al. 1994). Photons emerging from the liver are collected by the second bundle of optical fibres and detected by a photomultiplier tube (PMT) light detector (Elwell et al. 1994) (Figure 3.4). The incident and transmitted light intensities are recorded and from these the changes in the concentration of tissue chromophores (μmol/L) are calculated using an algorithm incorporating the known chromophores absorption coefficients and an experimentally measured optical pathlength.
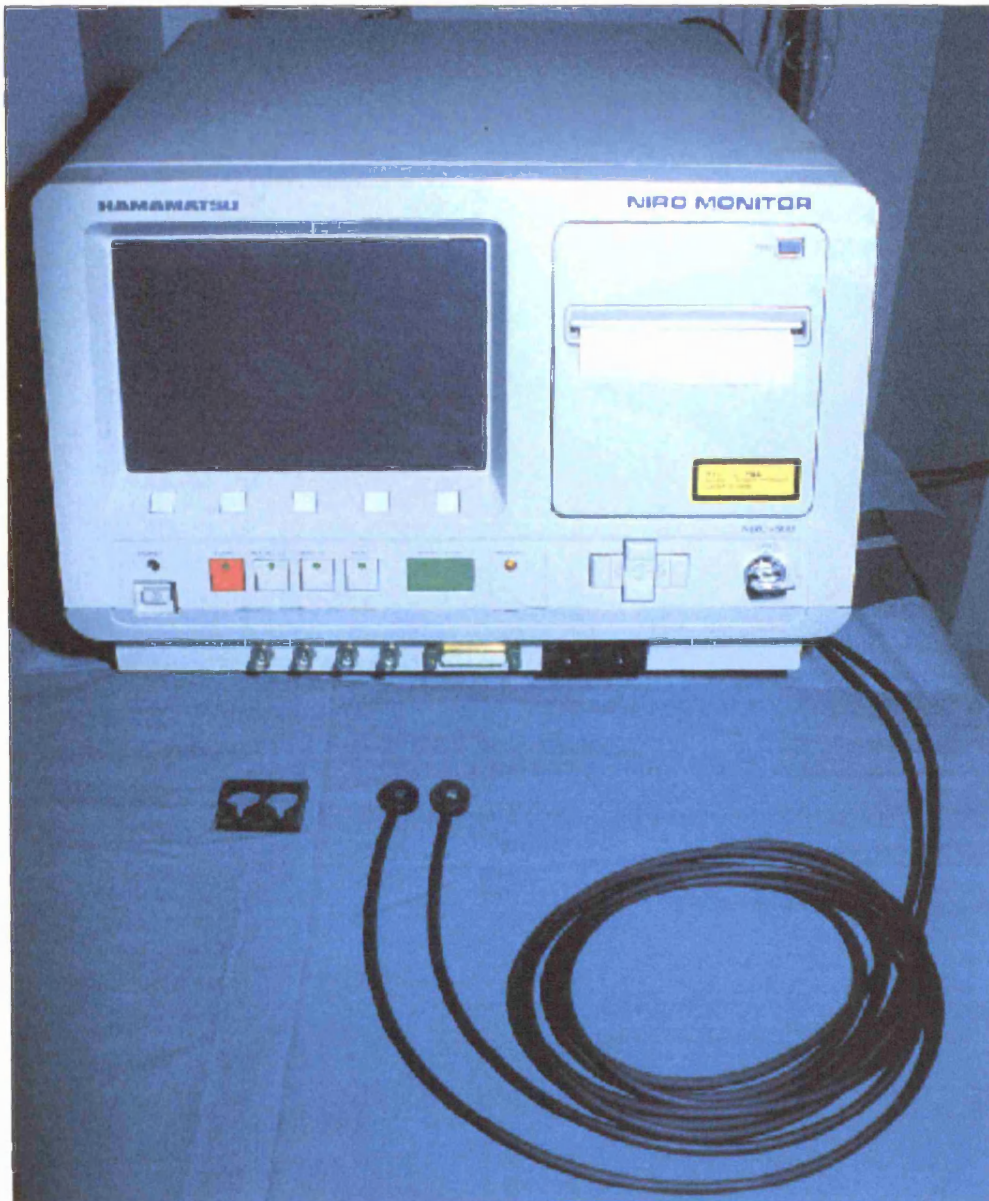
Figure 3.3: Near infrared spectrometer (NIRO 500, Hamamatsu Photonics KK,

Hamamatsu, Japan).
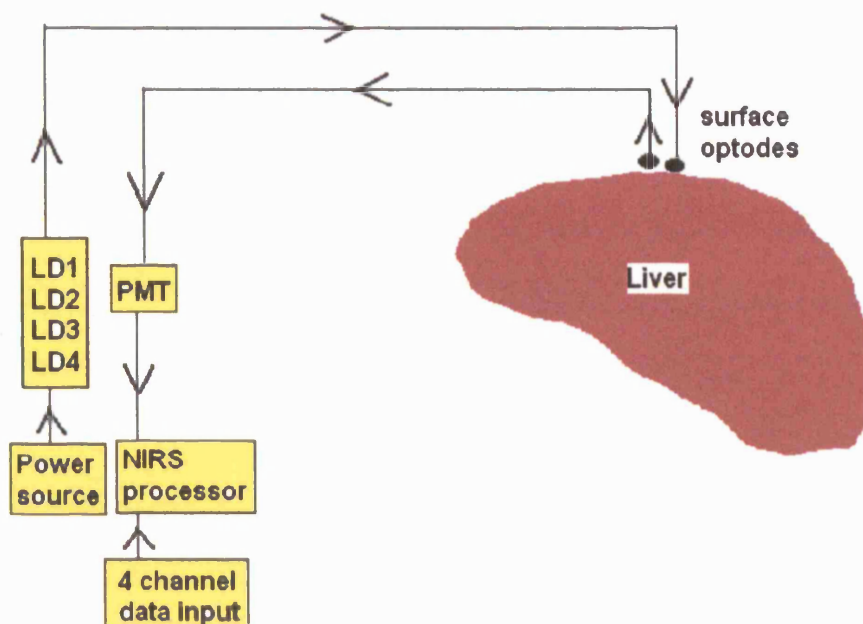
Figure 3.4: Schematic diagram of the NIRO 500 spectrometer. Four laser diodes (LD) are used as the monochromatic light source and a photomultiplier (PMT) is used to detect transmitted light from the tissue. Data from other monitors can be recorded simultaneously via the data input channels.

The standard NIRO 500 algorithm was developed using the wavelength dependant-pathlength for the brain tissue (Essenpreis *et al.* 1993). As a part of this study, modification of the NIRO 500 algorithm has been carried out in the Department of Medical Physics and Bioengineering, University College London for its hepatic application (Cope 1991). The absorption coefficient of a freshly dissected and viable blood free pig liver was measured. The absorption coefficient was found to be 0.04 mm^{-1} at 800 nm which is some four times larger than that of normally perfused brain tissue although the transport scattering coefficient was similar near 1.0 mm^{-1}. When the absorption coefficient of blood in a normally perfused liver was added to the blood free

absorption coefficient the overall absorption coefficient was near 0.1 mm^{-1} with the scattering coefficient essentially unchanged. The optical pathlength as a function of wavelength for normally perfused liver was then calculated incorporating the measured absorption coefficient and the scattering coefficient mathematically corrected for the contribution of haemoglobin absorption. The average blood content of the liver was assumed to be 12% by volume (Lautt and Greenway 1987) at an average haemoglobin saturation of 60%.

3.3.3 NIRS application in the rabbit, data collection, analysis

NIRS probes were mounted inside a probe holder and placed on the liver surface at a fixed site of the left lobe of the liver in all the animals in each experiment to avoid any anatomical variation which could influence in tissue oxygenation and blood volume. As a part of the modification of this instrument for use on the liver, a flexible rubber holder was made to hold the NIRS probes at a fixed spacing over the liver surface. This probe holder ensured that the sites of light entry and exit are maintained at a constant and known spacing distance which minimises the possibility of artefact due to changes in the distance between the probe ends. Also, it allows a satisfactory contact between the liver surface and the probe ends.

The NIRS includes the facility to set the attenuation and therefore chromophore concentration changes to zero with the NIRS initial setting. Since all the measurements are changes from an arbitrary initial zero, this function is important to ensure that artifacts such as system drift, optode movement, and excessive light having a minimal effect on the data. The NIRS data were continuously collected in a laptop computer connected to the NIRS. These data are the changes in light attenuation (optical densities:

OD) at four wavelengths due to absorption by the tissue chromophores. A software program called ONMAIN® (Hamamatsu Photonics K.K., Hamamatsu, Japan) was used to convert these data into changes in concentration changes of Cyt Ox (μmol/L) using the previously defined algorithm in the NIRO 500. This was then transferred to excel® data sheets (Microsoft Company, Seattle, USA) for analysis. The data at the relevant time points were collected as the mean of 2-minute data and calculated in regard to the baseline value at the start of the experiment.

3.4 Ultrasonic transit time flowmetry

Portal vein blood flow (PVBF) was measured using ultrasonic transit time flowmetry.

3.4.1 Basic principles

Using wide-beam illumination, two transducers pass ultrasonic signals back and forth, alternately intersecting the flowing liquid in the upstream and downstream directions. The transit time of such beam is a function of the volume flow intersecting this beam, regardless vessel dimensions or cross-sectional area. The flowmeter derives an accurate measure of the transit time it took the wave of ultrasound to travel from one transducer to the other. The difference between the upstream and downstream –integrated transit times is a measure of volume flow rather than velocity (Takata and Robotham 1992). The ultrasonic transit time flowmetry has been demonstrated to provide an accurate method for hepatic inflow measurement (Ayuse *et al.* 1994; Doi *et al.* 1988; Jakab *et al.* 1995).

3.4.2 Ultrasonic transit time flowmeter

PVBF was measured continuously using a dual transonic transit time flowmeter (HT207, Transonic Systems Inc, NY, USA). The flowmeter perivascular probe was placed around the portal vein. The accuracy of this technique is dependent on careful positioning and alignment of the probe with respect to the vessel. The vessel should be positioned within the central area of uniform ultrasonic intensity of the probe window i.e. away from the probe window edges, which have lower ultrasonic beam intensity (Takata and Robotham 1992). Also it requires accurate selection of the probe size which was determined by the outer diameter of the blood vessel.

3.4.3 Data collection, analysis

Data from the continuous measurement by the ultrasonic flowmeter were collected via the NIRS program. After conversion of the NIRS data to excel sheets, the ultrasonic flowmeter data at the relevant points were calculated as a 2-min data.

3.5 Liver transaminases

Alanine aminotransferase (ALT), aspartate aminotransferase (AST) and creatinine kinase (CK) activity were measured in serum. Blood samples were centrifuged at 2,000 g for 10 min at room temperature. Serum was separated from the samples and stored at $-20°C$ until assayed. Measurements were made using an automated clinical chemistry analyser (Hitachi® 747, Roche Diagnostics, Lewes, UK).

3.6 Histology

Small wedge biopsies were taken in baseline and at the end of the procedure from the ischaemic liver lobes. The liver tissue was fixed in neutral buffered formalin (10%),

embedded in paraffin and stained with haematoxylin and eosin (H&E). Sections were examined under microscope (digital light microscope CLF 60 optical system, Nikon UK Ltd, Surrey, UK). For assessment of the degree of injury a semi quantitative method was used in which a blinded liver pathologist estimated the number of neutrophils (PMN) in sinusoids, the aggregation of PMN, structural changes in Kupffer cells and the presence of necrosis and apoptosis in 5 high power fields per slide.

For steatotic livers formalin fixed but not paraffin embedded tissue was stained for fat using the Swank & Davenport modification of the Marchi method (Swank RL 1935). The grade of steatosis was analysed in a semi-quantitative manner: mild (<30 %), moderate (30-60 %) and severe (>60 %) using a clinically applied grading system (Adam *et al.* 1991).

3.7 Proton Nuclear Magnetic Resonance (^{1}H NMR) Spectroscopy

^{1}H NMR spectra of hepatic bile samples was obtained using spectrometer (Eclipse+ 500; JEOL Ltd., Tokyo, Japan) operating at 11.7 Tesla (500 MHz for ^{1}H). All Spectra were recorded at 25°C. 600 µL of bile was placed in a 5 mm tube with a 2 mm coaxial insert containing 100 µL of 1 mg/ml of sodium trimethylsilyl-[^{2}H$_4$]propionate (TSP) dissolved in deuterium oxide (^{2}H$_2$O, Fluorochem Ltd., Old Glossop, Derbyshire, UK) and set at 0.0 parts per million (ppm). A basic single pulse sequence was used to acquire the one-dimensional spectra using a 45° pulse (8.25 µs) with acquisition duration of 4.36 s and a relaxation delay of 5 s. To reduce the large water resonance signal a gated secondary irradiation at the water frequency was applied. Published peak assignments (Ellul *et al.* 1992; Melendez *et al.* 2001; Sequeira *et al.* 1994; Sequeira *et al.* 1995) were used. In brief, these were bile acid peaks (0.7-2.5 ppm), C-18 bile acids proton peaks (0.70 ppm), cholesterol peaks (0.90 ppm), PC tail group –(CH$_2$)$_n$ (1.20 ppm), lactate

methyl group (CH₃) (1.33 ppm), acetate (1.91 ppm), C-25 taurine methylene proton

peak (3.10 ppm), PC head group –N+(CH₃)₃ (3.20 ppm), C-26 taurine methylene proton

peak (3.50 ppm), C-25 glycine methylene proton peak (3.80 ppm), lactate (4.12 ppm).

Integration of the ^{1}H NMR spectra areas was measured relative to the TSP area and

obtained using MestRe-C software (version 2.3a, Departmento de Química Orgánica,

Universidade de Santiago de Compostela).

3.8 Capillary electrophoresis

The concentration of NO$^\bullet$ metabolites, nitrite (NO_2^-) and nitrate (NO_3^-), were

determined using capillary electrophoresis (Davies *et al.* 1999). Plasma samples were

centrifuged at 13000 rpm through a Vectrospin Micro 30 kDa ultra-filtration device

(Whatman International Limited, Kent, UK) for 30 min. A P/ACE System 5500 (CE

machine) equipped with a P/ACE diode array detector (DAD), automatic injector, a

fluid-cooled column cartridge and a System Gold data station (Beckman Instruments

Limited, Buckinghamshire, UK) was used for analysis. Samples were passed through a

50 µm x 47 cm (40 cm to detector) fused-silica capillary (Composite Metals Services,

Worcestershire, UK) with an electrolyte consisting of 150 mM sodium chloride / 5 mM

Tris-HCl at pH 7.4 with 2 mM TTAB. Prior to addition, the TTAB was passed through

a SAX anion exchange cartridge (Phenomenex Limited, Cheshire, UK) to replace the

bromine ions with hydroxide ions. The electrolyte was passed through a 0.45 µm

syringe filter (Whatman International Limited, Kent, UK) before use. The analysis was

performed at 25°C. The solutions were injected into the capillary by hydrodynamic

injection using a pressure injection of 0.5 psi for 15 s. The components in the samples

were separated using a voltage of ⁻10 kV. The DAD was set at 214 nm with a

bandwidth of 4 nm. The capillary was rinsed before each injection with 0.1 M NaOH

and electrolyte for 1 and 2 min, respectively. The sample concentrations of NO_2^- and NO_3^- were determined by comparison with standard solutions of sodium nitrite (0, 2, 4, 6, 8 and 10 μM) and sodium nitrate (0, 10, 20, 30, 40 and 50 μM), respectively. All chemicals were obtained from Sigma Chemicals Limited (Dorset, UK).

3.9 Electron Paramagnetic Resonance (EPR) Spectrometry

S-nitrosothiols (RSNOs) were measured in plasma by EPR spectrometry (Rocks et al. 2005) . With this technique the RSNOs are degraded using an alkaline pH (pH 10.5) in the presence of the spin trap complex N-methyl-D-glutamine dithiocarbamate $(MGD)_2$-Fe^{2+}.

Blood samples (1 mL) were added to EDTA-coated tubes containing 10 μL of N-ethylmalemide (NEM). The samples were centrifuged immediately at 3000 rpm for 10 minutes. The supernatant was aliquoted into small centrifuge tubes and snap frozen in liquid nitrogen and kept at -70 °C for up to 4 weeks.

During analysis plasma samples were placed in an evacuated glass vial, kept on ice and the concentration of RSNOs present was measured the same day. First, a solution of 0.2 M Caps buffer, adjusted to pH 10.5, was placed in a plain vacutainer (Becton-Dickinson UK Ltd., Oxford, UK) and deaerated for 15 min using nitrogen gas. A pH of 10.5 was used to in order to promote the decomposition of RSNOs to NO·, while avoiding the decomposition of nitrite to NO· that occurs under acidic conditions. The deaerated buffer was then used to prepare 20 mM ammonium ferrous sulfate solution in another

vacutainer deaerated with nitrogen gas. A gas-tight syringe was used to add the ammonium ferrous sulfate solution (500 µl) to MGD powder to form the $(MGD)_2$- Fe^{2+} complex (the final concentration of MGD and Fe^{2+} were 50 and 10 mM, respectively. $(MGD)_2$- Fe^{2+} solution (100 µl) was then removed using a gas-tight syringe and added to 100 µl of the biological sample or standard. These last two steps were performed using air-tight, evacuated, glass vials with a rubber septum in the cap. The NO released from the decomposed RSNOs reacts with $(MGD)_2$- Fe^{2+} to produce a paramagnetic complex, which is measured by EPR spectrometry. The optimum time to measure the $(MGD)_2$- Fe^{2+}-NO formed was determined by monitoring the decomposition of nanomolar concentrations of S-nitrosoglutathione and S-nitrosoalbumin over time. Consequently, the reaction mixtures were tested by EPR spectrometry 3 min after the biological fluid or standard had been added to the spin trap.

A calibration curve was constructed by adding $(MGD)_2$- Fe^{2+} to different concentrations of the standard, S-nitrosoglutathione. The final concentrations of S-nitrosoglutathione were 1000, 800, 600, 300 and 0 nM. The peak area of the $(MGD)_2$- Fe^{2+}-NO signal was plotted against the concentration of S-nitrosoglutathione added to the reaction mixture. In order to investigate if the yield of NO from S-nitrosoalbumin is similar to that of S-nitrosoglutathione, a preliminary experiment was performed. Human serum albumin sample containing a proportion of S-nitrosoalbumin was prepared by exposing a solution of albumin to acidified nitrite. This sample was analysed for RSNO content by the Saville reaction (Feelish and Stamler 1996; Saville 1958) using a GSNO standard curve (Gladwin *et al.* 2002). The same sample was then also analysed for RSNO content using the EPR assay calibrated using a GSNO standard curve as described above. On comparing the results from the Saville reaction with the results from the EPR

method, the value for RSNO content was in close agreement. This confirmed that the yield of NO from S-nitrosoalbumin was similar to the yield from GSNO.

A known amount of S-nitrosoalbumin was also spiked into the sample of whole human blood (immediately after collection onto EDTA and addition of NEM) to give a final concentration of 1 μM of added S-nitrosothiol). The sample was centrifuged as described above, and the resulting plasma sample was analysed by EPR spectrometry, providing a recovery of the added S-nitrosothiol of 71 %. This result is in agreement with a similar, previously reported, experiment in which a synovial fluid sample was spiked wit 1 μM (final concentration) of the low molecular weight S-nitrosothiol, GSNO, from which 71 % was recovered (Rocks *et al.* 2005).

The samples were analysed using a JEOL JES RE1X spectrometer (Jeol Ltd, Welwyn Garden City, UK) equipped with an ES-UCX2 cylindrical mode X-band cavity. Samples were analyzed at room temperature in a WG-LC-11 quartz flat cell (Wilmad Glass, Buena, NJ, USA). The instrument parameters differed for each set of experiments. The spectra were recorded and stored using an IBM-compatible computer with SpecESR software (version 1.0, 1998; JEOL limited, Hertfordshire, UK) and the mean peak area was determined. Water was taken through the same procedure to act as a control. The EPR spectrometer parameters were: microwave frequency 9.45 GHz, microwave power 20 mW, centre field 330 mT, sweep width ± 5 mT, sweep time 250 s, number of data points 8192, time constant 1 s, modulation frequency 100 kHz, and modulation width 0.4 mT. The spectra were taken as an average over 3 scans.

3.10 Dihydrorhodamine (DHR) oxidation

The oxidation of DHR 123 to rhodamine (RH) is partially peroxynitrite-dependent and has been used as a marker of peroxynitrite formation (Cuzzocrea *et al.* 2000a; Kooy *et al.* 1995). 7 h after reperfusion in the I/R group or the equivalent time in the sham group animals were injected with DHR 123 (2 μmol/kg in 0.8 mL normal saline 0.9 %, Sigma-Aldrich Company Ltd, Dorset, UK). Twenty minutes later plasma samples were collected for rhodamine fluorescence evaluation. For fluorescence measurements a fluorometer (Thermo Electron Corporation, Basingstoke, UK) was used at an excitation wavelength of 485 nm and emission wavelength of 538 nm. The concentration of RH formation was calculated using a standard curve obtained with authentic RH (1 to 40 nM, Sigma-Aldrich Company Ltd, Dorset, UK) prepared in plasma obtained from untreated rabbits.

3.11 Liver tissue Nitrotyrosine levels measured by mass spectrometry

Protein-bound nitrotyrosine levels were measured in liver tissue from rabbits at baseline and seven hours postreperfusion using an isotope-dilution mass spectrometric method (Frost *et al.* 2000; Moore and Mani 2002). Tissue was homogenised in a mixture of ice-cold saline (2 mL) and chloroform / methanol (2:1) containing 10 ng $^{13}C_9$-nitrotyrosine and the protein precipitate (middle layer) was isolated by centrifugation at 2000 x g for 30 minutes at 4 °C. The supernatant was used for measuring free nitrotyrosine (in ventricles) and the protein precipitates were lyophilized under vacuum for assessment of protein – bound nitrotyrosine. 1-1.5 mg of lyophilized protein was hydrolyzed for 15 hours at 120 °C in 1 mL 4 M sodium hydroxide following the addition of 10 ng $^{13}C_9$-nitrotyrosine and 10 μg of $^2H_4$-tyrosine as stable isotopic internal standards. These conditions prevent the artifactual nitration of tyrosine that occurs during acidic

hydrolysis conditions. Following two steps of solid phase extraction, nitrotyrosine and tyrosine were quantitated by stable isotope dilution gas chromatography / negative ion chemical ionization mass spectrometry. Results of protein bound nitrityrosine are expressed as a ratio of nitrotyrosine to tyrosine (pg/μg).

3.12 Data collection and statistical analysis

Data from the pulse oximeter, blood pressure and portal flow monitor, LDF and NIRS were collected continuously on a laptop computer. The data were averaged for two minutes before the induction of ischaemia (baseline), at the end of ischaemia and at the end of each hour of the reperfusion period. Changes in hepatic tissue oxygenation at the end of each period were calculated relative to baseline. Values are expressed as mean, standard deviation (s.d.). Student's t test for paired samples was used for comparison within the group, at different time points. Student's t-test for independent samples was used for comparisons between two groups and one-way ANOVA with Bonferroni adjustment for multiple comparisons, unless otherwise stated. $P < 0.050$ was considered statistically significant.

CHAPTER 4

ESTABLISHMENT AND REFINEMENT OF AN EXPERIMENTAL RABBIT MODEL FOR THE STUDY OF WARM LIVER ISCHAEMIA / REPERFUSION INJURY

4.1 Introduction

Liver I/R injury involves two phases, as mentioned previously, the early phase (within

two hours of reperfusion)(Jaeschke and Farhood 1991) and the late phase (after 4 h)

(Colletti *et al.* 1990; Jaeschke *et al.* 1990). The early phase is associated with Kupffer

cell-induced oxidative stress, while the late phase results mainly from the accumulation

of neutrophils and the production of ROS and RNS. The degree of injury is much more

extensive during the late phase. I/R pathophysiology is very complex with the activation

of different metabolic pathways and the release of mediators which induce liver injury

(Glantzounis *et al.* 2005a; Jaeschke 2003; Lentsch *et al.* 2000). Progress in the

development of effective therapeutic strategies for liver I/R injury requires further

clarification of the pathophysiology, and study of the mechanism and timing of any

beneficial effects.

The rabbit lobar liver I/R model has been used before for assessment of changes in

hepatic oxygenation measured by near infrared spectroscopy (El Desoky *et al.* 2001). In

that study anaesthesia was induced by 0.5 ml/Kg Hypnorm (fentanyl citrate and

fluanisone) and maintained with Hypnorm 2.5 mg/Kg intramuscularly and the inhaled

agent Halothane through face mask. Lobar ischaemia was applied in the median and left

liver lobe for 1 h followed by 7 h of reperfusion. Polyethylene catheters were inserted in

the femoral artery for monitoring of mean arterial blood pressure (MABP) and for blood

sampling and into the femoral vein for fluid administration.

In the present study the anaesthetic protocol was modified to allow a longer period of

study under stable anaesthesia. For maintenance of anaesthesia isoflurane 0.5-3% was

used though an anaesthetic circuit, along with 25-40 % of oxygen mixed with nitrus oxide (N_2O). The reason for this is that isoflurane is metabolised mainly in the lungs rather in the liver and avoids cumulative effects of hepatotoxicity. Also a tracheostomy was performed after the induction of anaesthesia with the placement of a paediatric size tube in the trachea. This offered better metabolic stability to the animals, by maintaining stable oxygen saturation and acid-base balance.

To assess both the early and the initial part of the late phase of liver I/R injury monitoring of the reperfusion period was extended up to 7 hours. During this period the rabbits remained under general anaesthesia. For technical reasons an ear marginal vein was used for administration of intravenous fluids instead of the femoral vein. The experiments were terminated by exsanguination.

The femoral arterial line was initially used for continuous monitoring of the arterial blood pressure and blood sampling. Use of a femoral arterial line is the standard method in experimental studies with rats (Martin *et al.* 1996; Munzenmaier and Greene 2006; Reinert *et al.* 2004; Torres-Reveron *et al.* 2006; Yoburn *et al.* 1984) and rabbits (Madsen *et al.* 1979; Mossberg and Taegtmeyer 1991; Nahoul and Gilbert 1991). It has also been used for studies focusing on liver function (El Desoky *et al.* 1999; Kotzampassi *et al.* 2003; Ueda *et al.* 1999) and liver I/R (El Desoky *et al.* 2001). However, the placement of the femoral arterial line could reduce limb blood supply with resulting systemic effects.

Prolonged limb I/R injury can produce reactive oxygen species (ROS) and consumption of endogenous antioxidants (Sun *et al.* 1999; Yassin *et al.* 2002) resulting in remote

organ injury including the heart, lungs, liver, bowel and kidneys. An experimental study with rats, showed that 4 h of bilateral limb ischaemia followed by 3 h reperfusion caused remote hepatic injury, induced mainly via activation of Kupffer cells. This process was complement dependent (Brock *et al.* 2001). Experimental and clinical studies (Corson *et al.* 1992; Edrees *et al.* 2003; Harkin *et al.* 2001; Yassin *et al.* 1996) have also shown that limb I/R injury can alter intestinal structure and permeability associated with systemic endotoxaemia and cytokines activation.

Previous studies have demonstrated systemic effects of complete limb I/R. Whether the reduction in limb perfusion encountered during femoral arterial line cannulation has a remote effect on organ function has not previously been reported; but it is clearly vital to experimental design of studies on liver function or hepatic I/R. The aim of the present study was to investigate the effect of a femoral arterial line on liver function, in a rabbit model of lobar liver I/R.

4.2 Materials and methods

4.2.1 Animal model

The study was conducted under a license granted by the Home Office in accordance with the Animals (Scientific Procedures) Act 1986. New Zealand white rabbits (3.4 ± 0.8 kg, n=24) were used. Anaesthesia was induced by intramuscular injection of 0.5 ml/kg Hypnorm (Fentanyl citrate and fluanisone, Janssen Animal Health Ltd., Buckinghamshire, UK). Following tracheostomy anaesthesia was maintained with Isoflurane (0.5-3%) through an anaesthetic circuit.

Body temperature was maintained at 37-38.5°C by a warming blanket (Homoeothermic blanket control unit; Harvard Apparatus, Southmatick, Massachusetts, USA). Haemoglobin saturation and heart rate were continuously recorded by a pulse oxymeter (Ohmeda Biox 3740 pulse oxymeter; Ohmeda, Louisville, Colorado, USA). For the placement of the arterial line in the femoral artery a 2-3 cm skin incision was performed in the right groin. After dissecting the subcutaneous tissue, the femoral structures were identified and the femoral artery was separated from the femoral vein and the femoral nerve. The distal part of the common femoral artery was ligated with 2/0 silk. A microvascular clip was applied in the external iliac artery. An arteriotomy was performed on the common femoral artery and a radiopaque catheter (20 GA) was inserted to the aorta and fixed with 2/0 silk. The femoral line was used for monitoring of the arterial blood pressure and collection of blood samples. For the placement of an ear arterial line, a 20G radiopaque catheter was placed in the right ear marginal artery and fixed with 3/0 Prolene. In both groups also an ear marginal vein was cannulated with radiopaque catheters (22 G) for the administration of anaesthetics and fluids. After the placement of the arterial and venous lines a laparotomy was performed through a roof top transverse incision. The ligamentous attachments from the liver to the diaphragm were divided and the liver was exposed. The bile duct was cannulated with a polyethylene catheter (PE-50, 0.58 mm inner diameter, Portex, Kent, UK) to measure bile flow.

Lobar ischaemia was induced by clamping the vascular pedicles of the median and left lobes of the liver, using a microvascular clip. This method produces a significant ischaemic insult without inducing mesenteric venous hypertension (Koo *et al.* 1992). After 60 min of ischaemia the vascular clip was removed and reperfusion was allowed

for 7 h. Normal saline 0.9% (15 ml/kg/h) was administered through the ear marginal vein to compensate for intraoperative fluid loss.

At the end of the experiment the animals were killed by exsanguination.

4.2.2 Experimental groups and protocol

Animals were allocated to four experimental groups (n=6 in each). Groups 1 and 2 underwent sham laparotomy but no liver ischaemia. In group 1 (sham femoral), an arterial line was placed in the right femoral artery while in group 2 (sham ear) this was placed in the right ear artery. In groups 3 and 4, liver lobar ischaemia was induced for 60 min followed by 7 h of reperfusion. In group 3 (I/R femoral), the arterial line was placed in the right femoral artery while in group 4 (I/R ear) this was placed in the right ear artery.

Blood and liver tissue samples were taken at baseline and at the end of 7 h reperfusion in the I/R groups or after the equivalent of the ischaemia (1 h) and reperfusion period (7 h) of the I/R animals in the sham groups for subsequent biochemical and histological studies.

4.2.3 Biochemical measurements

Serum alanine aminotransferase (ALT) and creatine kinase (CK) activities were measured using an automated clinical chemistry analyzer (Hitachi 747, Roche Diagnostics Ltd., Sussex, UK). Plasma lactates were measured in fresh blood samples using an automatic blood gas analyzer.

4.2.4 Bile flow

Bile was continuously collected from the common bile duct catheter and the volume was expressed as $\mu L/min/100g$ of wet liver weight.

4.2.5 Histopathology

Liver biopsies were taken from the ischaemic lobes at the end of the experiment. Formalin fixed paraffin embedded blocks of liver were cut at 4 microns and stained with haematoxylin and eosin (H & E) and reviewed by a pathologist, blinded to treatment of the animals. The tissue was semiquantitatively scored for the number of polymorphs in a high powered field using an Olympus BX50 microscope at x 400 magnification. An average of 5 fields was used. A score was devised; a score of 0-3 was given to the number of single PMNs in sinusoids (1: 1-5, 2: 5-20, 3: >20); a score 0-3 for aggregated PMNs (1: 1-2 clusters, 2: 2-5, 3 > 5). A comment was also made if the aggregated PMNs were associated with hepatocyte necrosis. The presence or absence of apoptosis, judged by shrunken, dark stained hepatocytes, was also made in a semiquantitative manner. Comment was made on presence or absence of Kupffer cell hyperplasia and if there were any other changes to hepatocytes, taking particular note of any zones affected. Immediately subcapsular areas and foci around large hepatic veins were not counted, as these would have shown a disproportionate amount of PMN infiltrate.

4.2.6 Statistical analysis

The results were expressed as mean ± Standard Deviation (SD). Comparisons within groups were made using the student's t test for paired samples, while comparisons

between groups were made using the student's t test for independent samples. P values less than 0.05 were considered to be significant.

4.3 Results

4.3.1 Systemic haemodynamics

Animals tolerated the prolonged general anaesthesia (>9h) and remained haemodynamically stable. In the sham fem group systemic mean arterial blood pressure (MABP) declined during the late phase of reperfusion compared to baseline, while in the sham ear group MABP remained relatively stable. However there were no statistically significant differences between the two groups (table 4.1). In the I/R groups MABP fell in the reperfusion period compared to baseline. This decline was more prominent for the I/R ear artery group (table 4.1).

Pulse rate (PR) slowed in the sham fem group, especially during the late phase of reperfusion compared to baseline. It remained stable in the sham ear group throughout the experiment. The pulse rate was not statistically different at any time point between the two groups (table 4.2). In the I/R fem group PR fell in the reperfusion period compared to baseline, while remained stable in the I/R ear group (table 4.2).

Oxygen saturation (SaO_2) remained stable through the experiment.

4.3.2 Biochemical measurements

There was a significant increase in serum ALT activity (Fig. 4.1), plasma lactate levels (Fig. 4.2) and CK activity (Fig. 4.3) in the I/R femoral line group compared to the I/R ear line group, at 7 h post-reperfusion. In the sham groups, serum ALT activity, plasma lactate levels and CK activity were also significantly higher in the femoral line

group compared to the ear line (Fig. 4.1-4.3). ALT was higher in the I/R groups compared to sham groups. Lactates and CK were very high in the sham femoral line group and actually higher compared to the ear line I/R group.

Table 4.1. Mean arterial blood pressure (MABP) in mm Hg, during the experiment. Blood pressure fell in the sham fem group during the experiment and remained relatively stable in the sham ear group. In the I/R groups there was also a drop during reperfusion from baseline and this drop was more prominent in the I/R ear line group. Results are expressed as mean ± SD. *p < 0.05, compared to baseline

Groups	Base-line	Ischaemia or equivalent	1st	2nd	3rd	4th	5th	6th	7th
Sham fem	73 ± 6	69 ± 7	67± 5	58 ± 3	64±3	61± 4	62± 5*	61 ± 2*	65 ± 7
Sham ear	67 ± 8	66 ± 8	60± 6	59 ± 7	60±6	59± 7	60± 5	63± 4	63 ± 8
I/R fem	69 ± 4	65 ± 5	58± 4	59 ± 5	58±9	59±12	60±10	62±10	62±10
I/R ear	64 ± 5	57 ± 5	56± 5	51 ± 7	49±9*	49±10*	49±11*	50±10*	50±12*

Table 4.2. Pulse rate (beats / min) during the experiment. Pulse rate remained stable during the experiment in the ear artery groups, while declined significantly from base line during the experiment in sham fem and the I/R fem groups. Results are expressed as mean ± SD. *p<0.05, compared to baseline

Groups	Baseline	Ischaemia or equivalent	1st	2nd	3rd	4th	5th	6th	7th
Sham fem	226±28	219±21	210±24	212±25	211±25	203±31*	198±29*	193±32*	191±31*
Sham ear	223±27	224±23	221±20	219±20	223±15	224±18	224±20	221±26	223±26
I/R fem	215±22	194±32	191±26	188±23	190±8	179±25*	175±14*	176±17*	171±18*
I/R ear	232±28	218±30	224±33	220±31	221±33	215±26	219±22	221±29	221±29
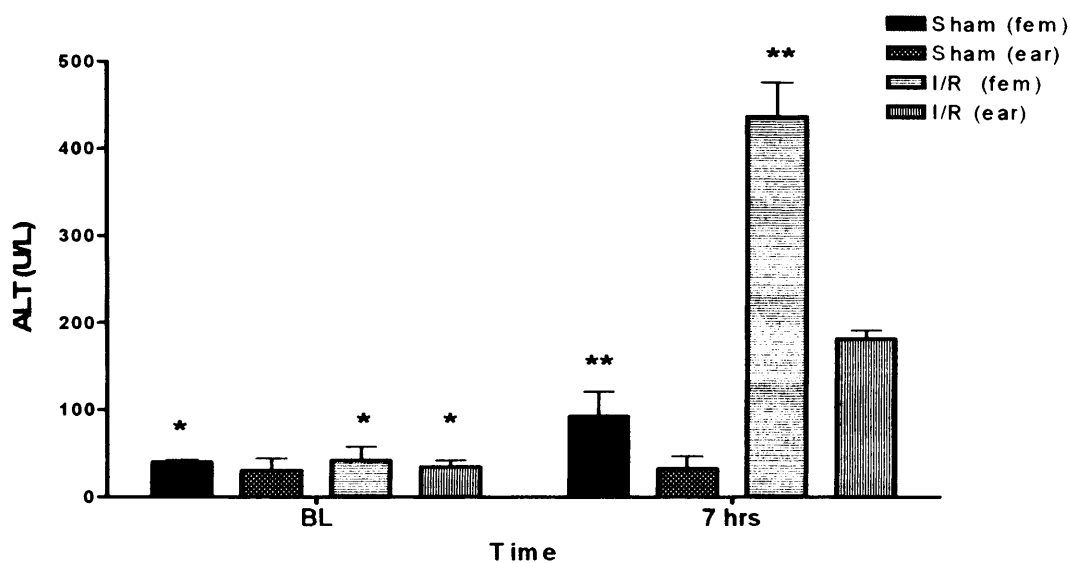
Figure 4.1 Serum alanine aminotransferase (ALT) activity (U/L) in femoral and ear arterial line sham and ischaemia-reperfusion (I/R) groups. There was a significant increase in ALT activity in all groups, except in the sham ear group at 7 hrs compared to baseline. The ALT levels were significant higher in the femoral line groups compared to the ear artery groups at 7 hrs post reperfusion. BL: baseline, *p<0.05, (7 hrs vs BL), **p<0.05 (femoral vs ear at 7 hrs).
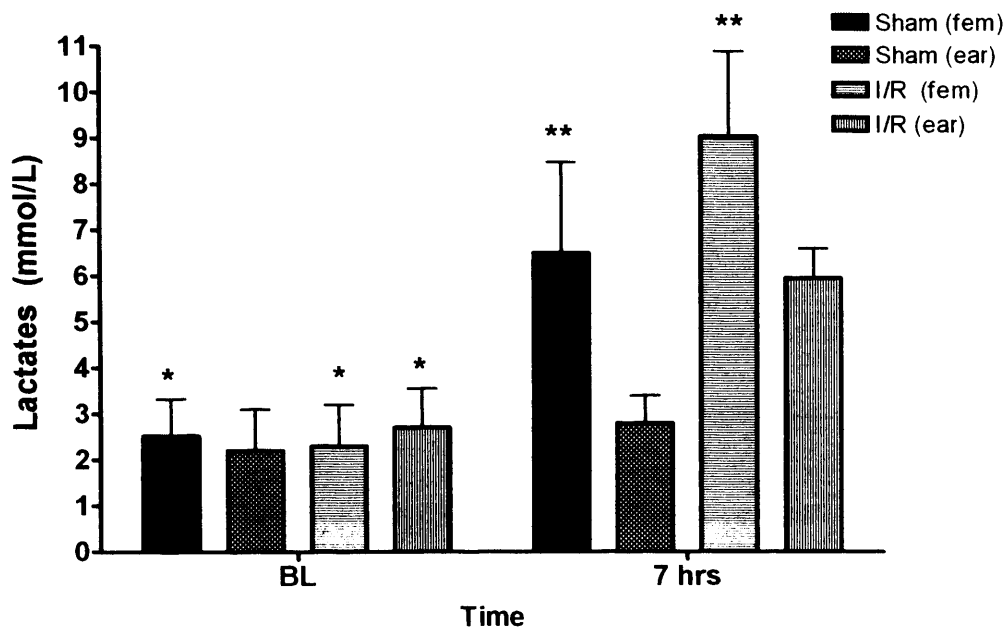
Figure 4.2. Plasma lactate (mmol/L) in femoral and ear arterial line sham and ischaemia reperfusion (I/R) groups. There was a significant increase in plasma lactate in all groups, except for the sham ear group at 7 hrs post- reperfusion compared to baseline. The plasma lactate levels were significant higher in the femoral line groups compared to ear line groups at 7 hrs post-reperfusion. BL: baseline, *p<0.05, (7 hrs vs BL), **p<0.05 (femoral vs ear at 7 hrs).

4.3.3 Bile production

Bile production was significantly decreased in both I/R groups at 7 h post-reperfusion compared to baseline but the decrease was more prominent in the I/R femoral line group (Figure 4.4). Bile flow was also significantly decreased at the end of the experiment, compared to baseline, in the sham femoral line group, while it remained unchanged in the ear artery group (Figure 4.4).
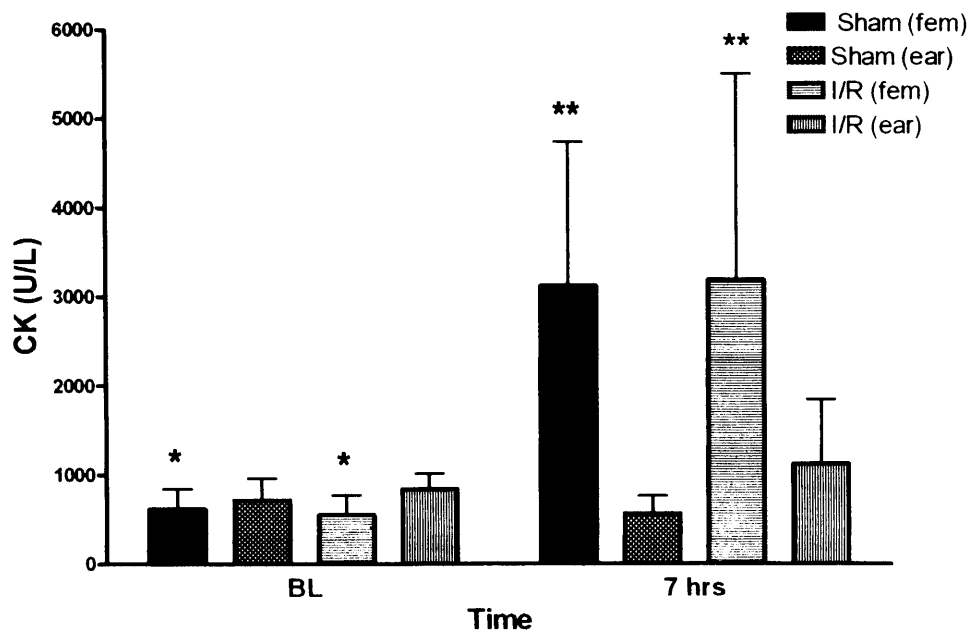
Figure 4.3. Creatine kinase (CK) activity (U/L) in the femoral and ear arterial line sham and ischaemia-reperfusion (I/R) groups. There was an increase in CK activity in all groups, except the sham ear group at 7 hrs post- reperfusion compared to baseline. The CK levels were significant higher in femoral line groups compared to the ear artery groups at 7 hrs post reperfusion. BL: baseline, *p<0.05, (7 hrs vs BL), **p<0.05 (femoral vs ear at 7 hrs).
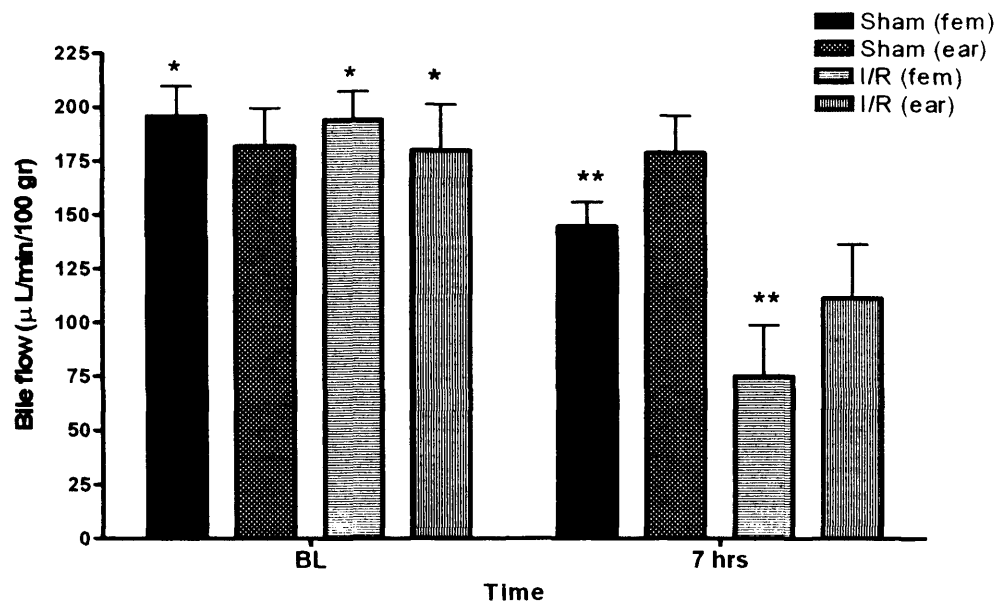
Figure 4.4. Bile flow (μL/min/100g) in femoral and ear arterial line sham and ischaemia-reperfusion (I/R) groups. There was a significant decrase in bile flow in all groups, except the sham ear group at 7 hrs post- reperfusion compared to baseline. Bile flow was significantly reduced in the femoral line groups compared to ear artery groups at 7 hrs post -reperfusion. BL: baseline, *p<0.05, (7 hrs vs BL), **p<0.05 (fem vs ear at 7 hrs).

4.3.4 Histopathology

Representative rabbit liver histology at baseline is shown in picture 4.5.
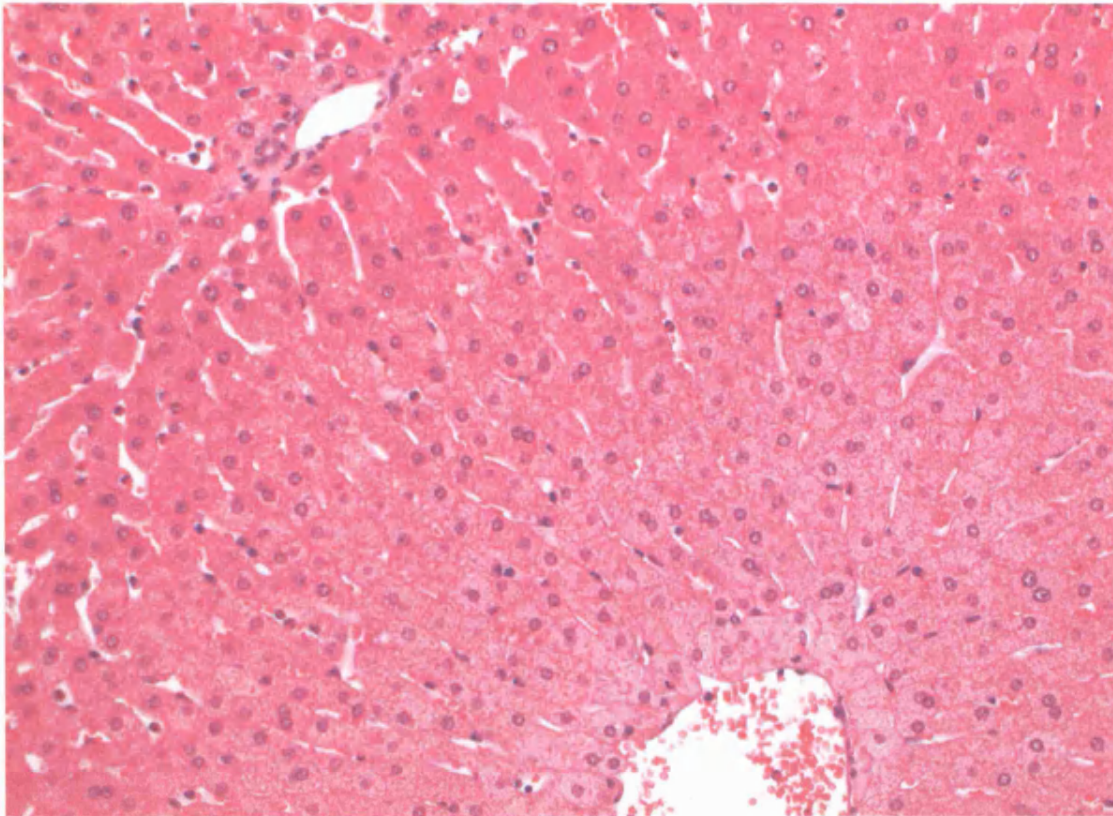
Figure 4.5 Representative photomicrograph of liver histology at baseline (H&E x20). It shows a small portal tract (top left) with normal intact hepatocyte plates and a terminal hepatic venule (bottom right).

The histological score regarding the number of single PMNs in sinusoids was 10 for the sham fem group, 9 for the sham ear group, 17 for the I/R fem group and 14 for the I/R ear group. The score regarding the aggregated PMNs was 8 for the sham fem group, 2 for the sham ear group, 15 for the I/R fem group and 11 for the sham ear group.

Hepatocellular necrosis, apoptosis and Kupffer cell hyperplasia was more severe in the I/R femoral line group compared to the I/R ear line group (Figure 4.6). There was also histological evidence of liver injury, at the end of the experiment in the sham femoral line group (Figure 4.7), while the liver morphology was preserved in the sham ear artery group without evidence of significant liver injury (Figure 4.7).
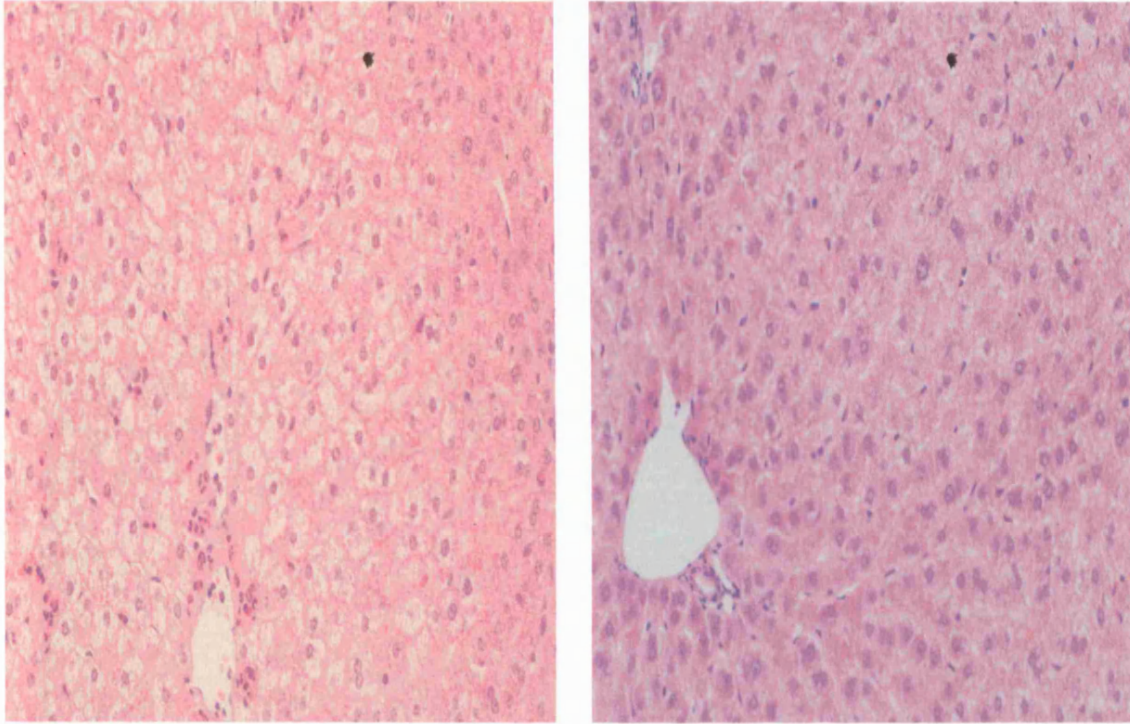
Sham-femoral   **Sham-ear**

Figure 4.6 Representative photomicrographs of liver histology at 7 h post- reperfusion in the femoral and ear arterial line sham groups. It shows neutrophil aggregation and hepatocellular necrosis (H&E x200) in the femoral line, while in the ear line shows well preserved hepatic structure without evidence of hepatocellular necrosis (H&E x200).
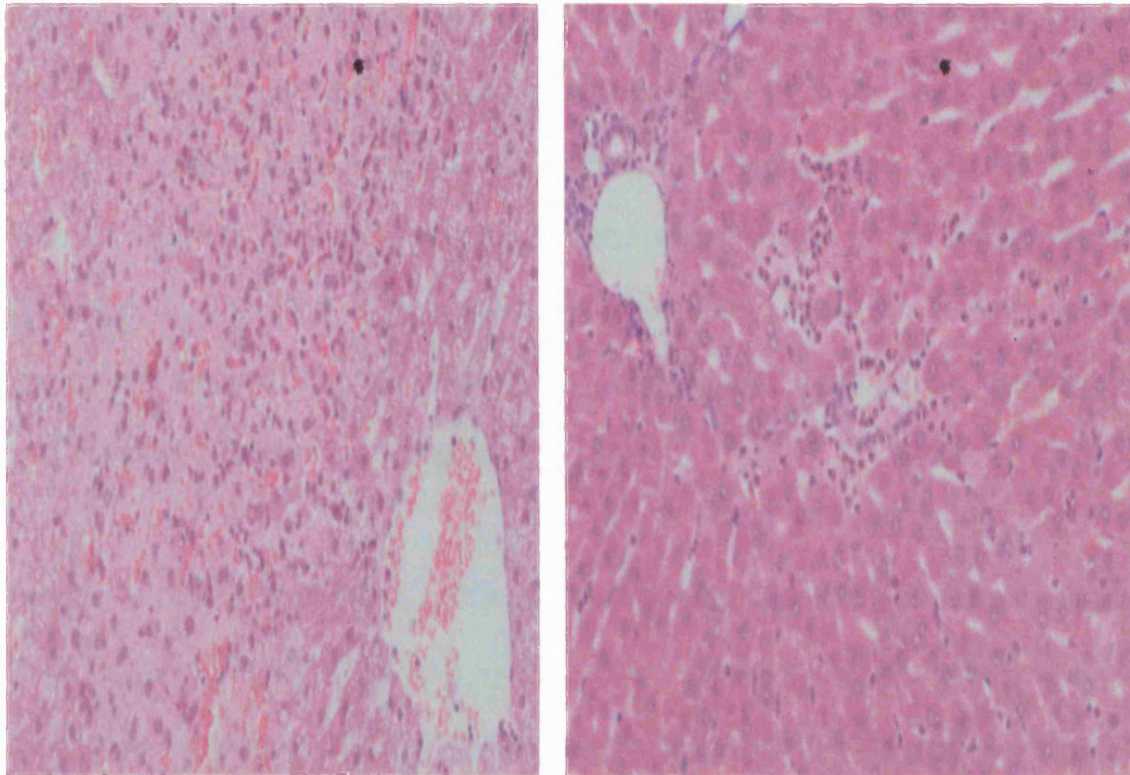
I/R-femoral   **I/R - ear**

Figure 4.7 Representative photomicrographs of liver histology at 7 h post-reperfusion in femoral and ear arterial line ischaemia-reperfusion groups showing massive neutrophil aggregation and extensive necrosis (H&E x200) in the femoral line group. The ear arterial line group shows less neutrophil aggregation and necrosis.

4.4 Discussion

This study has investigated the influence of femoral arterial line insertion to liver function in an experimental model of lobar liver ischaemia-reperfusion injury. Femoral line insertion induces limb skeletal muscle ischaemia. Skeletal muscle is relatively resistant to ischaemia for up to 2 h with minimal risk of ischaemic necrosis (Eckert and Schnackerz 1991). However with prolonged periods of ischaemia total muscle necrosis can result. The duration of ischaemia is considered to be the most important factor determining the outcome; after 6 h of ischaemia the incidence of death, limb loss or both increases (Kendrick et al. 1981).

Despite its clinical importance, relatively little is known about the systemic responses to acute ischaemic injury of skeletal muscle tissue. Acute interruption of arterial blood flow to the extremities may be associated with significant morbidity and mortality (Sun et al. 1999; Sun et al. 1998). Rhabdomyolysis, compartment syndrome and even circulatory shock can result from skeletal muscle ischaemia (Sirsjo et al. 1996; Sun et al. 1998). New Zealand white rabbits have previously been shown to tolerate 8 h of right hind limb ischaemia (Sun et al. 1998). Beyond this period there is very high mortality with major cellular destruction mainly due to systemic inflammatory response leading to multi-organ failure. There is experimental evidence suggesting that hepatic injury following a remote inflammatory insult occurs in a biphasic manner much like that of local liver injury (Brock et al. 2001; Jaeschke and Farhood 1991; Lichtman and Lemasters 1999).

The present study raises concerns for the interpretation of the results of experimental studies which have used an arterial femoral line, since it has shown that femoral artery cannulation produces remote systemic effects including liver injury. As shown above the femoral line groups (sham and liver I/R) had a significant increase in ALT activity compared to the respective ear artery groups. ALT principally resides within the cytoplasm of hepatocytes and its measurement in the serum may occur as a result of cell necrosis and /or an increase in membrane permeability (Rej 1989). Differences in the levels of ALT suggest that a large fraction of hepatocytes experienced some degree of injury. Our finding that ALT activity was significantly increased compared to baseline in the femoral artery sham group is a clear indication that limb ischaemia causes remote hepatic injury.

CK has three isoenzymes: CK-MM located in skeletal muscles, CK-MB found predominantly in cardiac tissue and CK-BB found in brain (Sun *et al.* 1998). CK elevations are indicators of tissue ischaemia or injury. In the present study CK was significantly higher in the femoral line groups compared to the ear line groups. This would suggest that the CK originated mainly from the ischaemic limb.

Lactate is the final product of anaerobic glucose metabolism. Tissue ischaemia or hypoxia can lead to increased production of lactates. In the present study the increase in lactates was mainly due to muscle hypoxia, as demonstrated by the high lactate levels in the femoral sham group. Lactate levels were also increased by liver I/R in the ear line group, but less compared to the femoral arterial line group. The increase in lactates was exacerbated by impaired liver function. The increased levels in I/R femoral group over

sham femoral would suggest that this group has increased production of lactates and reduced clearance.

Bile excretion is regarded as a good marker of hepatocellular function and bile flow is decreased with liver injury (El Desoky *et al.* 2001). In the present study bile flow remained unchanged over the experimental period in the ear artery sham group while decreased significantly from baseline to the end of the experiment in the sham femoral line group.

The present study has not investigated the mechanisms by which limb ischaemia can cause remote liver injury. Oxidative stress, release of cytokines, activated neutrophils and endotoxaemia are the pathogenetic factors associated with remote organ injury in limb I/R injury (Kyriakides *et al.* 2000). An experimental study of rabbit hind limb ischaemia for 12 h followed by 4 h of reperfusion, showed that ischaemic injury significantly increased luminal amplified tert-butylhydroperoxide-initiated chemiluminescence, a marker of free radical production. Reperfusion of the ischaemic tissue tended to bring the values back towards baseline levels (Sun *et al.* 1999). The oxidative stress was most pronounced in the heart tissue, followed by the spleen, skeletal muscle, lung, liver and kidney. The authors suggested that the main cause of mortality was cardiac tissue injury.

Several studies have suggested that cytokines including Inteleukin-6 (IL-6) are released directly from the endothelium of injured tissue within hours of injury or surgery (Roumen *et al.* 1993). These cytokines circulate on reperfusion and can mediate remote organ injury. An experimental study where rats underwent 3 h bilateral hind limb

ischaemia followed by 1 h of reperfusion, produced a systemic inflammatory response as demonstrated by the increase in plasma tumour necrosis factor-alpha (TNF-α) and IL-6 concentrations with liver, kidney and lung dysfunction (Yassin *et al.* 2002). Uncontrolled production of cytokines can induce a systemic inflammatory response that could inflict fatal damage and contribute to the development of multiorgan dysfunction syndrome (MODS) (Haupt *et al.* 1997; Schlag and Redl 1996).

Experimental studies of hindlimb I/R found that polymorphonuclear leukocytes (PMN) play a central role in organ damage (Klausner *et al.* 1988; Kyriakides *et al.* 2000). After ischaemia, local tissue reperfusion generates a number of inflammatory mediators that activate circulating PMNs and remote endothelium. Remote organ stimulation by circulating mediators leads to generation of chemoattractants, which are responsible for PMN recruitment and activation (Klausner *et al.* 1989; Welbourn *et al.* 1991). In fact PMN activation before the ischaemic insult moderates both local and remote organ injury (Klausner *et al.* 1988). An experimental study where mice underwent 2-h bilateral tourniquet hind-limb ischaemia followed by 3 h of reperfusion showed that remote liver injury is mediated by activated neutrophils and is complement dependent (Kyriakides *et al.* 2000). In our study by using a semiquantative histological system we found that in the femoral line groups, there were more PMNs in the sinusoids and also more aggregates PMNs compared to the respective ear line groups. This was correlated with more extensive necrosis and more significant Kupffer cell hyperplasia.

Although previous studies have shown that remote injury after limb ischaemia occurs mainly during limb reperfusion, the present study has shown that cannulation of the femoral artery can cause liver injury without reperfusion. Collateral circulation could

occur through the iliolumbar artery, deep femoral artery, medial and lateral circumflex femoral arteries of the ischaemic limb with internal iliac and deep femoral arteries of the nonischemic leg thus allowing a reperfusion phenomenon (Sun *et al.* 1999). Cannulation of the femoral artery may be creating a condition of low flow I/R injury. The mechanism proposed in the present study is that low flow I/R can cause remote liver injury through the release of mediators from the ischaemic limb.

4.5 Conclusions

Limb ischaemia resulting from femoral artery cannulation can produce remote liver injury. The use of the ear artery instead avoids remote hepatic injury. Experimental studies aiming at studying liver function should avoid femoral cannulation.

4.6 Acknowledgements

4.7 Chapter summary

In this work an experimental rabbit lobar liver ischaemia-reperfusion model was evaluated. This model aimed to monitor both early and initial part of late phase of reperfusion (up to 7 hrs). The effect of femoral arterial line placement on liver function was studied. Femoral arterial lines are commonly used for continuous monitoring of arterial blood pressure in experimental studies. However, placement of the catheter in the femoral artery could produce acute limb ischaemia with associated systemic effects. 4 groups of animals (n=6 each) were studied: Groups 1 and 2 (Sham) underwent laparotomy but no liver ischaemia. In groups 3 and 4 (I/R) liver lobar ischaemia was induced for 60 min followed by 7 h of reperfusion. In groups 1 and 3 the arterial line was placed in the femoral artery whereas in groups 2 and 4 in the ear artery. Liver function was assessed by serum alanine aminotransferase (ALT) activity, bile flow, plasma lactate levels and histology. The results were expressed as mean ± standard deviation (SD).

All animals tolerated the prolonged anaesthesia well. Blood pressure declined during the experiment mainly in the I/R groups. PR also slowed during the experiment, mainly in the I/R groups. Oxygen saturation remained stable during the experiment. ALT and lactate levels were significantly higher in the I/R femoral line group compared to the I/R ear line group, at 7 h post-reperfusion and bile production was significantly lower (75±9.6 vs 112±10 µL/min/100g liver weight). Histopathology showed more extensive hepatocellular necrosis and neutrophil accumulation in the I/R femoral line group compared to I/R ear line group. The sham femoral group showed liver injury which was more marked than the ear line group (all $p < 0.05$). In conclusion, femoral artery cannulation induces remote liver injury most probably through the systemic effects of low blood flow limb I/R injury. The use of femoral arterial lines should be avoided in studies concerning liver function.

CHAPTER 5

CHANGES IN BILE COMPOSITION FOLLOWING HEPATIC WARM ISCHAEMIA / REPERFUSION: AN EXPERIMENTAL EVALUATION USING PROTON MAGNETIC RESONANCE SPECTROSCOPY

5.1 Introduction

In the previous chapter a rabbit lobar liver I/R model was evaluated and refined. In this chapter changes in bile composition during liver I/R, will be studied using proton nuclear magnetic resonance (^{1}H NMR) spectroscopy. Bile formation is an active secretory process involving bile salt dependent and independent mechanisms (Bowers *et al.* 1987). Bile excretion is used as an indicator of liver function and recovery following ischaemia in experimental studies (Bowers *et al.* 1987; Glanemann *et al.* 2003; Jamieson *et al.* 1988; Sumimoto *et al.* 1988; Terajima *et al.* 2000) and clinical liver transplantation (Boyer 2002). However, little is known about changes in bile composition during liver ischaemia and following reperfusion.

Bile formation is an osmotic filtration-driven process that depends on the proper function of transport systems in both the basolateral and apical or canalicular membranes of hepatocytes (Boyer 1996; Trauner and Boyer 2003). Bile secretion is driven by active excretion of organic solutes into the bile canaliculus, followed by passive inflow of water, electrolytes, and non-electrolytes from hepatocytes and across semi-permeable tight junctions (Trauner and Boyer 2003).

Liver ischaemia reduces energy dependant transport processes and is therefore associated with reduced bile flow (Smith and Blandford 1995). Bile flow recommences on reperfusion, and has thus been considered to be a marker of liver function and used to assess clinical liver transplantation studies (Sequeira *et al.* 1995; Small *et al.* 1969; Stark *et al.* 1985). However the changes in bile composition during ischaemia and reperfusion have not been widely investigated.

In recent years, high resolution ^{1}H NMR spectroscopy has been established as a powerful technique to explore the biochemical composition of biological fluids such as plasma, bile, seminal and synovial fluids, in various pathological conditions (Ishikawa *et al.* 1999). This technique is rapid, non-invasive and non-destructive and can detect metabolites present at the millimolar concentration. In bile, it has been used to investigate biliary lipids (Vejchapipat *et al.* 2002) and conjugated bile acids (Scarpa *et al.* 2001). In the liver, NMR has also been used to assess glutamine metabolism (Hayakawa *et al.* 1997), energy status after warm ischaemia (Powell J 1990), and to study effects of I/R on liver cell membranes (Melendez *et al.* 2001; Paczkowska *et al.* 2003). A preliminary study of ^{1}H NMR analysis of bile after liver transplantation has been reported by Powell et al in 1990, suggesting that this technique might help to distinguish ischaemia from rejection post-transplant by detecting large resonance peaks for the ischaemic metabolites lactate and acetate (Powell J 1990). More recently, there has been growing interest in attempting to predict graft function by analyzing bile production immediately after transplantation (Vilca *et al.* 2004).

Melendez et al in 2001 published a small pilot study consisting of only four liver transplants in which they analyzed donor organ bile before and after transplant. One recipient had primary graft non-function, another had early graft dysfunction and two had good early graft function. ^{1}H NMR analysis of bile demonstrated that good graft function was associated with rapid clearance of sugar peaks derived from University of Wisconsin solution, lower lactate and an increase in PC head group and bile acid levels (Melendez *et al.* 2001).

With advances in MR imaging technology, spectroscopy is an increasingly available option. In vivo spectroscopic assay of gall bladder has recently been reported (Prescot *et al.* 2003). The obvious implication is for the use of in vivo bile spectroscopy to assess liver function in a non-invasive manner. However, controlled experiments to determine the effect of first warm and then cold I/R injury on bile constituents are needed.

The aim of this pilot study was to examine bile composition changes during liver ischaemia and reperfusion in an established model of warm lobar liver I/R injury (Glantzounis *et al.* 2004) to determine any compositional changes during I/R. In the present study the femoral arterial line was used for arterial blood pressure measurement and blood withdrawal, since this study was performed in the first stages of this thesis and the detrimental effect of femoral artery cannulation to remote organs was not clear. However this is unlikely to affect the bile spectroscopy changes with time.

5.2 Materials and methods

5.2.1 Model of lobar ischaemia/reperfusion

The study was conducted under a license granted by the Home Office in accordance with the Animals (Scientific Procedures) Act 1986. New Zealand white rabbits (3.8 ± 0.5 kg) were used in a model of lobar ischaemia (Glantzounis *et al.* 2004) . Anaesthesia and surgical procedure are described in section 3.1. Briefly, anaesthesia was induced by an intramuscular injection of 0.5 ml/kg fentanyl citrate and fluanisone (Hypnorm). Following tracheostomy anaesthesia was maintained with isoflurane (0.5-3%) through

an anesthetic circuit. Haemoglobin saturation and heart rate were continuously recorded by a pulse oxymeter. One catheter (20 G) was inserted into the right femoral artery for arterial blood pressure monitoring and collection of blood samples. The ear marginal vein was cannulated with another catheter (22 G) for the administration of anaesthetics and fluids.

Laparotomy was performed through a midline incision. The ligamentous attachments from the liver to the diaphragm were divided and the liver was exposed. The bile duct was cannulated with a polyethylene catheter for measurement of bile flow and collection of samples for ^{1}H NMR spectroscopy.

5.2.2 Experimental groups and protocol

Two groups of animals were used; the I/R group (n=6) had lobar liver ischaemia induced by clamping the vascular pedicles of the median and left lobes of the liver, using an atraumatic microvascular clip. After 60 min of ischaemia the vascular clip was removed and reperfusion was allowed for 7 h. During the I/R period, the abdomen was covered with a plastic wrap to prevent fluid evaporation. The sham control group (n=6) underwent laparotomy for 9 hours, without hepatic inflow occlusion. In both groups 10 ml/kg/h of 0.9 % NaCl was administered iv to compensate for intraoperative fluid loss.

5.2.3 Blood sampling for liver function

Arterial blood samples (1 ml each) were taken before the induction of liver ischaemia (baseline), at the end of ischaemia (60 min) and 2, 5 and 7 h after reperfusion for measurement of alanine aminotransferase (ALT) and aspartate aminotransferase (AST)

activity. An equal volume of normal saline was used to replace the volume of the blood taken. Serum was separated from the samples and stored at -20°C until assayed. The measurements were done using an automated clinical chemistry analyzer (Hitachi 747, Roche Diagnostics Ltd., Sussex, UK).

5.2.4 Liver Histopathology

Small wedge biopsies were taken at the end of the procedure from the ischaemic lobes. Formalin-fixed liver tissue samples were embedded in paraffin and stained with haematoxylin and eosin for subsequent microscopy (digital light microscope CLF60 optical system, Nikon UK Ltd, Surrey, UK). For assessment of the degree of injury a semi quantitative method was used in which a blinded liver pathologist estimated the number of neutrophils (PMN) in sinusoids, the aggregation of PMN, the hypertrophy of Kupffer cells and the presence of necrosis and apoptosis in 5 high power fields per slide

.

5.2.5 Measurement of cytochrome oxidase activity

The effect of I/R on intracellular liver tissue oxygenation was measured by near infrared spectroscopy (NIRS) as described in section 3.3. The optical-fibre bundles (probes) were positioned on the left lobe of the liver, with a 10 mm separation. A flexible probe holder was used to ensure that probes have a satisfactory contact with the liver surface and a fixed inter-probe spacing. A NIRS algorithm was developed to measure continuously changes in the redox state of Cu_A centre of Cyt Ox in $\mu Mol/L$. The pre-ischemic baseline was taken as baseline against which changes recorded.

5.2.6 Measurement of bile flow and bile composition with ^{1}H NMR spectroscopy

The common bile duct was cannulated with a polyethylene catheter (PE-50, Portex, Kent, UK) for continuous collection of bile. Bile samples were collected continuously and aliquoted in separate containers at the end of baseline (before the I/R procedure), following 60 min ischaemia and hourly during reperfusion in the I/R group or the equivalent time in the sham group. The total bile volume collected was expressed as μL/min/100g of liver wet weight and then each aliquot was analyzed by ^{1}H NMR spectroscopy. Spectra of hepatic bile samples were obtained using a ^{1}H NMR spectrometer (Eclipse+ 500; JEOL Ltd., Tokyo, Japan) operating at 11.7 T (500 MHz for ^{1}H). All Spectra were recorded at 25°C. 600 μL of bile was placed in a 5 mm tube with a 2 mm coaxial insert containing a reference standard of 100 μL of 1mg/ml of sodium trimethylsilyl-[^{2}H$_4$]propionate (TSP) dissolved in deuterium oxide (^{2}H$_2$O, Fluorochem Ltd., Old Glossop, Derbyshire, UK) and set at 0.0 parts per million (ppm). A basic single pulse sequence was used to acquire the one-dimensional spectra using a 45° pulse (8.25 μs) with an acquisition duration of 4.36 s and a relaxation delay of 5s. To reduce the large water resonance signal a gated secondary irradiation at the water frequency was applied. Published peak assignments (Melendez et al. 2001) were used. In brief, these were lactate methyl group peaks (1.33 ppm), acetate peaks (1.91 ppm), PC head group $-N+(CH_3)_3$ (3.20 ppm) and C-25 glycine methylene proton peak (3.80 ppm), as shown in figure 5.1. Integration of the ^{1}H NMR spectra areas was measured relative to the reference standard TSP area at baseline and obtained using MestRe-C software (version 2.3a, Departmento de Química Orgánica, Universidade de Santiago de Compostela).

5.2.7 Data collection and statistical analysis

Values are expressed as mean ± standard deviation. Spectroscopy values are shown as a percentage change of baseline values. For statistical analysis Student's paired and unpaired t-test was used for comparisons within and between groups. Simple and polynomial regression analysis was used for correlations. $P<0.05$ considered significant.
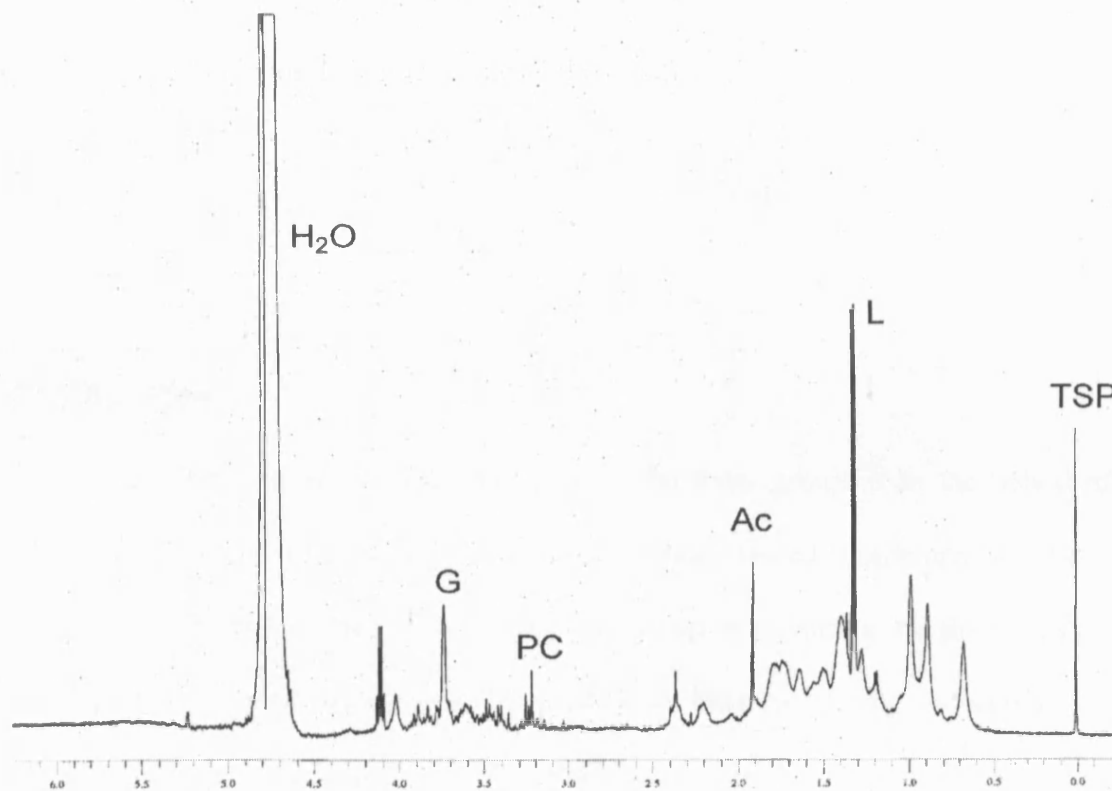
Figure 5.1 Proton nuclear magnetic resonance (^{1}NMR) spectrum of baseline bile. L=lactate, G=glycine, PC=phosphatidylcholine, TSP=sodium trimethylsilyl [^{2}H$_4$]propionate standard, Ac=acetate, H$_2$O=Water

5.3 Results

5.3.1 Liver biochemistry

In the sham-operated controls, ALT rose significantly at 5 and 7 h post-reperfusion compared to baseline ($p < 0.05$). A similar effect was seen in AST levels. In the I/R group there was a marked significant increase in ALT and AST at 2, 5 and 7 h reperfusion as shown in figures 5.2 and 5.3, respectively.

5.3.2 Liver Histopathology

Examination of liver histology revealed polymorphonuclear leukocyte (PMN) accumulation in sinusoids, PMN aggregation, Kupffer cell hypertrophy, necrosis and apoptosis in the I/R group compared to the sham control group as shown in figure 5.4.

5.3.3 Bile Flow

There was a gradual decrease in bile flow in the sham group, over the period of the experiment compared to baseline, and this difference reached significance at 7 hrs post-reperfusion (P=0.045). Bile flow in the I/R group was similar to sham controls at baseline but reduced significantly to 26.6% of baseline during ischaemia (60 ± 19 $\mu L/min/100g$ vs. 202 ± 32 $\mu L/min/100g$; P=0.0022). Bile flow increased on reperfusion to but remained significantly less than sham as shown in figure 5.5. Mean total bile flow for the three reperfusion time points together was 45 ± 9% of baseline values.

5.3.4 Changes in cytochrome oxidase redox state

During ischaemia there was a significant decrease in the redox state of Cyt Ox Cu_A which was maximal at 15-20 minutes after induction of ischaemia (Table 5.1). After reperfusion, in the I/R group, initially Cyt Ox Cu_A redox state returned towards baseline values during the first two hrs of reperfusion. However, subsequently Cyt Ox Cu_A redox state decreased again and reached levels lower than the ischaemic levels by the fifth hour of reperfusion. There was also a decrease in Cyt Ox Cu_A redox state in the sham group during the experiment compared to baseline.

Table 5.1 Changes in the redox state of Cu_A centre of cytochrome oxidase (in μMol / L) in the sham and ischaemia-reperfusion (I/R) groups during the experiment, compared to baseline. Results are presented as mean (SD). * $p < 0.05$ vs. baseline and sham group.

Groups	Baseline	Ischaemia	2nd hour	5th hour	7th hour
Sham	0.00	-7.5 ± 4.6	-1.3 ± 1.9	-3.7 ± 1.4	-6.5 ± 1.5
I/R	0.00	-23.1 ± 3.5*	-11.1 ± 3.8*	-22.3 ± 11.6*	-24 ± 7.2*
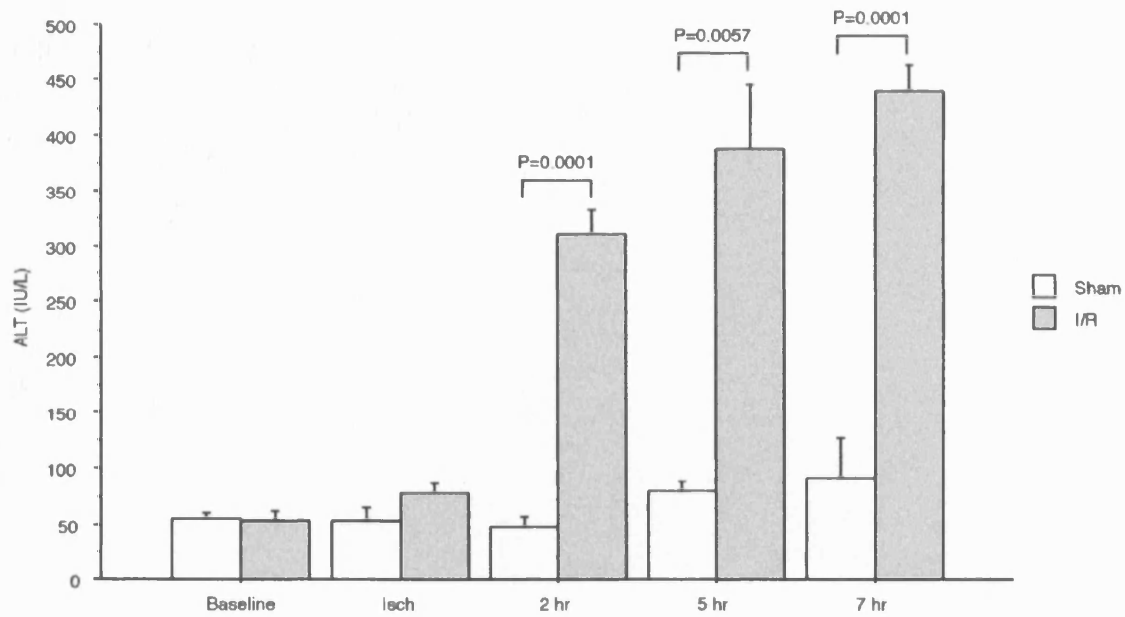
Figure 5.2 Changes in serum alanine aminotransferase (ALT) activity during ischaemia-reperfusion.
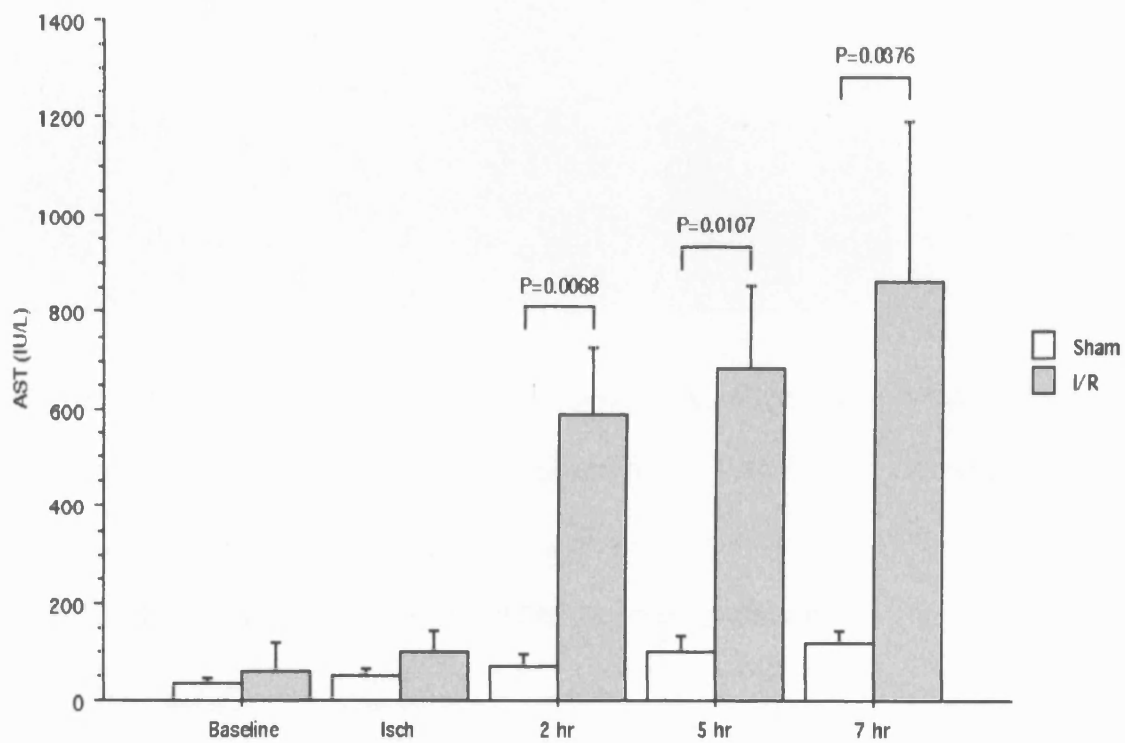
Figure 5.3 Changes in serum aspartate aminotransferase (AST) activity during ischaemia-reperfusion.
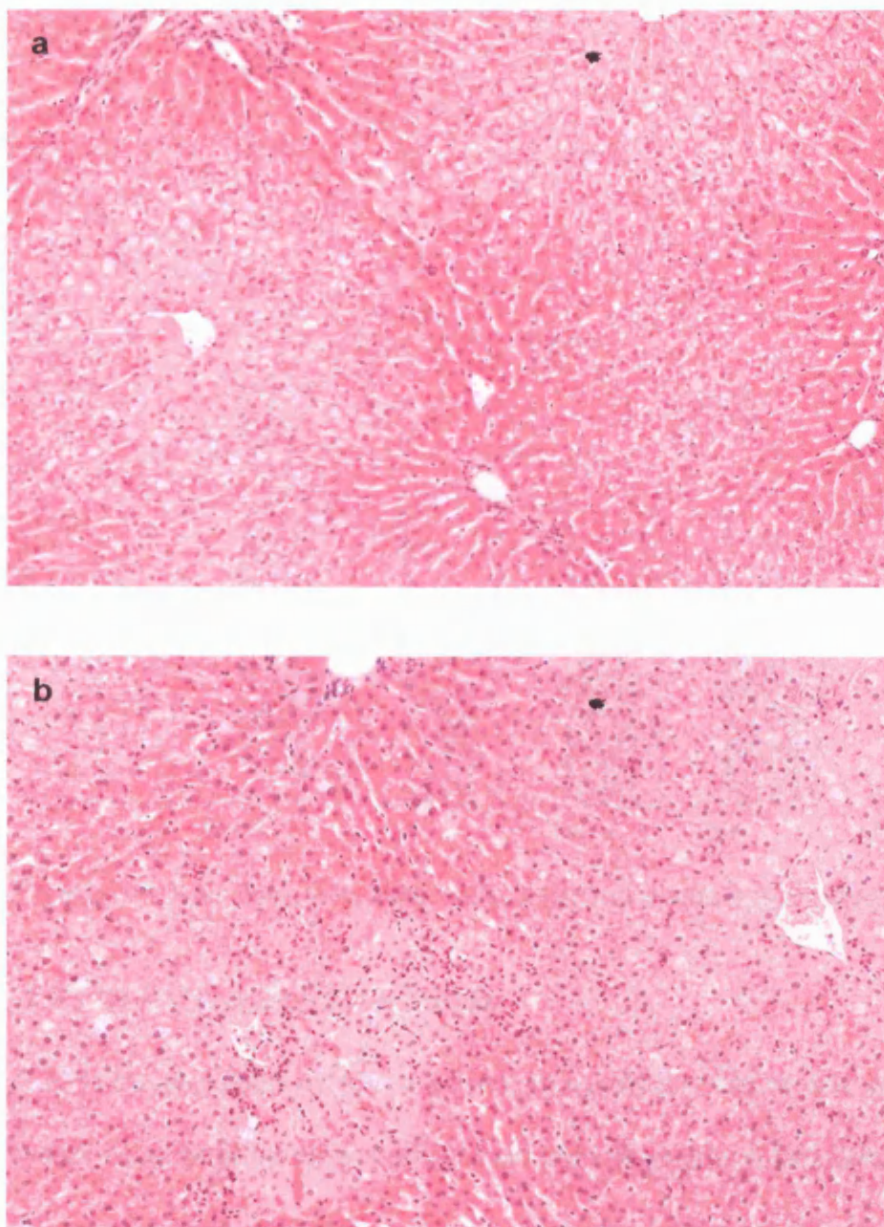
Figure 5.4 Light microscopy of liver changes during ischaemia-reperfusion (I/R). Light microscopy (x100) stained with hematoxylin and eosin showing extensive polymorphonuclear leukocyte (PMN) accumulation in sinusoids, PMN aggregation and necrosis in the I/R group (b) compared to the sham group (a).
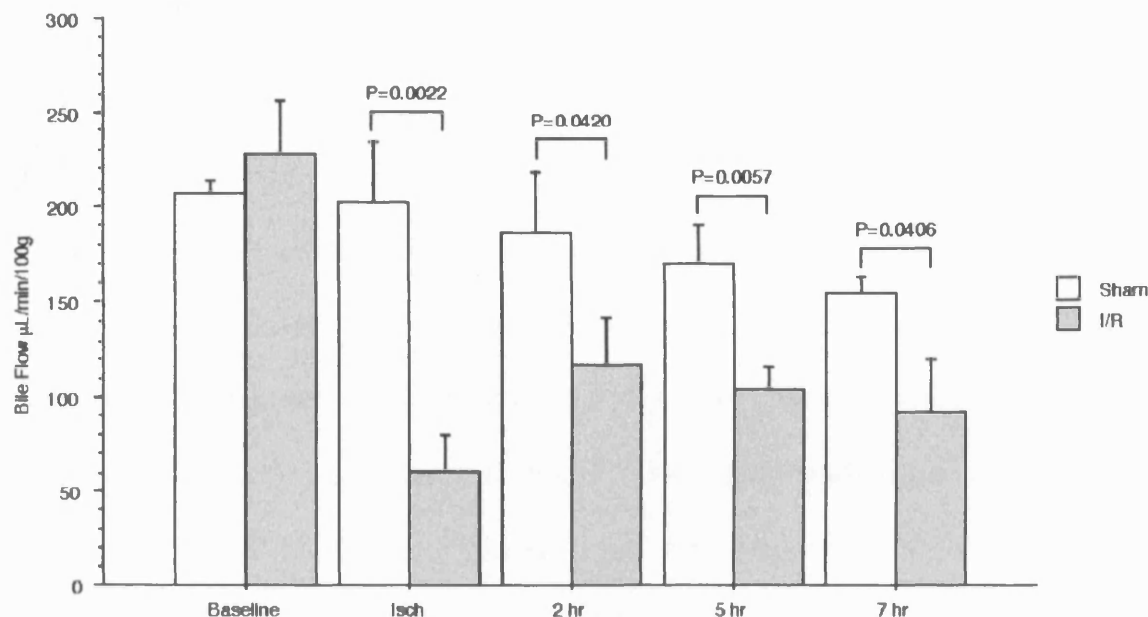
Figure 5.5 Bile flow following warm liver ischaemia-reperfusion

5.3.5 ^{1}H NMR spectroscopy of bile

A baseline spectrum of bile is shown in figure 5.1. There was no difference in biliary lactate concentration between the sham and I/R groups at baseline. Biliary lactate levels were significantly elevated in the I/R group at 5 h post-reperfusion to 165.44±22.97% of initial baseline values (P=0.0036 vs. sham controls), followed by a decline at 7 h to 89.13±29.91% of baseline values as shown in figure 5.6.

Analysis and integration of peaks corresponding to phosphatidylcholine (PC) head group (figure 5.7) revealed that ischaemia produced a two-fold increase (213.04±64.06% of baseline vs. 82.13±31.91% of baseline in sham controls, P=0.0105). Mean values for PC over all time points tended to be higher in the I/R group compared to the sham group (P<0.0001 as assessed by ANOVA).

Acetate levels were reduced compared to baseline throughout the period of the experiment in both the I/R and sham groups (figure 5.8 and 5.9). The reduction was significantly greater in the I/R group by 5 h post-reperfusion (50.75 ± 13.24 % vs. 70.35 ± 6.86%, P=0.0391) and 7 h post-reperfusion (37.47 ± 7.49 % vs. 69.36 ± 13.29 %, P=0.0058) (I/R vs. sham).

Correlation of ^{1}H NMR bile spectroscopy data with other parameters revealed that acetate decreased with increases in ALT (R=0.762, P<0.0001) (figure 5.10). Acetate increased with increases in bile flow (R=0.458, P=0.0109) and the redox state of Cyt Ox (R=0.650, P=0.0001) as shown in figure 5.11. Phosphatidylcholine increased with increases in ALT (R=0.428, P=0.0295) and decreased with increases in bile flow (R=0.699, P<0.0001) and redox state of Cyt Ox (R=0.644, P<0.0001). Lactate increased with increases in AST (R=0.445, P=0.0200) and tended to decrease with increase in the redox state of Cyt Ox (R=0.319, P=0.0857), however these results did not reach statistical significance.
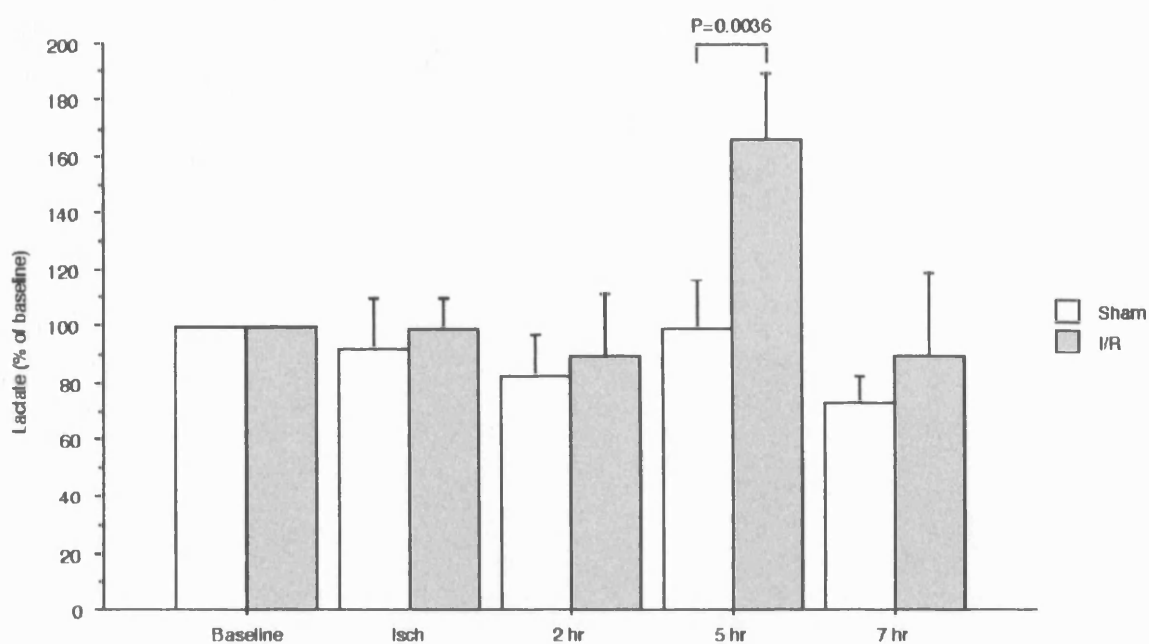
Figure 5.6 Bile lactate levels following warm liver I/R. Lactate levels were calculated by integration of peaks at 1.3 ppm and expressed as a percentage of baseline values (*p < 0.05, I/R vs. sham).
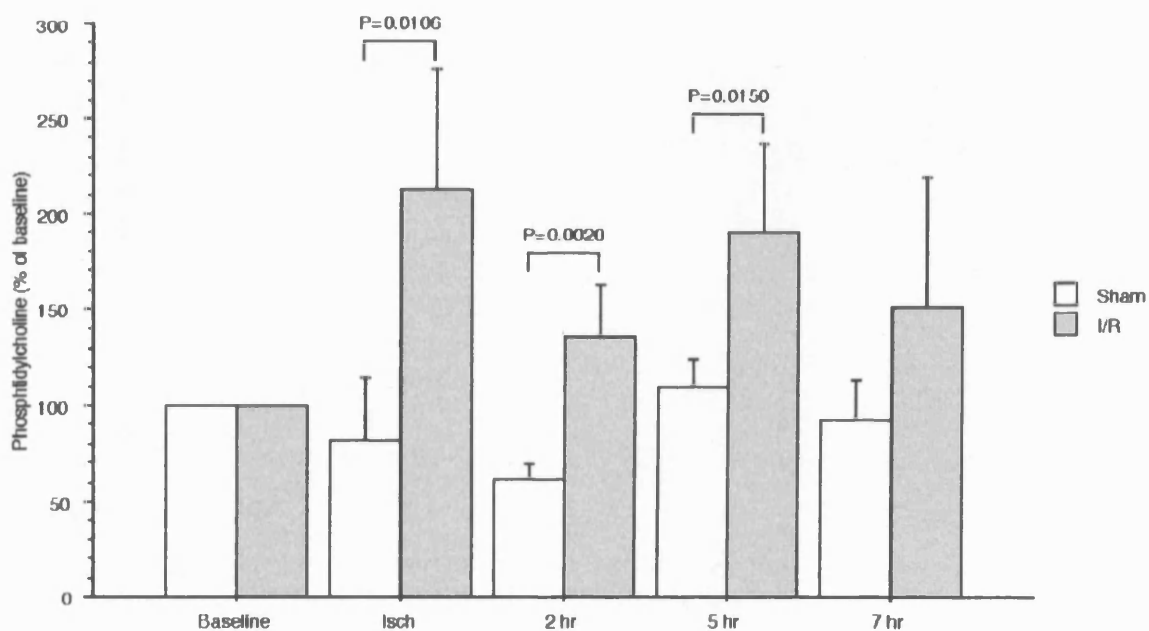
Figure 5.7 Bile phosphatidylcholine (PC) levels following warm liver ischaemia-reperfusion. PC levels were calculated by Integration of peaks at 3.2 ppm and expressed as a percentage of baseline values. (*p < 0.05, I/R vs. sham).
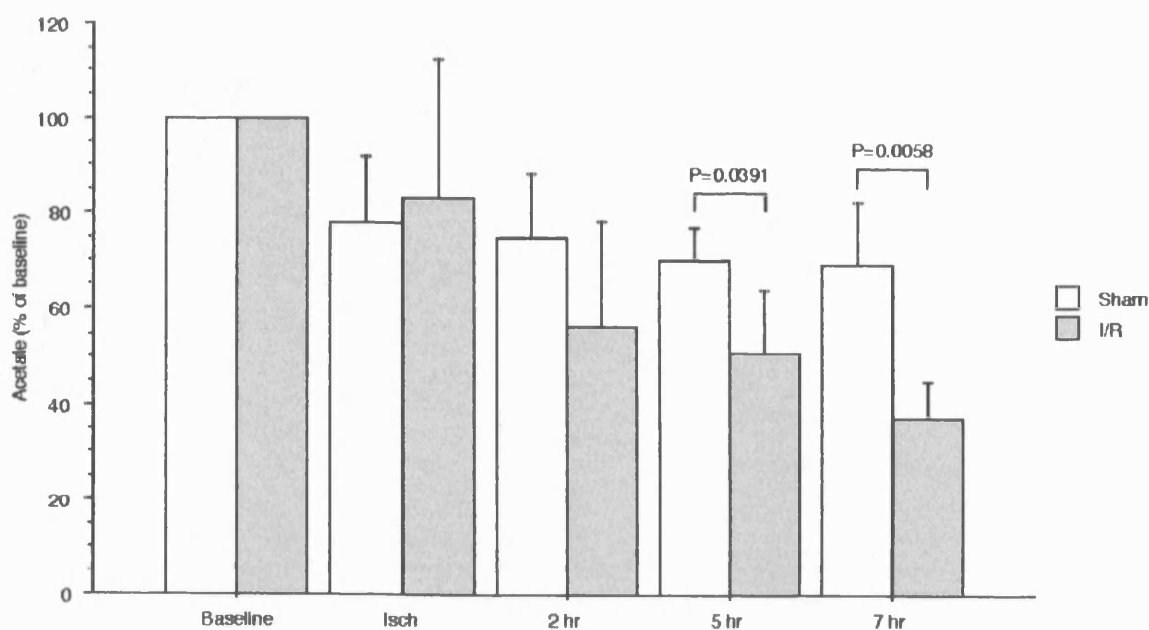
Figure 5.8 Bile acetate levels following warm liver ischaemia-reperfusion. Acetate levels were calculated by integration of acetate peaks at 1.91 ppm and expressed as a percentage of baseline values. (*p < 0.05, I/R vs. sham).
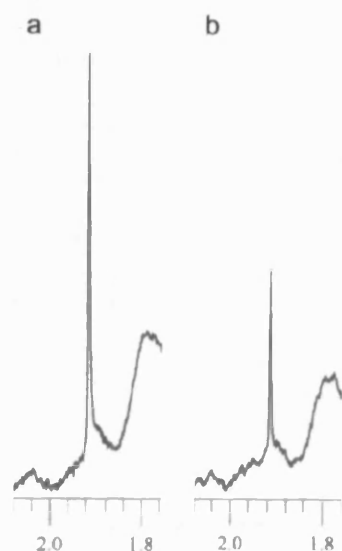
Figure 5.9 ^{1}H NMR bile spectra of acetate peaks (2.0-1.84 ppm) scaled to TSP of I/R group at (a) baseline and (b) after 7 hours of reperfusion showing significant reduction.
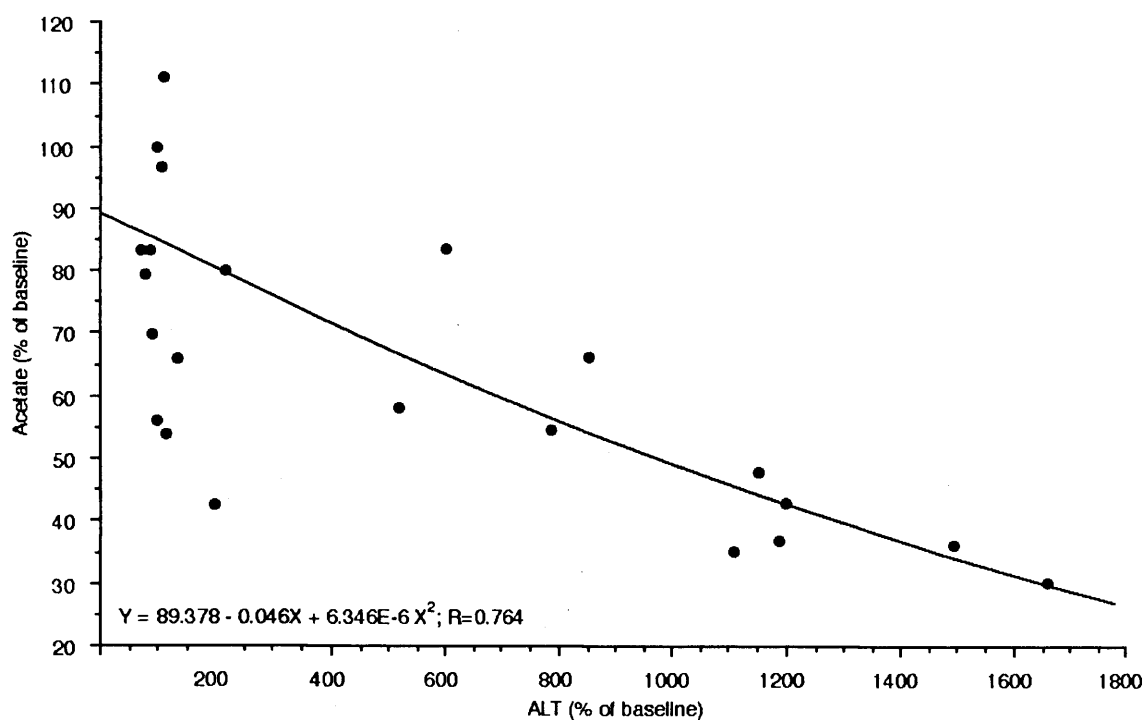
Figure 5.10. Correlation of ALT with billiary acetate (p < 0.05).
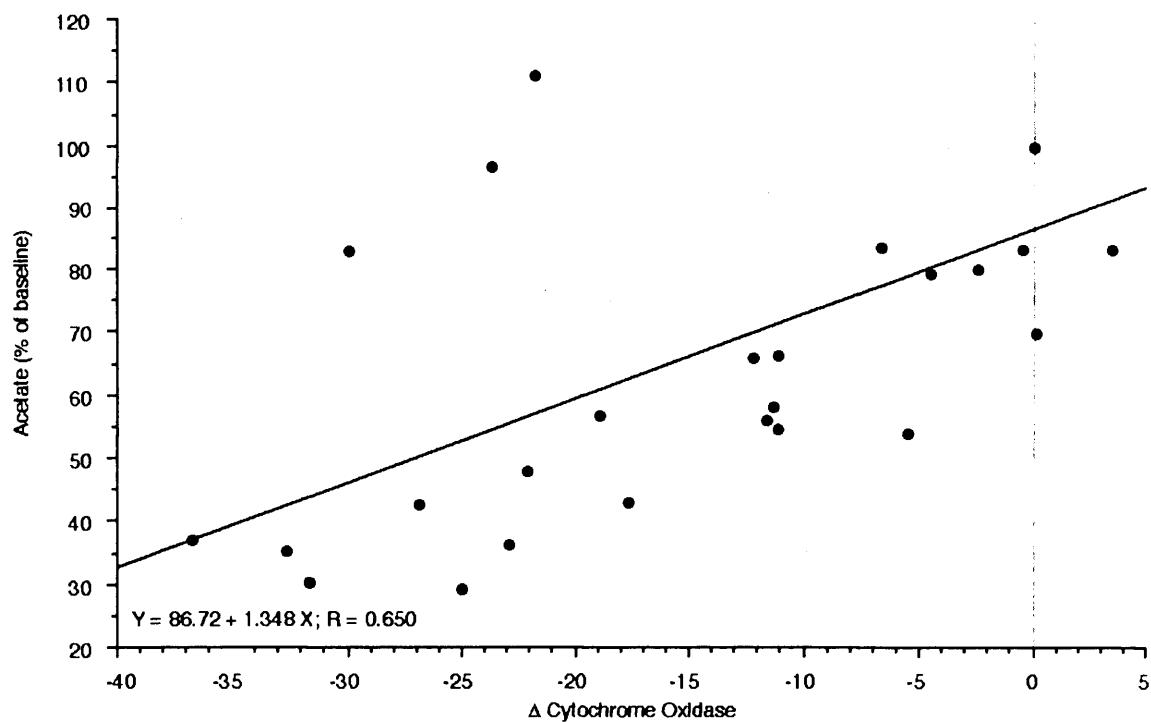
Figure 5.11. Correlation between changes in cytochrome oxidase redox state and biliary acetate (p < 0.05).

5.4 Discussion

This is the first time that ^{1}H NMR analysis of bile has been applied to a controlled experimental model of warm liver I/R. Liver warm I/R injury can be divided into an early phase which is associated with Kupffer cell activation and a cytokine surge (Jaeschke et al. 1988; Jaeschke 1998) and a late phase (> 4 h) which is mainly due to the accumulation of neutrophils and is associated with more extensive injury (Jaeschke et al. 1990; Lentsch et al. 2000).

In the present study, a rabbit lobar I/R model was used with reperfusion period of seven hours (Glantzounis et al. 2004). This model permitted the analysis of bile constituents in both the early phase and the beginning of the late phase of I/R injury. The lobar I/R model rather than total hepatic ischaemia was used as this avoids the production of acute portal hypertension and intestinal ischaemia. Moreover, lobar clamping avoids the cessation of biliary drainage, a characteristic of the total hepatic ischaemia model (Jaeschke et al. 1988), permitting the effects of I/R on biliary composition to be evaluated. As common bile duct cannulation results in the collection of bile from both the ischaemic and non-ischaemic lobes we compared the findings from the I/R group with a sham (non-ischaemic) group to ensure that these changes in bile composition were the result of the I/R procedure. Although previous studies have shown no difference in bile salt secretion or any other measured parameters, besides a decrease in bile flow, between ischaemic and non-ischaemic lobes in a similar lobar ischaemia rat model (Accatino et al. 2003). Tracheostomy and ventilation stabilized the model haemodynamically and permitted bile constituents to be analyzed into the late phase of I/R. An inhaled anaesthetic agent isoflurane, which is mainly metabolized in the lungs

rather than in the liver, was also used for maintenance of anaesthesia, in order to avoid cumulative effects or hepatotoxicity.

The hepatocyte is a polarized epithelial cell with sinusoidal and canalicular plasma membrane domains. Hepatic uptake of biliary constituents is initiated at the sinusoidal membrane, which is in direct contact with portal blood plasma via the fenestrae of the sinusoidal endothelial cells and the space of Disse (Nathanson and Boyer 1991). For cellular uptake of taurine or glycine conjugated bile acids, an active transport mechanism is required (Trauner and Boyer 2003; Wolkoff and Cohen 2003) as hepatic uptake of bile salts occurs against a 5-10 fold concentration gradient between the portal blood plasma and the hepatocyte cytosol (Bowers *et al.* 1987; Glanemann *et al.* 2003). Canalicular excretion of biliary constituents represents the rate-limiting step of bile secretion since biliary constituents are excreted against high concentration gradients into bile. ATP-dependent transport systems in the liver transport biliary constituents against steep concentration gradients across the canalicular membrane that are often in the range of 100- to 1,000-fold and are driven by ATP hydrolysis (Fisher *et al.* 1999; Sumimoto *et al.* 1988).

Bile flow decreased and ALT activity increased mildly over the duration of the experiment in the sham-operated group suggesting minimal operative and anaesthetic trauma to liver function over this lengthy surgical procedure combined with remote liver injury from limb ischaemia, caused by femoral artery cannulation as previously discussed (Chapter 4). However, the 6-10 fold rise in ALT following reperfusion in the I/R group suggests significant I/R injury.

Cytochrome oxidase is the terminal complex of the mitochondrial respiratory chain. It takes electrons from cytochrome c and catalyses the reduction of oxygen to water with the concomitant synthesis of ATP through the oxidative phosphorylation process (El Desoky et al. 2001; Hafez et al. 2004). In hepatocytes, approximately 90% of the oxygen is consumed by mitochondrial cytochrome oxidase. Absorption of the NIR light by Cyt Ox occurs primarily at the Cu_A centre within Cyt Ox. There is a decrease in signal intensity on reduction of this centre. The redox state of Cyt Ox Cu_A is dependent on cellular oxygen availability. In the presence of oxygen, electron transfer occurs and the enzyme becomes oxidised, whereas lack of oxygen, results in a decreased flow of electrons and Cyt Ox becomes reduced. The increase in the reduction state of Cyt Ox reflects severe cellular hypoxia. NIRS has been used to measure liver tissue oxygenation changes following warm liver I/R injury (El Desoky et al. 2000). There was a significant decrease in the redox state of Cyt Ox Cu_A following ischaemia that was still persistent at 5 h and 7 h following reperfusion in the I/R group.

The drop in bile flow during ischaemia corresponds to the exclusion of liver mass in the I/R group during this phase. A post-reperfusion decrease in bile flow has been reported previously (Fisher et al. 1999; Sumimoto et al. 1988) and bile excretion has been used as an indicator of liver function and recovery following ischaemia in experimental studies (Sumimoto et al. 1988) and clinical liver transplantation (Fisher et al. 1999). ^{1}H NMR spectroscopy of bile revealed changes in the concentration of some bile constituents. Bile lactate were elevated towards the beginning of the late phase of I/R where there is significant neutrophil activation. It has been observed that the injury produced during the beginning of the late phase is a more extensive hepatocellular injury in comparison to the early phase (Glantzounis et al. 2004; Lentsch et al. 2000).

Neutrophil sequestration, sinusoidal narrowing and vasoconstriction combine to form a 'no-reflow paradox' whereby hepatocytes are subjected to a further perfusion deficit and persistent ischaemia (Menger *et al.* 1992; Peralta *et al.* 2000). This may explain the observed peak in lactate at this late time-point. Lactate levels have been shown to become elevated in bile even when they are not elevated in serum (Nishijima *et al.* 1997). ATP degradation after ischaemia leads to the activation of glycolysis, resulting in the net formation of lactate. Serum lactate concentration has been shown to increase progressively following liver ischaemia, primarily from glycolytic/gluconeogenic pathway. This was verified by measuring glucose-6-phosphate and fructose-6-phosphate which were shown to increase with I/R injury (Peralta *et al.* 2000).

Phosphatidylcholine was raised following ischaemia and reperfusion at 2 h and 5 h. The main source of phosphatidylcholine in cells is in the plasma membrane, which suggests that the increase during ischaemia and again at 5 h post-reperfusion may be associated with an increase in cellular breakdown and membrane lipid peroxidation. Increased biliary phosphatidylcholine has also been observed in other experimental studies (Suzuki *et al.* 2000) and in association with poor graft function (Melendez *et al.* 2001).

Acetate was observed to drop continuously from baseline throughout the experiment in the I/R group. Hepatocytes are one of the few cells that can utilize fatty acids in the production of energy through the β-oxidation helix (Ambrose and Easty 1977). This reaction requires ATP at its initiation. Post-reperfusion ATP is consumed to clear excess lactate and pyruvate and may not be readily available for fatty acid oxidation. The ATP reduction as a result of I/R may result in decreased β-oxidation and acetate production is

reduced. Biliary acetate decreased with increases in ALT and AST, established markers of hepatocyte damage, and increased with increases in bile flow and redox state of Cyt Ox Cu_A, accepted indicators of liver function and viability.

In conclusion, bile spectroscopy has demonstrated significant changes in bile composition during I/R. These changes are evident despite a constant, albeit reduced, post-reperfusion rate of bile flow. Having identified significant changes in lactate, PC and acetate with I/R, further studies are required to evaluate and quantify these changes.

5.5 Chapter summary

Ischaemia-reperfusion (I/R) injury in liver resection and transplantation is associated with poor liver function and reduced bile excretion. Compositional changes in bile following liver I/R are of growing interest. A rabbit model was used and two groups of animals were studied. In the I/R group, lobar liver ischaemia was induced in New Zealand white rabbits (n=6) by inflow occlusion to the median and left liver lobes, under general anesthesia, for 60 min, followed by 7 h reperfusion. The sham group (n=6) underwent laparotomy for 9 hours but no liver ischaemia. In both groups the bile duct was cannulated and bile was collected continuously and blood was collected for analysis of transaminases. Bile composition was determined by proton nuclear magnetic resonance (^{1}H NMR) spectroscopy. Liver oxygenation was determined throughout the experiment using near infrared spectroscopy. During ischaemia bile excretion decreased (60 ± 19 µL/min/100g vs. 202 ± 32 µL/min/100g; P=0.0022) and biliary phosphatidylcholine increased (213.04 ± 64.06% of baseline vs. 82.13 ± 31.91% of baseline, P=0.0105) in the I/R group. With reperfusion in the I/R group, bile flow returned to 45 ± 9% of baseline, ALT and AST were raised at 7 h (P=0.0001 and P=0.0376 respectively), biliary lactate was elevated at 5 h post-reperfusion to 165.44 ± 22.97% of baseline (P=0.0036). Acetate levels were significantly decreased at 7 h post-reperfusion (37.47 ± 7.49% vs. 69.36 ± 13.29%, P=0.0058). In conclusion, ^{1}H NMR spectroscopy has demonstrated significant changes in bile composition associated with I/R injury and could be applied as marker to assess the effectiveness of therapeutic strategies and also for early diagnosis of liver dysfunction (e.g. post liver transplantation).

CHAPTER 6

THE EFFECT OF CONTINUOUS N-ACETYLCYSTEINE

INFUSION ON LIVER FUNCTION DURING WARM ISCHAEMIA-

REPERFUSION IN NORMAL LIVER

6.1 Introduction

In the previous chapter significant changes were found in bile volume and composition during hepatic I/R. This chapter studies the effect of the antioxidant N-acetysteine, in rabbits with normal liver, undergoing liver I/R. As mentioned before, liver I/R injury occurs during major liver surgery and transplantation or following haemorrhagic shock and subsequent fluid resuscitation (Huguet *et al.* 1994; Henderson 1999; Vedder *et al.* 1989). Extracorporeal circulation used in cardiac or vascular surgery is also associated with low-flow I/R of the liver (Pannen 2002). When the degree of injury is severe it may result in both liver failure (Huguet *et al.* 1994) and remote organ failure in the lungs, heart and the systemic circulation (Matuschak 1994; Lichtman and Lemasters 1999). The pathophysiology of liver I/R involves the activation of many metabolic pathways and the release of mediators that induce liver injury (Lentsch *et al.* 2000). There is growing evidence that there are two distinct phases of liver injury after warm ischaemia and reperfusion (Colletti *et al.* 1990; Jaeschke *et al.* 1990; Jaeschke and Farhood 1991; Lentsch *et al.* 2000). The initial phase (within two hours of starting reperfusion) is characterized by Kupffer cell-induced oxidative stress (Jaeschke and Farhood 1991). The late phase (after four hours) mainly results from the accumulation of neutrophils and is associated with more extensive injury (Jaeschke *et al.* 1990). Main contributors to the liver damage are ROS and RNS, which are released in both phases of reperfusion and produce significant oxidative stress (Jaeschke 2000). Mammals have a complex antioxidant system to protect themselves from such stress. One of the most important components of the intracellular antioxidant system is glutathione, a powerful active radical scavenger that is depleted during severe liver I/R injury (Liu *et al.* 1994).

N-acetylcysteine (NAC) is a thiol-containing compound used in the management of fulminant liver failure after paracetamol overdose (Prescott *et al.* 1979; Chyka *et al.* 2000). One mechanism by which NAC acts, is by entering cells and undergoing hydrolysis to cysteine, a glutathione precursor capable of rapidly replenishing depleted intracellular reduced glutathione concentrations (Halliwell B and Gutteridge JMC 1999). NAC also scavenges several ROS and RNS directly (Cotgreave 1997). Experimental studies in which NAC has been used to reduce warm liver I/R injury have produced conflicting results (Chavez-Cartaya *et al.* 1999; Koeppel *et al.* 1996) and the use of antioxidants for liver I/R still requires study of the mechanism and timing of any beneficial effects.

The present study evaluated the effect of NAC administration on liver function in both the early phase and initial part of the late phase of warm liver I/R injury. A rabbit lobar I/R model was used in which several markers of liver function were monitored continuously or at intervals for seven hours after the ischaemic injury. For continuous monitoring of arterial blood pressure and blood withdrawal the right femoral artery was cannulated. This was done because the effects of limb ischaemia to remote organs was not apparent at the time this study was performed.

6.2 Materials and methods

6.2.1 Animal model-measurements

The study was conducted under a license granted by the Home Office in accordance with the Animals (Scientific Procedures) Act 1986. New Zealand white rabbits with mean (s.d.) body weight (BW) 3.8 (0.5) kg; $n = 18$) were used. The mean (s.d) liver weight (LW) was 90 (15) and the mean (s.d) LW/BW ratio (%) was 2.5 (0.26). Anaesthesia and surgical

procedure are described in section 3.1. Briefly anaesthesia was induced by intramuscular injection of 0.5 ml/kg Hypnorm and maintained with 0.5–3 % isoflurane through an anaesthetic circuit following tracheostomy. A radio-opaque catheter 20 gauge (GA) was inserted into the right femoral artery for monitoring of arterial blood pressure and collection of blood samples. Ear marginal veins were cannulated with radio-opaque catheters (22 GA) for the administration of anaesthetics, fluids and medication.

Laparotomy was performed through a midline incision. Lobar ischaemia was induced by clamping the vascular pedicles of the median and left lobes of the liver, using an atraumatic microvascular clip. After 60 min of ischaemia, the vascular clip was removed and reperfusion was allowed for 7 h. At the end of the experiment the animals were killed by exsanguination.

Three groups of animals were used. In the NAC group 150 mg/kg NAC (Parvolex[®]; Medeva Pharma, Ashton-under-Lyne, Lancashire, UK) in 20 ml 5 % dextrose was infused intravenously through the ear vein over the 15 min before reperfusion and maintained at 10 mg per kg per h in 5 % dextrose (10 ml/hour) during the 7 h reperfusion period. In the I/R group 20 ml five per cent dextrose was infused intravenously 15 min before reperfusion and continued at a dose of 10 ml/h during the reperfusion period. Animals in the sham operation group underwent laparotomy but no liver ischaemia. In all groups 0.9 % sodium chloride was administered intravenously at 10 ml per kg per h to compensate for intraoperative fluid loss.

Laparotomy and baseline measurements were completed over one hour. In the sham group monitoring continued for the equivalent of the ischaemia and reperfusion periods in treated animals (total monitoring period 9 h).

Portal flow was monitored continuously using a perivascular transonic flow probe 3 mm in diameter (see section 3.4).

Arterial blood samples (1 ml each) were taken before the induction of liver ischaemia (baseline), at the end of ischaemia (60 min) and two, five and seven hours after reperfusion for measurement of alanine aminotransferase (ALT) activity. Serum was separated from the samples and stored at $-20°C$ until assayed. Measurements were made using an automated clinical chemistry analyser (Hitachi® 747, Roche Diagnostics, Lewes, UK).

Hepatic microcirculation was measured by a surface laser Doppler flowmeter (LDF), following the procedure described in section 3.2.

Intracellular hepatic tissue oxygenation was measured by near-infrared spectroscopy (NIRS). Basic principles of NIRS are described in section 3.3. For continuous monitoring of hepatic tissue cytochrome oxidase redox state, NIRS probes were positioned flat on the surface of the left lobe of the liver 10 mm apart. A flexible probe holder was used to ensure satisfactory contact with the liver surface with fixed interprobe spacing. NIRS measurements during ischaemia and reperfusion were expressed relative to baseline values before vascular occlusion.

A bolus of 0.5 mg/kg ICG (Cardiogreen®, 90 per cent dye content; Sigma Chemical Company, Poole, UK) was given after seven hours of reperfusion. ICG was dissolved in sterile water (50 mg per 10 ml) and administered via the marginal ear vein over 20 sec. Continuous measurement of hepatic ICG by NIRS produced a concentration–time curve. This curve was analysed to produce two exponential rate constants: α, which represented hepatic ICG uptake from the plasma to the hepatocytes and β, which represented hepatic ICG excretion from the liver by cytoplasmic transport and biliary excretion (Shinohara *et al.* 1996).

6.2.2 Data collection and statistical analysis

Data from the pulse oximeter, blood pressure and portal flow monitor, LDF and NIRS were collected continuously on a laptop computer. The data were averaged for two minutes before the induction of ischaemia (baseline), at the end of ischaemia and at the end of each hour of a seven hour reperfusion period. Changes in hepatic tissue oxygenation at the end of each period were calculated relative to baseline. Values are expressed as mean (s.d.). Student's *t* test for paired samples and one-way ANOVA with Bonferroni adjustment for multiple comparisons were used for statistical analysis. $P < 0.050$ was considered significant.

6.3 Results

6.3.1 Systemic haemodynamic variables

In all three groups systemic mean arterial blood pressure fell in the reperfusion period compared with baseline. These changes were not significantly different between groups (Figure 6. 1). In all groups the pulse rate slowed during the course of the experiment in

comparison with baseline measurements. The pulse rate stayed closer to baseline in the

NAC group than the I/R group; this difference was significant in the fifth (P = 0.026), sixth

(P = 0.025) and seventh (P = 0.016) h of reperfusion (Figure 6.2).
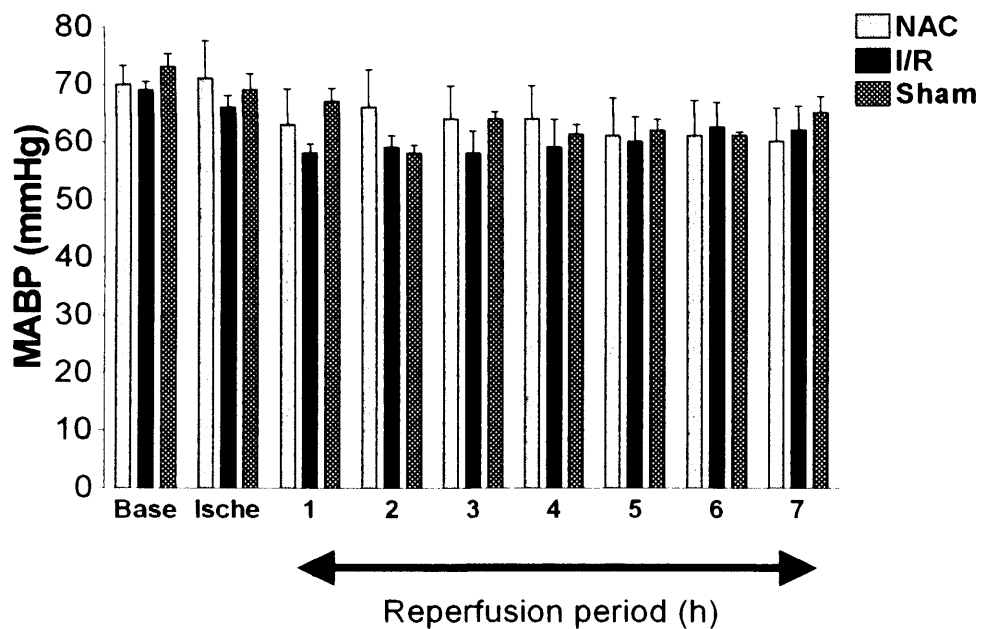
Figure 6.1 Mean arterial blood pressure (MABP) during ischaemia and reperfusion. Values are mean (s.d.). NAC, N-acetylcysteine; I/R, ischaemia–reperfusion. There were no significant differences between groups
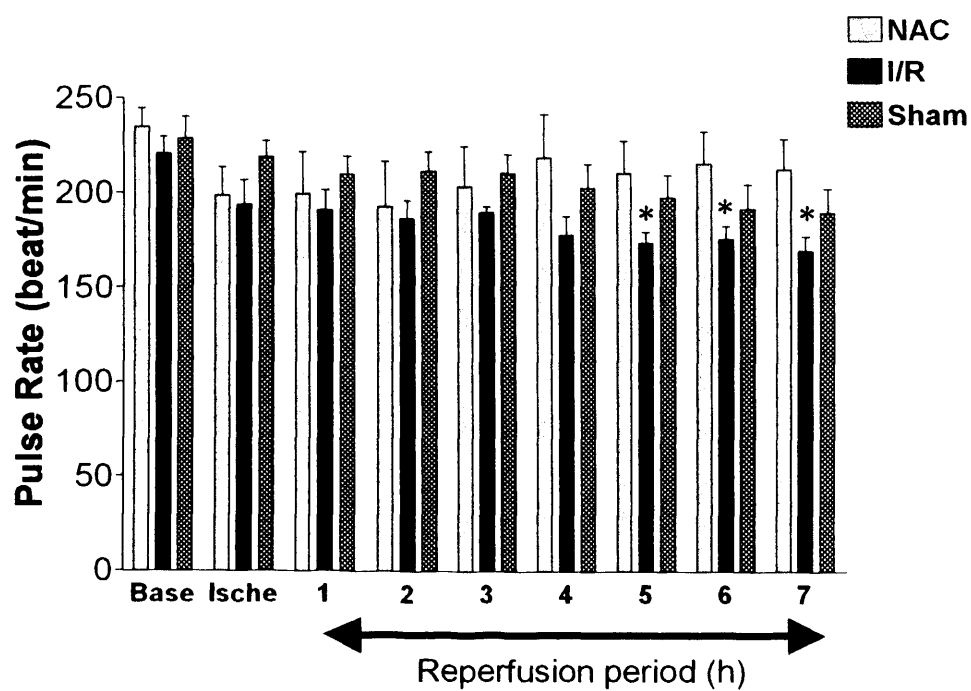
Figure 6.2 Pulse rate during ischaemia and reperfusion. Values are mean (s.d.). NAC,

N-acetylcysteine; I/R, ischaemia–reperfusion. *$P < 0.050$ *versus* I/R group (one-way

ANOVA with Bonferroni adjustment for multiple comparisons)

6.3.2 Hepatic haemodynamic parameters

There were no differences in portal blood flow values between the groups at baseline. During the period of ischaemia the portal flow was reduced in both the NAC (P = 0.003) and I/R (P = 0.001) groups, compared with baseline values. The reduction started one minute after the induction of ischaemia and was maximal by ten minutes. During the rest of the experiment, the portal flow fluctuated in all three groups. In the sham group there was an increase in portal flow rate. Portal flow was significantly greater in the sham group in comparison to that in the NAC and I/R groups during the first (P = 0.037), sixth (P = 0.025) and seventh (P = 0.033) hours of reperfusion (*Fig. 6.3*).
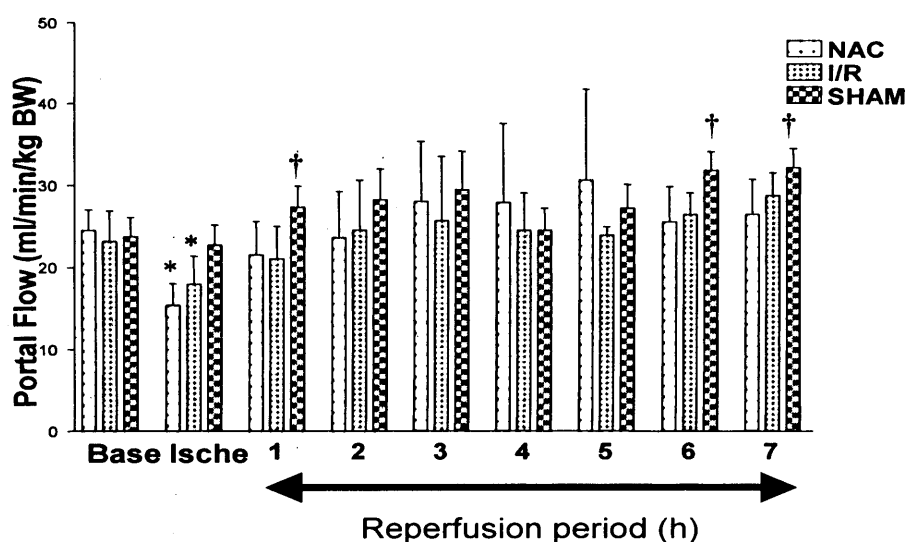
Figure 6.3   Portal flow during ischaemia and reperfusion. Values are mean (s.d.). NAC, *N*-acetylcysteine; I/R, ischaemia–reperfusion. *P < 0.050 *versus* baseline (Student's *t* test for paired samples); †P < 0.050 *versus* NAC and I/R groups (one-way ANOVA with Bonferroni adjustment for multiple comparisons)

6.3.3 Liver function tests

Mean serum ALT activity at baseline was within the normal range, with no significant differences between the three groups (*Fig. 4*). After reperfusion, the mean ALT activity in the NAC and I/R groups was significantly higher than that in the sham-operated group. The serum ALT activity was less severe in the NAC group than I/R group in the fifth (P = 0.007) and seventh (P = 0.021) hours of reperfusion (*Fig. 4*). By the end of the experiment ALT activity had increased twofold in the sham-operated group compared with baseline values (*Fig. 6.4*).
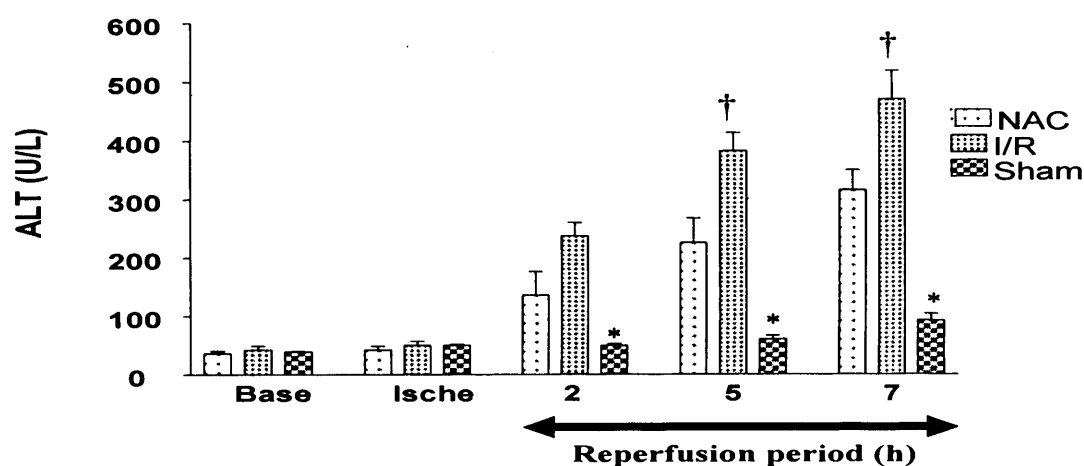
Fig. 6.4 Serum alanine aminotransferase (ALT) activity during ischaemia and reperfusion. Values are mean (s.d.). NAC, N-acetylcysteine; I/R, ischaemia–reperfusion. *P < 0.050 *versus* NAC group (one-way ANOVA with Bonferroni adjustment for multiple comparisons)

6.3.4 Hepatic microcirculation

There was no significant difference in the hepatic microcirculation over the nine hour sham operation. Following I/R, flow in the microcirculation was reduced in the NAC and I/R groups. NAC treatment was associated with an increased flow in the hepatic microcirculation after the third hour of reperfusion compared with that in the I/R group, but this was not statistically significant until the fifth hour ($P = 0.036$) (*Fig. 6.5*).
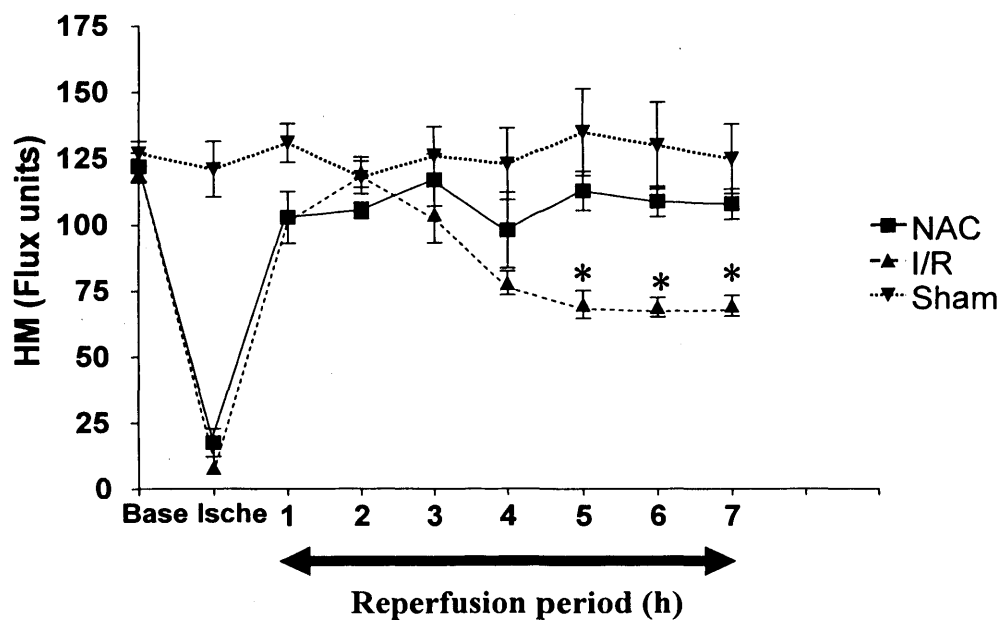
Fig. 6.5 Hepatic microcirculation during ischaemia and reperfusion. Values are mean (s.d.). NAC, N-acetylcysteine; I/R, ischaemia–reperfusion. *P < 0.050 *versus* NAC group (one way ANOVA with Bonferroni adjustment for multiple comparisons). There were no significant differences in the hepatic microcirculation during the experiment in the sham-operated group (Student's t test for paired samples)

6.3.5 Hepatic tissue oxygenation

During ischaemia there was a significant decrease in cytochrome oxidase redox state, which was maximal at five to ten minutes after induction of ischaemia (*Fig. 6.6*). After reperfusion, cytochrome oxidase redox state returned towards baseline values during the first hour in the I/R group, but subsequently decreased. In the NAC group, redox state of Cyt Ox also fell with ischaemia but was significantly better compared to the I/R group in the second (P = 0.023), sixth (P = 0.005) and seventh (P = 0.004) hours after reperfusion (*Fig .6.6*). Levels in the NAC group were similar to those in the sham group in the sixth and seventh hours (*Fig. 6.6*).
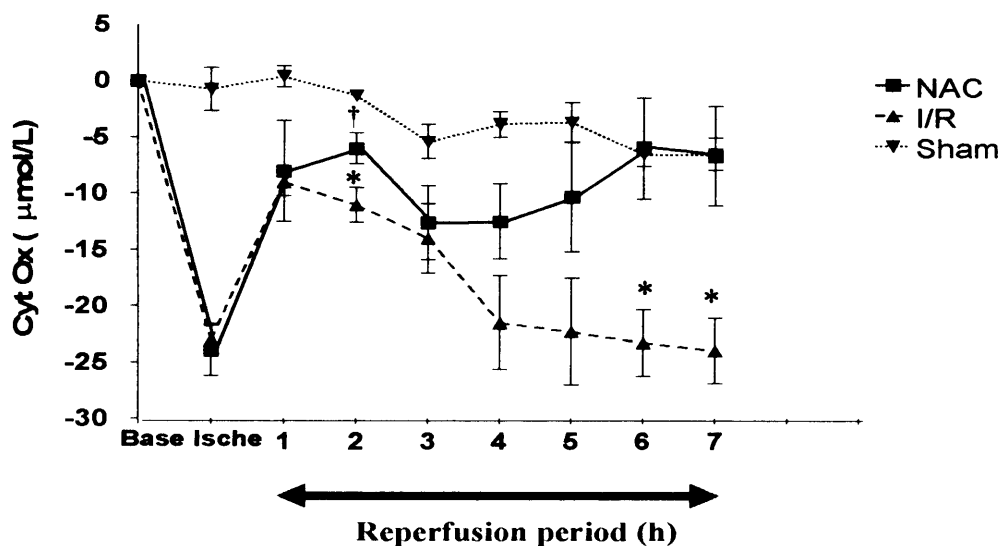
Fig. 6.6 Changes in cytochrome oxidase redox state relative to baseline during ischaemia and reperfusion. Values are mean (s.d.). NAC, *N*-acetylcysteine; I/R, ischaemia–reperfusion. *P < 0.050 *versus* NAC group (one-way ANOVA with Bonferroni adjustment for multiple comparisons)

6.3.6 Indocyanine green (ICG) clearance

ICG uptake (α) and excretion (β) rates in NAC and I/R groups were reduced by I/R, in comparison to values in the sham group (*Table 6.1*). NAC produced a significant improvement in both ICG uptake and excretion at seven hours after ischaemic injury compared with rates in the I/R group (*Table 6.1*).

Table 6.1. Hepatic indocyanine green (ICG) uptake and excretion rates.

GROUPS	α (min^{-1})	β (min^{-1})
NAC	2.245 ± 0.32*	0.024 ± 0.007[†]
I/R	0.848 ± 0.394*	0.005 ± 0.001[†]
Sham	2.565 ± 0.488	0.064 ± 0.019

Values are mean ± (SD.) of six animals in each group. α: Hepatic ICG uptake rate; β: hepatic ICG excretion rate; I/R: ischaemia / reperfusion, *p= 0.001, NAC vs I/R group, [†]p=0.032, NAC vs I/R group. One way analysis of variance (ANOVA) with Bonferroni adjustment for multiple comparisons were used.

6.4 Discussion

The late phase of warm liver I/R injury is considered to start at four hours after reperfusion and is mainly due to release of ROS and proteases from activated neutrophils. This produces more extensive hepatocellular injury than the early phase (Jaeschke *et al.* 1990; Harbrecht *et al.* 1993).

The present study investigated continuous infusion of NAC in a liver I/R model, which allowed hepatic function and haemodynamics to be evaluated in both the early and initial late phase of liver I/R injury. Liver microcirculation and intracellular oxygenation were continuously recorded during a reperfusion period of 7 h. An inhaled agent (isoflurane), which is mainly metabolized in the lungs rather the liver, was used for maintenance of anaesthesia, to avoid cumulative effects or hepatotoxicity. The sham group allowed the effects of prolonged anaesthesia and surgical trauma alone to be evaluated. The duration of general anaesthesia was similar to that for major human liver surgery (liver resection and transplantation).

In the sham-operated control group, arterial blood pressure dropped, ALT activity was significantly increased and hepatic intracellular oxygenation was significantly decreased during the period of anaesthesia compared to baseline. This was the effect of remote liver injury due to limb ischaemia caused from the canulation of femoral line. Also the anaesthetic agent and operative trauma could affect liver function during the lengthy surgical procedure.

Analysis of the changes in microcirculation and intracellular oxygenation during the seven hour reperfusion period suggests that the initial part of the late phase starts from the third hour after reperfusion and reaches a peak at the sixth hour. A previous study (Jaeschke *et al.* 1990) suggested that the late phase starts 6 h after reperfusion, based on the measurement of hepatocellular necrosis and neutrophil infiltration. Histological changes indicative of liver injury are likely, however, to be detected later than changes in the hepatic microcirculation or tissue oxygenation.

Portal flow values were calculated with respect to bodyweight to eliminate variations related to animal size. There was a significant decrease in portal flow during ischaemia in the I/R and NAC groups. This decrease may result in a reduced metabolic need of the liver. Similar findings have been reported in previous studies (El Desoky *et al.* 1999). During reperfusion there was fluctuation in portal flow in all three groups. In the sham group, the trend in portal flow was for a gradual increase during reperfusion, which might have been an effect of isoflurane which increases hepatic blood flow and oxygen supply despite lowering systemic arterial pressure (Bernard *et al.* 1991; Gatecel *et l.* 2003).

During inflow occlusion in the I/R group, the hepatic microcirculation dropped acutely almost to zero. There was a short-term recovery during the first two hours of reperfusion, followed by a further decrease. Mechanisms that might contribute to this impairment of sinusoidal perfusion include sinusoidal endothelial cell swelling with luminal narrowing (Vollmar *et al.* 1994) and sinusoidal vasoconstriction mediated by an altered endothelin–nitric oxide balance (Pannen *et al.* 1998; Pannen 2002; Scommotau *et al.* 1999). The increased expression of adhesion molecules, with subsequent leucocyte and endothelial cell

interaction, may also play a crucial role in these changes in the microcirculation, especially during the late phase of reperfusion injury (Vollmar *et al.* 1995). It has been reported that the changes in microcirculation during the immediate reperfusion period in liver transplantation correspond directly with the maximum postoperative enzyme release from the liver (Klar *et al.* 1997) and are of value in assessing and predicting the degree of liver injury (Pannen 2002).

Cytochrome oxidase is the terminal electron carrier of the mitochondrial respiratory chain that catalyses the reduction of oxygen to water with the concomitant synthesis of adenosine triphosphate through the oxidative phosphorylation process (Capaldi 1990). In hepatocytes, approximately 90 per cent of the oxygen is consumed by mitochondrial cytochrome oxidase(Capaldi 1990). Changes in cytochrome oxidase redox state are indicative of the level of intracellular oxygenation and mitochondrial activity (Seifalian *et al.* 2001b; Koti *et al.* 2002). The degree of impairment of the hepatic mitochondrial redox state determines the survival rate of patients after haemorrhagic shock and resuscitation (Nakatani *et al.* 1995). In the present study, during ischaemia there was a significant fall in cytochrome oxidase redox state in the I/R group, reflecting severe cellular hypoxia. Levels returned towards baseline during the first hour of reperfusion but subsequently decreased and by 7 h had reached levels similar to those observed during ischaemia. This further decline in cytochrome oxidase indicates persistent tissue and cellular hypoxia, that correlates well with the changes in hepatic microcirculation during the late phase of reperfusion injury.

ICG uptake is related to liver blood flow and excretion is related to hepatocellular injury (El Desoky *et al.* 1999). Direct measurement of ICG by NIRS may avoid the inaccuracies

inherent in assessing liver function from peripheral blood clearance of ICG (Ott *et al.* 1994). In the present study I/R produced a significant reduction in both the uptake and excretion of ICG compared with levels in the sham-operated group.

The effect of NAC on liver I/R injury was evaluated using a dose of NAC similar to that used for patients with fulminant hepatic failure due to paracetamol overdose (Harrison *et al.* 1991). The administration of NAC maintained the pulse rate close to baseline levels, reduced liver injury (as indicated by ALT serum activity) and improved flow in the microcirculation and intracellular tissue oxygenation. All these effects were obvious by the fifth hour of reperfusion. NAC also improved ICG clearance at seven hours after reperfusion. In another experimental study, in which a rat lobar liver I/R model was used, no benefit was shown with NAC administration in warm I/R injury (Chavez-Cartaya *et al.* 1999), although a shorter ischaemic period was used (45 minutes) and the NAC (300 mg/kg) was administered intramuscularly before ischaemia, which may have resulted in a less reliable systemic distribution or a shorter-term effect. The effect of NAC on the hepatic microcirculation in the late phase of reperfusion has not been reported previously. Previous experiments were limited to the first 1 to 2 h after reperfusion and the results were conflicting (Chavez-Cartaya *et al.* 1999; Koeppel *et al.* 1996).

The effect of NAC on cytochrome oxidase level in the altered parenchymal microcirculation with I/R was examined, with clear evidence of effects on the delivery and metabolism of oxygen in the hepatic parenchyma. This study did not assess the delayed effect of NAC (at 24 or 48 h), as the model used was not appropriate for these longer periods.

The mechanism underlying the protective effect of NAC in the late phase of warm I/R injury was not established in this study. However, NAC is a precursor of glutathione regeneration (Halliwell B and Gutteridge JMC 1999), is a direct scavenger of free radicals (Cotgreave 1997), inhibits inducible nitric oxide synthase expression (Hur *et al.* 1999) and inhibits the expression of intercellular adhesion molecule one and vascular cell adhesion molecule one (Weigand *et al.* 2001).

Further studies are required to assess the effect of NAC at later time points and to elucidate the underlying mechanisms of action. Use of other thiols such as bucillamine (Amersi *et al.* 2002) or a combination of NAC with other antioxidants such as melatonin (Sener *et al.* 2003) may further reduce liver I/R injury. A large randomized clinical trial will be required to demonstrate a clear benefit of NAC administration in human liver warm I/R.

6.5 Chapter summary

N-acetylcysteine (NAC) may modulate the initial phase (less than two hours) of warm liver ischaemia–reperfusion injury (I/R) but its effect on the late phase remains unclear. The present study investigated the role of NAC during the early and late phases in a rabbit lobar I/R model.

Liver ischaemia was induced by inflow occlusion to the median and left liver lobes for 60 minutes, followed by 7 h of reperfusion. In the NAC group ($n = 6$), NAC was administered intravenously at 150 mg per kg over the 15 min before reperfusion and maintained at 10 mg per kg per hour during reperfusion. In the I/R group ($n = 6$), 20 ml five per cent dextrose was infused over the 15 min before reperfusion and continued at a rate of 10 ml/h. Animals in a sham operation group ($n = 6$) underwent laparotomy but no liver ischaemia. All animals were killed at the end of the experiment.

Intracellular tissue oxygenation was improved after the second hour of reperfusion in animals treated with NAC compared with that in the I/R group ($P = 0.023$). Hepatic microcirculation improved after the fifth hour of reperfusion ($P = 0.036$) and liver injury was reduced after the fifth hour, as indicated by alanine aminotransferase activity ($P = 0.007$) and indocyanine green clearance (uptake, $P = 0.001$; excretion, $P = 0.032$).

In conclusion the main protective effect of NAC becomes apparent five hours after hepatic ischaemic injury.

CHAPTER 7

THE EFFECT OF N-ACETYLCYSTEINE ADMINISTRATION ON

LIVER FUNCTION IN HEPATIC ISCHAEMIA-REPERFUSION IN

STEATOTIC LIVER

7.1 Introduction

Having demonstrated a significant reduction in liver I/R with a continuous infusion of NAC in normal liver (chapter 6), a controlled study of the administration of NAC in steatotic liver I/R was planned. Hepatic steatosis occurs with obesity, alcohol abuse and metabolic disorders (Neuschwander-Tetri and Caldwell 2003), and an incidence up to 11% has been reported in autopsy studies on accidental deaths (Underwood Ground 1984). It is now well recognised that steatotic livers are more susceptible to I/R injury than normal livers (Selzner and Clavien 2001). The mechanisms involved are still poorly understood but, as fat accumulates within the hepatocytes, cell volume increases resulting in a decreased sinusoidal space (Hakamada *et al.* 1997) and impaired microcirculatory blood flow (Selzner and Clavien 2001). Sinusoidal blood flow can be reduced by 50% in fatty livers (Ijaz *et al.* 2003; Seifalian *et al.* 1999). Steatosis represents a risk factor in liver surgery, with a mortality rate up to 14% in patients undergoing major hepatic resections (Behrns *et al.* 1998). Similarly, the use of steatotic grafts for transplantation is associated with a much higher risk of primary graft non-function or dysfunction (Busuttil and Tanaka 2003).

As a period of ischaemia is often necessary in liver surgery, and is inevitable in organ retrieval and transplantation, pharmacological modulation could potentially protect steatotic livers, minimizing the detrimental effects of I/R injury.

N-acetylcysteine (NAC) is a thiol-containing compound that interacts and detoxifies free radicals by non-enzymatic reactions, and is deacetylated to form cysteine, which supports biosynthesis of glutathione, one of the most important components of the intracellular antioxidant system (Cotgreave 1997). Nakano et al. demonstrated that perfusion with NAC

prior to organ retrieval reduced the extent of I/R injury after 24 h of cold storage in an isolated perfused rat steatotic liver (Nakano *et al.* 1997). Bucillamine, another thiol antioxidant, has recently been shown to prevent reperfusion injury in rat models of liver transplantation with both normal and fatty livers (Amersi *et al.* 2002).

In chapter 6 it was shown that continuous infusion of NAC during reperfusion, in rabbits with normal liver undergoing 1 h of lobar ischaemia, reduces significantly liver injury (Glantzounis *et al.* 2004).

The aim of the present study was to determine whether administration of NAC to rabbits with fatty livers reduced liver I/R injury.

7.2 Material and methods

7.2.1 Animal model

Details of exprerimental procedure are described in section 3.1. 12 New Zealand white rabbits with a mean body weight of 3.8 ± 0.5 kg were used. Steatosis was induced by feeding the animals with a high cholesterol (2%) diet for 8 weeks.

One radiopaque catheter (20 GA) was inserted into the ear artery to collect blood samples and connected to a pressure transducer to monitor mean arterial blood pressure (MABP) and heart rate. Ear marginal veins were cannulated in both ears with radiopaque catheters (22 GA) for the administration of fluids and drugs. Normal saline 0.9 % was infused at a rate of 15 ml/kg/h to replace the intraoperative fluid losses.

Laparotomy was performed through a bilateral subcostal (roof-top) incision. The ligaments from the diaphragm to the liver were divided and the liver was fully exposed. The bile duct was cannulated with a polyethylene catheter (PE-50, 0.58 mm inner diameter, Portex, Kent, UK). Bile flow was measured and calculated as $\mu L/min/100g$ of liver wet weight. Following dissection of the portal vein, a perivascular Doppler probe (HT207; Transonic Medical System Inc, Ithaca, NY) was positioned around it to monitor the portal blood flow. Lobar ischaemia was induced by clamping the vascular pedicles of the median and left lobes of the liver, using an atraumatic microvascular clip. The microvascular clip was removed after 60 min of ischaemia and reperfusion was allowed for 6 h.

7.2.2 Experimental groups and protocol

Two groups of steatotic animals (n=6 each) were used.

In group A (NAC) 150 mg/kg of N-acetylcysteine (Parvolex, Medeva Pharma Limited, Lancashire, UK) in 20 ml of 5% Dextrose was infused intravenously through the ear vein over the 15 minutes immediately before reperfusion and maintained at 10 mg/kg/h in 5% Dextrose during the 6 hours reperfusion period.

In group B (I/R) 20 ml of 5% Dextrose was infused intravenously 15 min before reperfusion and continued at a rate of 10 ml/kg/h during the reperfusion period.

In both groups, after laparotomy and a 10 minutes period of stabilization, systemic and hepatic haemodynamics, oxygen saturation, body temperature and bile flow were continuously recorded. Arterial blood samples were taken before the induction of liver ischaemia (baseline) and at 2, 5 and 6 h after reperfusion for measurement of alanine aminotransferase (ALT) activity. An equal volume of normal saline was used to replace the

volume of the blood taken. Serum was separated from the samples and stored at -20°C until assayed. The measurements were done using an automated clinical chemistry analyser (Hitachi 747, Roche Diagnostics Ltd, Sussex, UK). A biopsy was taken from the left liver lobe at baseline and 6 h after reperfusion. Formalin-fixed liver tissue samples were embedded in paraffin and stained with haematoxylin and eosin for subsequent microscopy (digital light microscope CLF60 optical system, Nikon UK Ltd, Surrey, UK). Formalin fixed and not paraffin embedded tissue was stained for fat using the Swank & Davenport modification of the Marchi method (Swank RL 1935).

7.2.3 Measurement of blood flow in the hepatic microcirculation

To determine the effect of NAC in the hepatic microcirculation a Laser Doppler Flowmeter was used as described in section 3.2.

7.2.4 Bile flow and composition

To determine the effect of NAC therapy on bile production and excretion, bile flow was measured and bile composition analysed with proton nuclear magnetic resonance spectroscopy (^{1}HNMR) using a quantitative method. Bile samples were taken at baseline and each hour thereafter and stored at -80°C. Bile volume was expressed as μL/min/100g of liver weight.

^{1}HNMR analysis was performed on an 11.7 Tesla (500 MHz for protons) spectrometer (Varian Unity +; Varian, Palo Alto, CA, USA) at 25°C. Bile was thawed at room temperature and placed in 5mm NMR tube. For a field / frequency lock a coaxial capillary

insert was used (Wilmad, Buena, NJ, USA). This capillary insert was filled with a

deuterium oxide solution of sodium $[^2d_4]$ - trimethysilylpropionate (TSP) that acted both as

a chemical shift reference and quantification standard. This capillary was calibrated by

using a series of known concentration solutions of deoxycholate and a calibration curve

was obtained. This was then used for quantification of bile components (lactate, acetate,

pyruvate and phosphatidylcholine) which enabled an accurate comparison of bile levels

between the groups. One dimensional NMR spectra were obtained at 500 MHz with a

sweep width of 6 kHz. Presaturation of bile was carried out to attenuate the intensity of

water signal. The spectra were analysed using software from MestRe-C version 3.1.1

(Universidade de Santiage de Compostela, Spain). All spectra were integrated using a fixed

range for each peak and published peak assignments (Melendez *et al.* 2001) shown in table

1.

7.2.5 Dihydrorhodamine 123 (DHR 123) oxidation

To determine the effect of NAC on the production of ROS and RNS such as peroxynitrite,

the in vivo oxidation of dihydrorhodamine (DHR) 123 to rhodamine (RH) was studied (see

also section 3.10). DHR 123 is a chemical compound and its oxidation to RH is partially

peroxynitrite-dependent (Szabo, Salzman, and Ischiropoulos 1995). RH is a fluorescent

substance that gives a characteristic colour to plasma, easily detected by fluorescence. 6 h

after reperfusion animals were administered DHR 123 intravenously (2μmol/kg in 0.8 ml

N. saline 0.9 %). Twenty minutes later plasma samples were collected for rhodamine

fluorescence evaluation. For fluorescence measurements a fluorometer was used at an

excitation wavelength of 485 nm and emission wavelength of 538 nm. The concentration of

RH formation was calculated using a standard curve obtained with authentic RH (1 to 40 nM) prepared in plasma obtained from untreated rabbits.

7.2.6 Data collection and statistical analysis

Data from the pulse oximeter, blood pressure monitor, transonic flowmeter and LDF were continuously recorded on a laptop computer. The data were averaged for 2 min before the induction of ischaemia (baseline), at the end of ischaemia, and each hour of a 6 h reperfusion period. The results are presented as mean (standard deviation). Student's t test for paired samples was used to detect differences in values within the same group. The differences in values between the two groups were calculated using the Student's t-test for unpaired samples. $P < 0.05$ was considered statistically significant. All statistical analysis was done using SPSS for Windows version 11.0 (SPSS Inc).

7.3 Results

7.3.1 Macroscopic appearance, histopathology

All animals fed with a high-cholesterol (2%) diet for 8 weeks developed moderate hepatic steatosis.The animals tolerated the high cholesterol diet with no mortality. At laparotomy the animals showed fatty deposition in the skin, liver and spleen. The liver was enlarged, yellowish in colour, with rounded edge and firm consistency (Figure 7.1). The mean liver weight was 130 ± 11 gr, while the LW / BW ratio (%) was 3.6 ± 0.33. Histology revealed centrilobular steatosis with macrovesicular fat accumulation (Figures 7.2 and 7.3). Mild to moderate polymorphonuclear infiltration was evident in the liver tissue at the end of reperfusion.
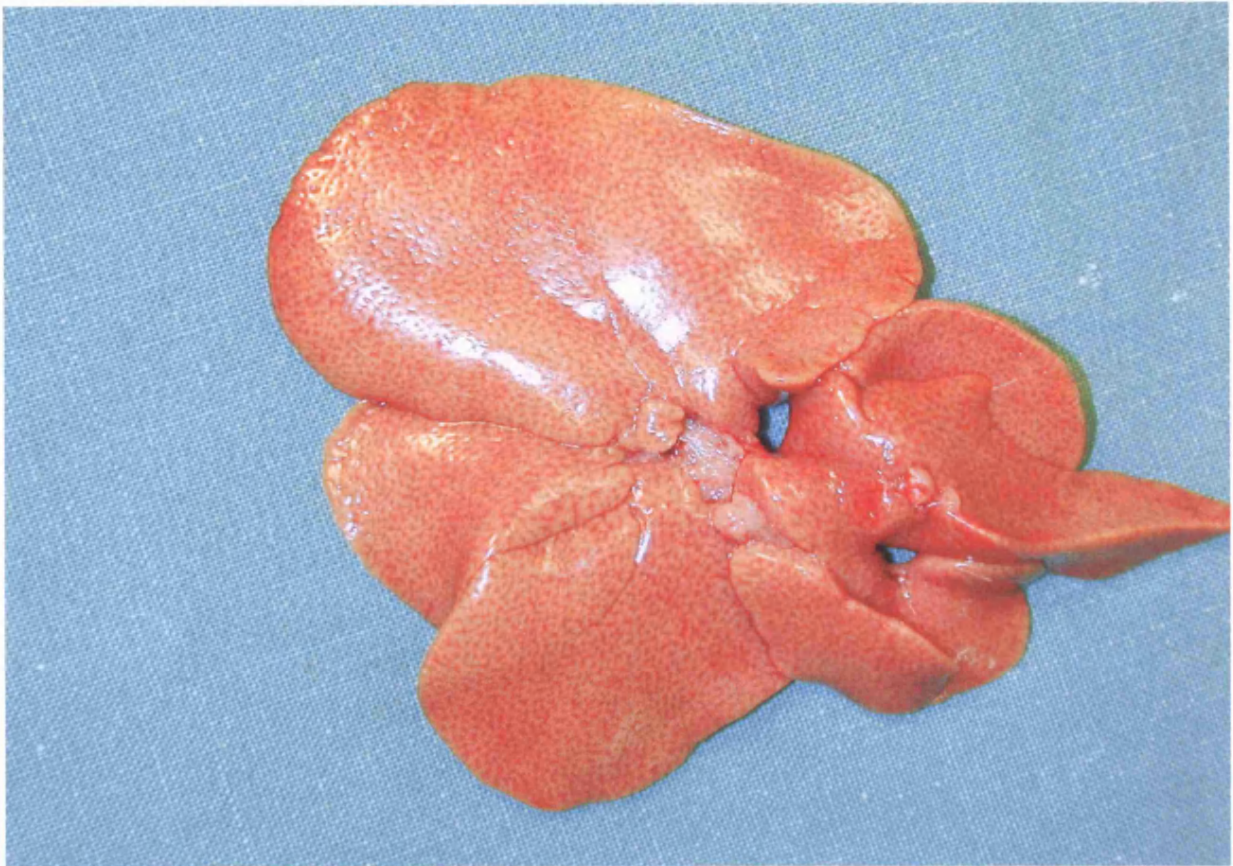
Figure 7.1 Macroscopical appearance of steatotic rabbit liver (after 8 weeks of high cholesterol diet). The liver is enlarged, yellowish in colour with rounded edges.
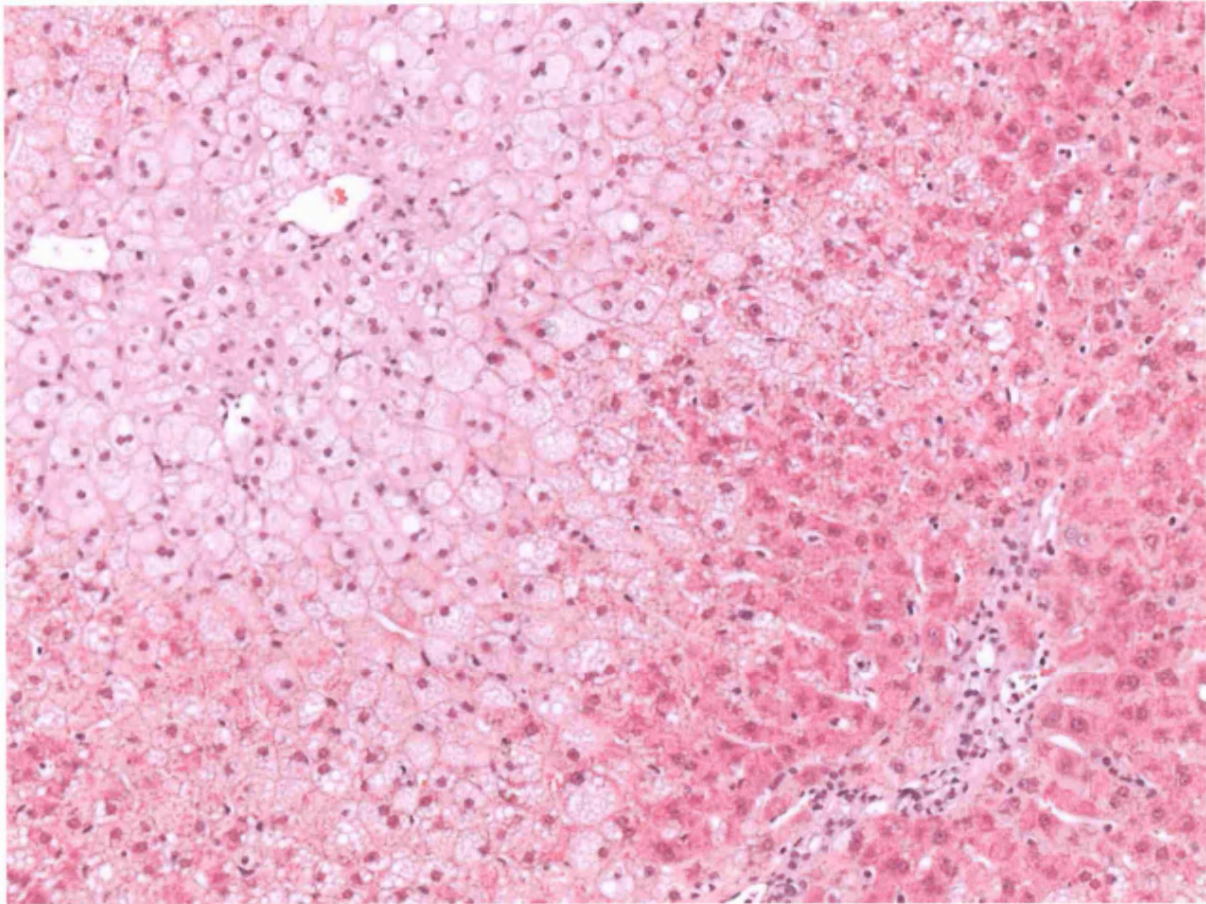
Figure 7.2 Histological features of steatosis with high cholesterol diet for 8 weeks. Macrovesicular fat accumulation in hepatocytes and Kupffer cells mainly in the pericentral area can be seen.
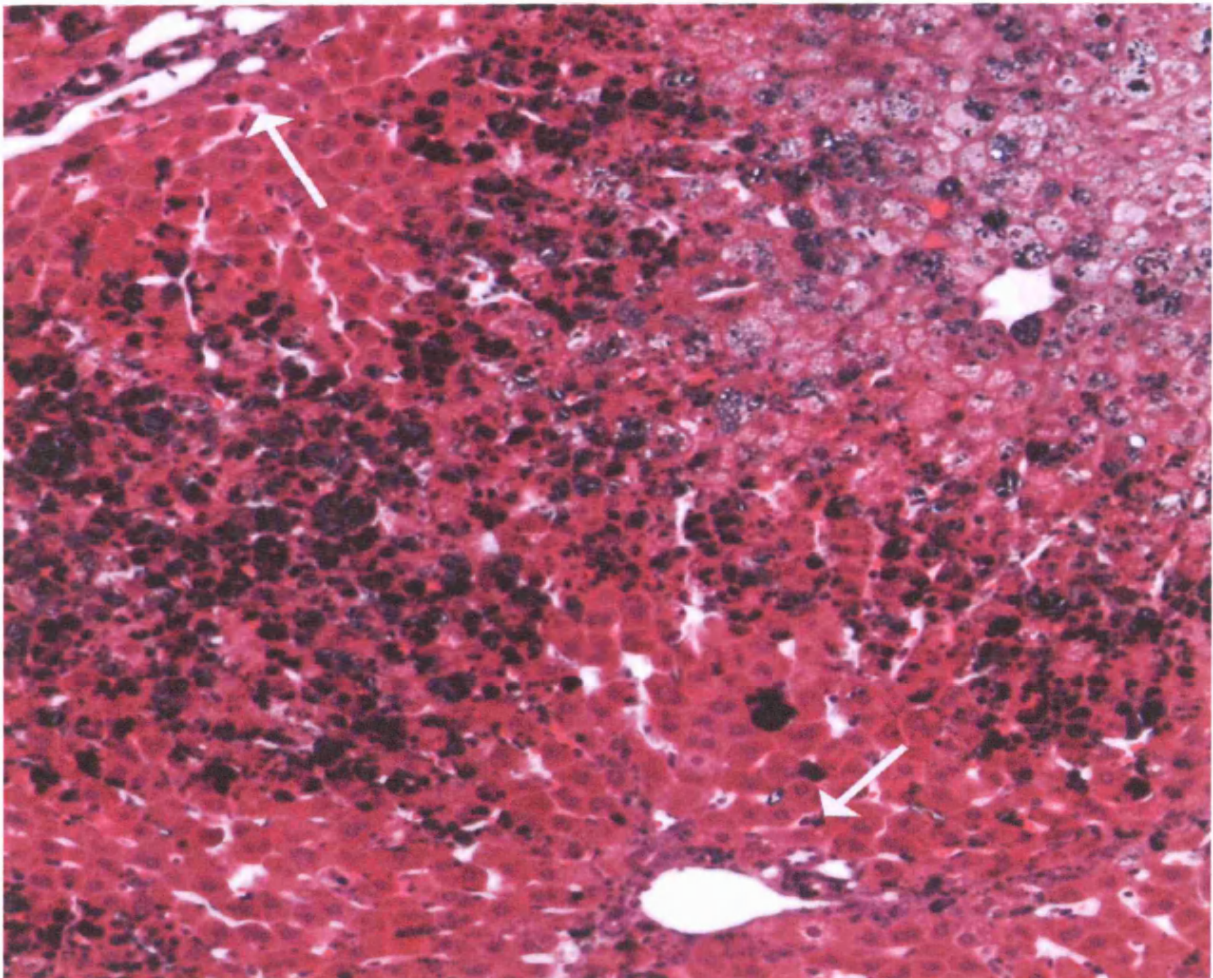
Figure 7.3 Section of liver stained with a special stain (Marchi method) showing a moderate amount of centrilobular fat (stained black). The arrows indicate two portal tracts (magnification 100x).

7.3.2 Systemic haemodynamics

In both groups MABP and heart rate fell in the reperfusion period compared to baseline. There was no significant difference in MABP, heart rate and oxygen saturation values between the two groups through out the experiment.

7.3.3 Hepatic haemodynamics

Portal flow values are demonstrated in figure 7.4. There was no significant difference in baseline values between the two groups. Portal flow was higher in NAC group after the third hour of reperfusion and this difference reached significance at the 6th h: 67.2 ± 7.2 vs. 52.4 ± 9.7 ml/min, (NAC vs. I/R, p=0.025).
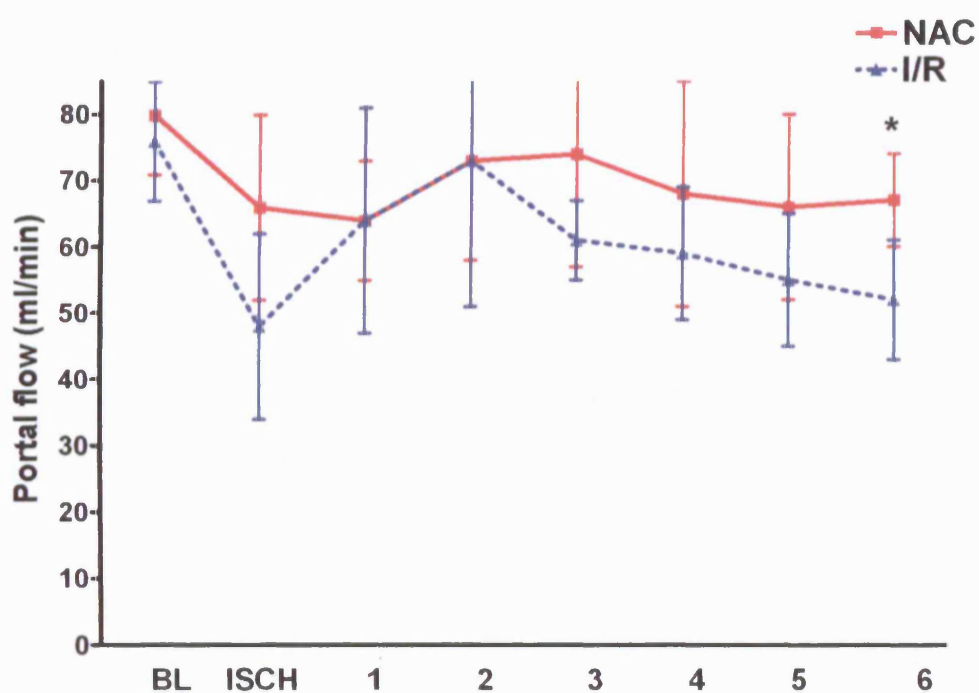
Figure 7.4. Portal flow in NAC and ischaemia-reperfusion (I/R) groups (mean, SD). Portal flow was significantly higher in the NAC group compared to the I/R group at the 6th h post-reperfusion (*p<0.05).

7.3.4. Hepatic Microcirculation (HM)

During ischaemia LDF values were significantly reduced in both groups and became almost nil (Figure 7.5). Following reperfusion in the I/R group, flow in the hepatic microcirculation was significantly reduced from baseline by 5th h: 86 ± 9 vs. 60 ± 9 flux units, (BL vs. 5th h, p=0.03) and 6th hour post-reperfusion: 86 ± 9 vs. 64 ± 10 flux units (BL vs. 6th h, p=0.04). In the NAC group LDF values were similar between baseline and reperfusion (Figure 7.5).

Flow in hepatic microcirculation was better in the NAC group in comparison to I/R group after the 3rd h of reperfusion and this difference became significant at the 5th h: 87 ± 21 vs. 60 ± 9 flux units, (NAC vs. I/R, p=0.03) and at 6th h: 89 ± 16 vs. 64 ± 10 flux units (NAC vs. I/R, p=0.02).

7.3.5 Liver function tests

No significant difference between baseline ALT values in the two groups was recorded (Figure 7.6). ALT values were significantly greater in the I/R group at 5th h: 154 ± 62 vs. 229 ± 49 U/L, (NAC vs. I/R, p=0.04) and 6th h of reperfusion: 168 ± 74 vs. 270 ± 54 U/L, (NAC vs. I/R, p=0.02) (Figure 7.6).
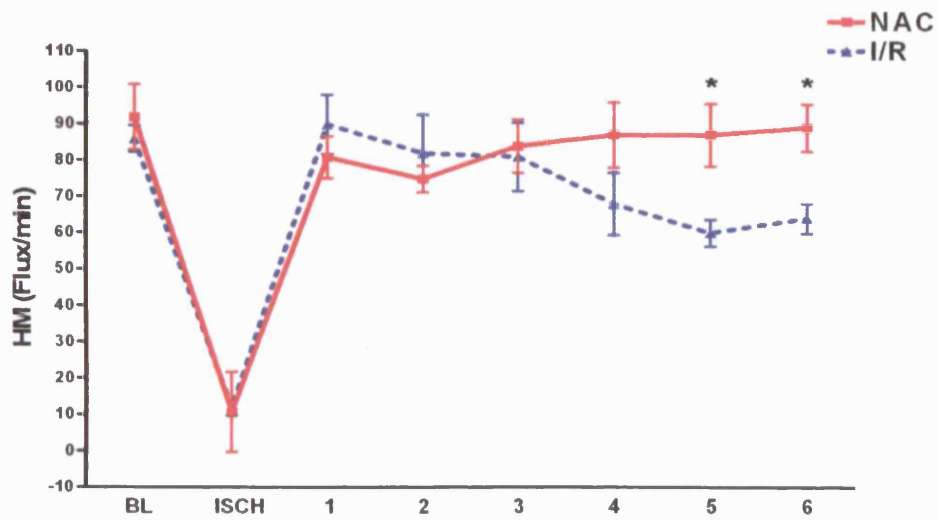
Figure 7.5. Hepatic microcirculation (HM) in NAC and I/R groups (mean, SD). HM was significantly better in NAC group compared to the I/R group at 5th and 6th h post-reperfusion (p<0.05)
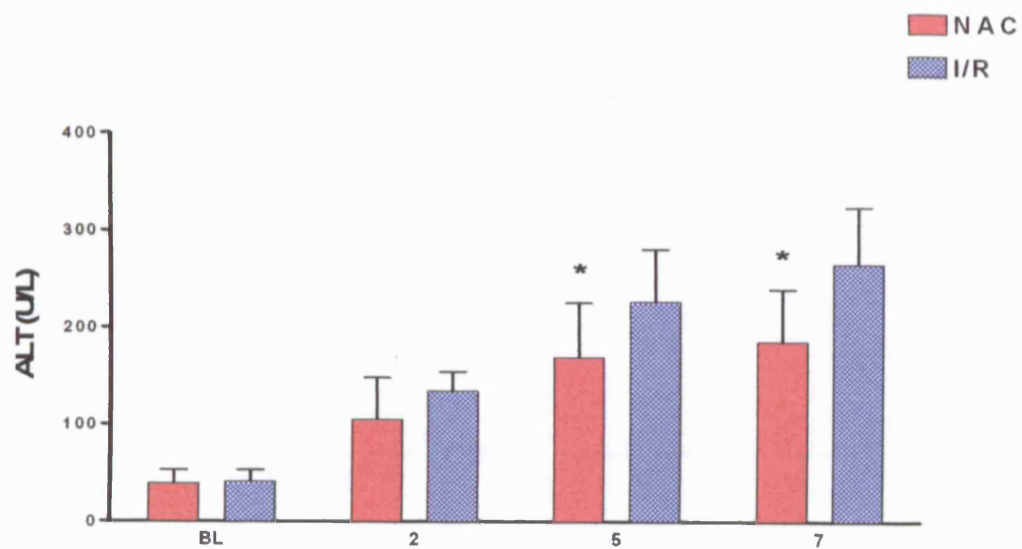
Figure 7.6. Serum alanine aminotransferase (ALT) activity (mean, SD). ALT activity was significantly lower in NAC group compared to the I/R group at 5th and 6th h post-reperfusion (*p<0.05).

7.3.6 Bile flow and Proton nuclear magnetic resonance (^{1}HNMR) spectroscopy

Baseline bile flow was similar in the two groups (Figure 7.7). Bile flow was higher in the NAC treated animals during the reperfusion period. This difference almost reached significance at the 5th hour: 115 ± 29 vs. 89 ± 10 μL/min/100 g of liver weight, (NAC vs. I/R, p=0.06) and became significant at the 6th h of reperfusion: 116 ± 25 vs. 83 ± 19 μL/min/100 g of liver weight (NAC vs. I/R, p=0.035) (Figure 7.7).
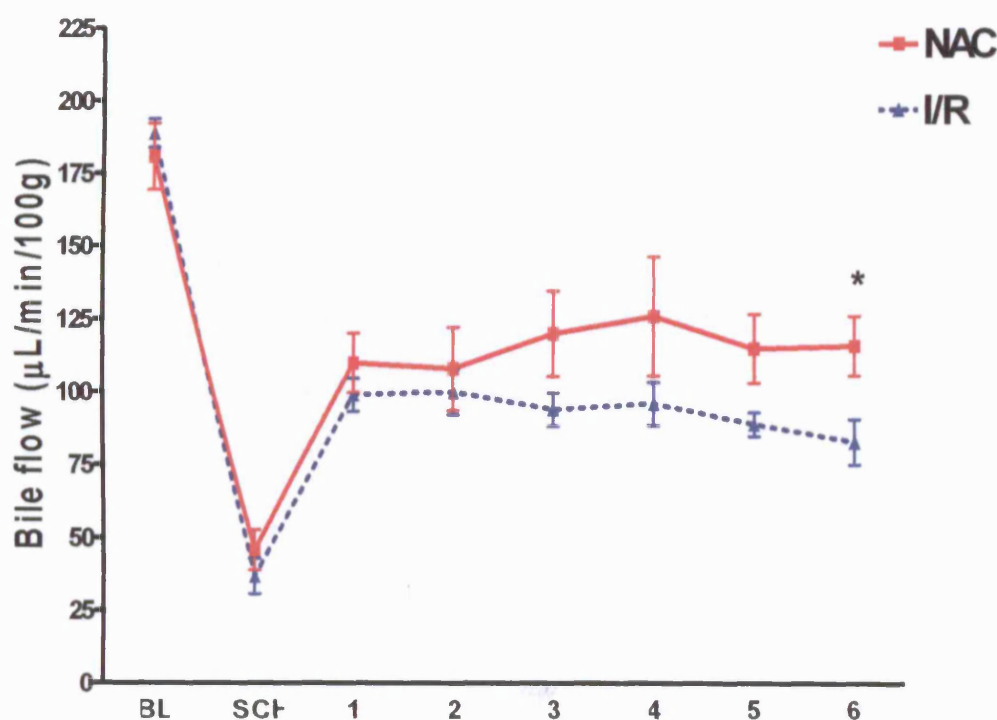
Figure 7.7. Bile flow (mean, SD). Bile flow was significantly higher in the NAC group compared to the I/R group at 6th h post-reperfusion (*p<0.05)

Baseline control bile spectra are shown in figure 7.8. The results of the integration of the area under curve for the assigned peaks are shown in table 7.1. Bile lactate levels were lower, across all time points, in the I/R group compared to NAC group (Table 7.2). Bile acetate levels rose in the NAC group to almost two fold the baseline level at 6 h post-reperfusion while in the control group they dropped to almost half their baseline levels. Acetate levels were significantly higher in the NAC group compared to the I/R at 5 h and 6 h post reperfusion (p=0.021) as shown in table 7.2.

Pyruvate levels were significantly higher in the NAC group compared to controls at 2 and 5 h post reperfusion (p=0.009 and p=0.021 respectively, Figure 7.9 and table 7.2).

During ischaemia there was a two fold rise in phosphatidylcholine (PC) levels in the I/R group compared to only a slight elevation in the NAC group (p=0.047). On reperfusion PC levels fell gradually in controls while in the NAC group PC levels rose to peak after 2 h reperfusion then fell to close to baseline values at 6 h reperfusion.

Table 7.1: ^{1}HNMR Peak Assignments

Peak Assignments	ppm	Range of Integration
TSP	0.0	-0.02-0.02
C-18 bile acids proton peak	0.7	0.61-0.73
Lactate methyl group (CH$_3$)	1.3	1.25-138
Acetate methyl group (CH$_3$)	1.9	1.89-1.95
Pyruvate methyl group (CH$_3$)	2.3	2.34-2.38
PC head group –N+(CH$_3$)$_3$	3.2	3.20-3.27
Anemeric glucose proton peak	5.2	5.20-5.25

ppm: parts per million

TSP: sodium [^{2}d$_4$]- trimethysilylpropionate

PC: phosphatidylcholine

Table 7.2: Results of peak integration of ^{1}HNMR bile spectra. Results are presented as mean ± standard deviation.

	Group	Baseline	Ischaemia	2 h post-reperfusion	5 h post-reperfusion.	6 h post reperfusion
Lactate	I/R	11.73±6.14	12.98±9.02	11.29±6.24	11.32±7.21	12.88±9.73
	NAC	13.84±3.93	17.22±0.75	17.46±3.02	14.34±4.88	15.16±3.56
	P	0.465	0.564	0.117	0.564	0.248
Acetate	I/R	1.20±0.40	1.24±0.34	0.77±0.25	0.51±0.21	0.74±0.32
	NAC	1.07±0.19	0.70±0.01	1.04±0.26	1.24±0.37	1.99±0.36
	P	0.754	0.083	0.175	0.021	0.021
Pyruvate	I/R	0.98±0.39	1.07±0.53	0.84±0.32	0.57±0.40	0.89±0.43
	NAC	1.37±0.19	1.76±0.09	1.86±0.31	1.39±0.36	1.57±0.82
	P	0.175	0.105	0.009	0.021	0.248
PC head group	I/R	3.75±1.62	8.38±1.92	5.68±2.22	6.26±5.24	4.11±2.89
	NAC	2.42±0.95	2.58±0.30	4.66±2.62	2.75±0.71	2.62±0.63
	P	0.175	0.047	0.347	0.248	0.772

NAC: N-acetylcysteine

PC: phosphatidylcholine
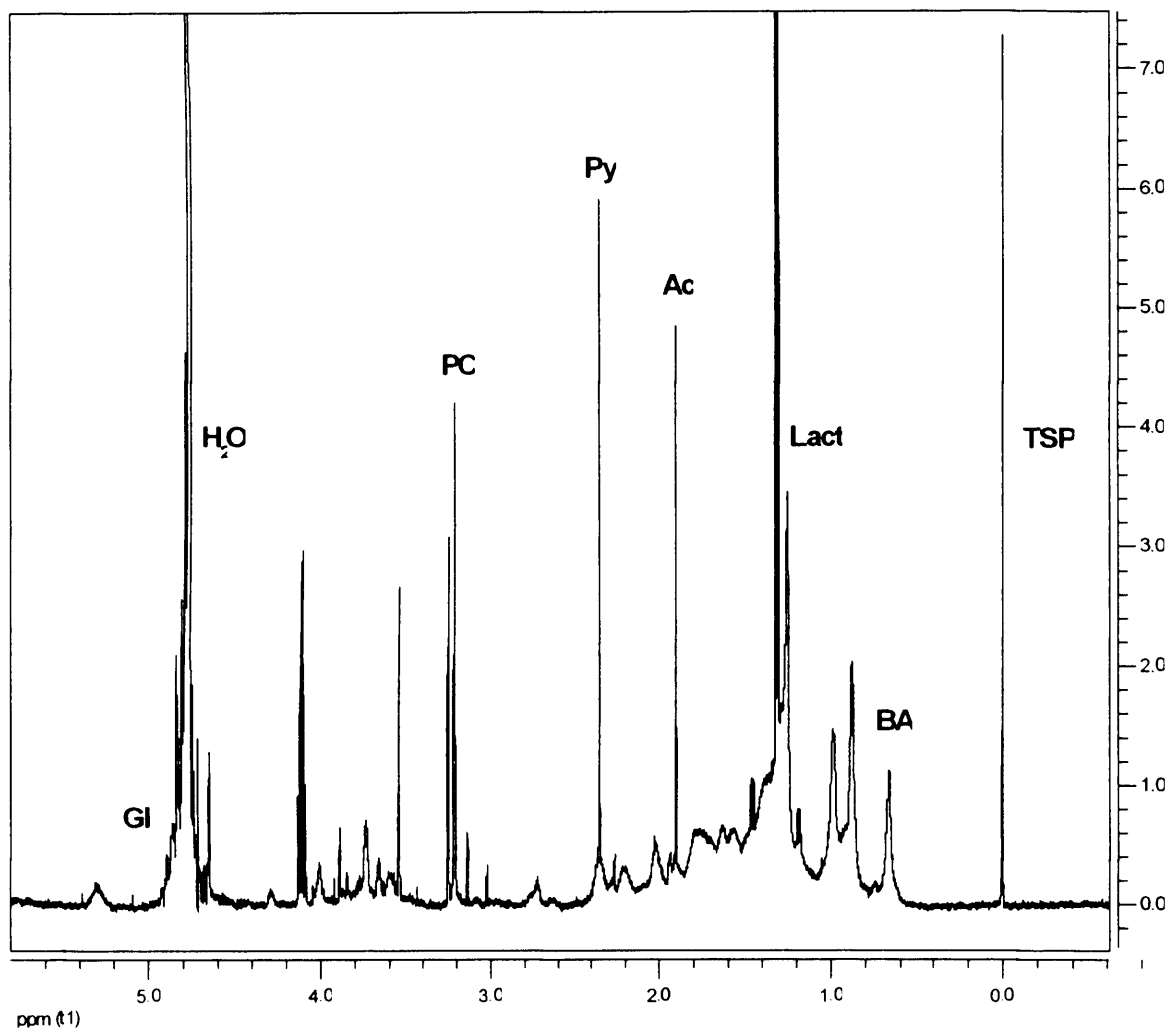
Figure 7.8. Proton nuclear magnetic resonance (^{1}H NMR) spectra of baseline control bile. TSP=sodium trimethylsilyl-$[^2$H$_4]$propionate standard, BA=bile acid, Lact=lactate, Ac=acetate, Py=pyruvate, PC=phosphatidylcholine head group, H$_2$O=water, Gl=glucose
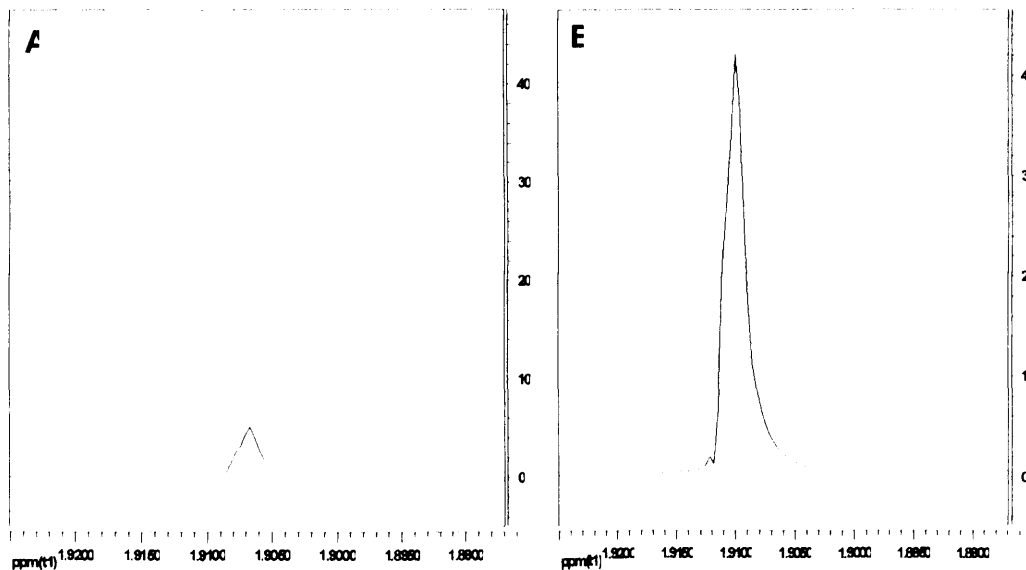
Figure 7.9. Proton nuclear magnetic resonance (^{1}H NMR) spectra of acetate levels following warm ischaemia-reperfusion.. ^{1}H NMR spectra of acetate peaks in representative samples from (a) the I/R and (b) NAC group at 6 h post-reperfusion showing significant difference.

7.3.7. DHR oxidation

Rhodamine values were significantly lower in NAC group than in I/R group, indicative of lower concentration into systemic circulation of RNS and ROS in the NAC group at 6 h post-reperfusion: 1.16 ± 0.28 vs. 2.85 ± 1.11 nM, (NAC vs. I/R, p=0.005) (Figure 7.10).
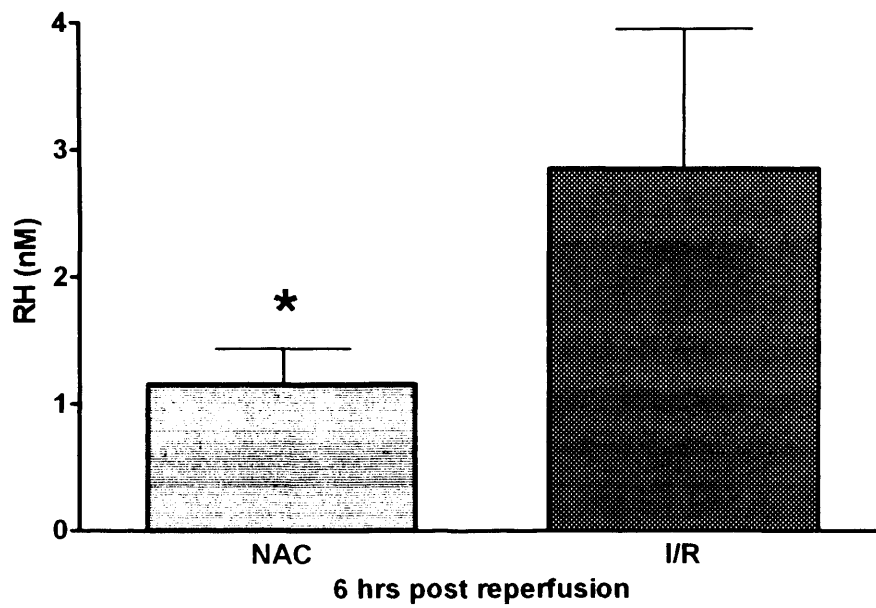
Figure 7.10. Rhodamine (RH) in nM at 6 h post reperfusion. Results are presented as mean (SD). The RH levels were significantly lower in the NAC group compared to I/R (p<0.005).

7.4 Discussion

This study has shown that NAC administration reduces I/R injury in the steatotic liver following a 1 h period of partial inflow occlusion, and the benefit is mainly apparent in the late phase of I/R injury. Although there have been previous in-vitro studies on the effect of NAC on hepatic I/R injury (Nakano *et al.* 1997) this is the first in vivo study to investigate the effect of NAC in warm liver I/R injury in steatotic livers.

Rabbits were fed with high-cholesterol diet (2%) for 8 consecutive weeks in order to induce moderate steatosis (Seifalian *et al.* 2001a). With this model the liver weight and liver weight / body weight (LW/BW) ratio are significantly greater in animals with liver steatosis compared to lean animals (as shown in chapters 6 and 7) (Seifalian *et al.* 2001a). Unlike the commonly used low-choline methionine diet, which produces periportal fatty infiltration (Hui *et al.* 1994; Teramoto *et al.* 1994), with this model a central lobular deposition of fat is observed, similar to that found in the majority of human fatty livers, such as in diabetes, obesity and alcoholism (Neuschwander-Tetri and Caldwell 2003). Liver steatosis reduces flow in the hepatic microcirculation and mitochondrial ATP generation (Selzner and Clavien 2001). Fat accumulation in the cytoplasm of the hepatocytes causes compression or even complete occlusion of the sinusoidal spaces with shunting of a proportion of total hepatic blood flow (THBF) (Teramoto *et al.* 1993). Our group previously demonstrated in an experimental model, that in moderate steatosis, the THBF was reduced by 30% and flow in hepatic microcirculation was 50% of normal controls (Seifalian *et al.* 1999).

A rabbit model was used instead of a rat model since the size of rabbits is more convenient for the placement of probes, bile flow measurement and blood uptake at different time points. The period of hepatic inflow ischaemia (1 h) is also similar to the warm ischaemia time during human liver resection and transplantation. In this model, partial ischaemia was obtained by interrupting the blood flow to the median and left lobes of the liver. Maintaining splanchnic blood flow through the right and the caudate lobe minimises portal vein stasis and intestinal venous congestion, reducing the risk of portal bacteraemia and haemodynamic instability (El Desoky *et al.* 2001; Koo *et al.* 1992).

Several studies on liver I/R injury have focused on the initial reperfusion phase (Demir and Inal-Erden 1998; Nakano *et al.* 1995), which is characterised by oxidative stress induced by Kupffer cells and occurs in the first 2 h (Jaeschke and Farhood 1991). In the present study the observation time was extended to 6 h as a distinct late phase of warm I/R injury develops 4 hours after reperfusion, and is primarily caused by activated neutrophils with release of ROS and proteases (Harbrecht *et al.* 1993; Jaeschke *et al.* 1990).

NAC was chosen as an antioxidant to modulate hepatic I/R injury as it is routinely administered to patients with acute liver failure secondary to acetaminophen (paracetamol) overdose (Harrison *et al.* 1991), and has been used in other clinical conditions where oxidant damage was the putative or known mechanism of injury, such as adult respiratory distress syndrome (ARDS) (Suter *et al.* 1994) and I/R after cardiac ischaemia-reperfusion (Ceconi *et al.* 1988; Tossios *et al.* 2003) . Recent work from our group has also shown beneficial effects of NAC in normal liver following one hour of lobar ischaemia (Glantzounis *et al.* 2004) . The dose of NAC administered is that used in clinical practice to

treat patients with acute liver failure (Harrison *et al.* 1991). The beneficial effect of NAC was demonstrated by improvement in liver microcirculation, bile production and composition, associated with reduced hepatocellular injury.

Several studies have validated the use of LDF to measure changes in hepatic microcirculation (Chavez-Cartaya *et al.* 1994; El Desoky *et al.* 2001; Seifalian *et al.* 1998). During partial inflow occlusion, parenchymal perfusion was greatly reduced but was still recordable. This low level of perfusion has also been reported in other studies and can be caused by a random wandering motion of red cells and breathing movements (Vollmar *et al.* 1994). In the NAC group hepatic microcirculation returned to baseline values at the end of reperfusion suggesting that NAC has reversed the perfusion failure of the late phase of I/R injury. This correlates well with the return of portal blood flow rates towards baseline at the end of the reperfusion period in the NAC group.

In order to adequately investigate hepatic injury and function, changes in serum ALT activity, bile flow and composition were studied. ALT is relatively liver specific and reflects the loss of membrane integrity and release of cytoplasmic content into the circulation (Lemasters *et al.* 1983). Bile production is a reliable marker of liver function (Bowers *et al.* 1987). The NAC group had reduced hepatocellular injury as indicated by lower ALT values following reperfusion, and improved biliary excretion, an energy dependent process, resulting in increased bile drainage volumes. Comparable rise in ALT values has been observed during reperfusion in steatotic animals subject to a similar ischaemic insult (Nakano *et al.* 1997).

This is the first study utilising ^{1}H NMR analysis of bile to study the effect of NAC on steatotic livers in a controlled experimental model of liver I/R. The use of spin echo spectra allowed the interference from broad lipid signals to be reduced and more clearly identify and comparatively quantify bile constituents. Continuous bile flow was recorded in both the I/R and NAC groups, and ^{1}H NMR spectroscopy revealed fluctuations in the concentration of several bile constituents. More specifically NAC administration enhanced the biliary excretion of acetate, pyruvate and lactate.

Acetate is produced as a result of fatty acid β-oxidation in the liver (Ambrose and Easty 1977). This reaction requires ATP at its initiation. Following I/R injury ATP is consumed to clear excess lactate and pyruvate and may not be readily available for fatty acid oxidation. The I/R-induced reduction or depletion of ATP may result in a decreased ability to utilize β-oxidation causing acetate production to falter. The higher bile acetate levels in the NAC treated livers would suggest improved ATP production and energetics. The increased excretion of pyruvate and lactate in the bile of NAC treated livers is difficult to interpret. Steatotic livers have lower ATP levels after stress compared to normal livers and mitochondrial injury has been proposed as one of the causes of reduced hepatocellular ATP stores in steatosis (Neuschwander-Tetri and Caldwell 2003). The main source of PC in the hepatocytes is the cell membrane. The increase following ischaemia correlates well with the theory of cellular breakdown and membrane lipid peroxidation (Suzuki *et al.* 2000).

In the present study NAC administration reduced significantly the oxidation of DHR 123 to RH. The formation of RH is partially peroxynitrite dependent, although other oxidants such

as hydrogen peroxide in the presence of horseradish peroxidase and hypochlorous acid (HOCl) can oxidise DHR 123 to RH (Halliwell B and Gutteridge JMC 1999) ROS and peroxynitrite can cause cellular injury and necrosis through several mechanisms including peroxidation of membrane lipids, protein denaturation and DNA damage (Halliwell B and Gutteridge JMC 1999). Oxidation of DHR 123 to RH has been reported in endotoxemia, hemorrhagic shock and splanchnic I/R (Szabo *et al.* 1995). Although the protective effect of NAC in DHR oxidation has been demonstrated in in-vitro studies (Kooy *et al.* 1994) and in splanchnic artery occlusion (Cuzzocrea *et al.* 2000a), this is the first report of a reduced DHR oxidation by NAC infusion in warm I/R in steatotic livers.

In conclusion, the administration of NAC, prior to and during reperfusion, reduced the extent of I/R injury in the steatotic liver. This was apparent during the late phase of reperfusion and was demonstrated by increased portal blood flow and liver parenchymal perfusion. Bile excretion was increased and acute liver injury was reduced. This improvement was associated with reduced levels of reactive oxygen and nitrogen species. A trial of NAC infusion in patients with hepatic steatosis undergoing liver resection or in patients undergoing liver transplantation with steatotic grafts is required to confirm these benefits in clinical practice.

7.5 Acknowledgements

I am very grateful to Mr John Difford for his technical advice regarding histology staining.

7.6 Chapter summary

Steatotic livers are more susceptible to ischaemia-reperfusion (I/R) injury. Patients with hepatic steatosis have increased morbidity and mortality following liver surgery and steatotic grafts have higher primary dysfunction after liver transplantation. The aim of the present study was to evaluate the in-vivo effect of N-acetylcysteine (NAC) on hepatic function in both the early and initial late phase of warm liver I/R injury in steatotic rabbits.

12 New Zealand rabbits were fed with high cholesterol (2%) diet for eight weeks. I/R group (n=6) underwent lobar liver ischaemia for 1 h followed by 6 h of reperfusion. In the treated group (NAC, n=6) an intravenous infusion of NAC was administered prior and during the 6 h reperfusion period. Systemic and hepatic haemodynamics were monitored continuously. Alanine aminotransferase activity and bile production were measured. Magnetic resonance spectroscopy (^{1}HNMR) was used to analyse bile composition. The oxidation of dihydrorhodamine (DHR) to rhodamine (RH) was used as a marker of production of reactive oxygen and nitrogen species.

Histology demonstrated moderate centrilobular hepatic steatosis. NAC administration improved significantly portal flow, hepatic microcirculation, bile composition and bile flow at 6th hour of reperfusion. NAC administration was also associated with less hepatocellular injury as indicated by ALT serum activity and reduced the oxidation of DHR to RH after 6 h of reperfusion.

In conclusion NAC administration reduced the extent of I/R injury on the steatotic liver, particularly during the late phase of reperfusion.

CHAPTER 8

THE EFFECT OF N-ACETYLCYSTEINE ON ENERGY

METABOLISM AND NITRIC OXIDE ACTIVITY DURING HEPATIC

ISCHAEMIA-REPERFUSION

8.1 Introduction

N-acetylcysteine (NAC) is a low molecular weight thiol used successfully in the treatment of fulminant hepatic failure due to acetaminophen overdose (Aitio 2006; Chyka *et al.* 2000; Prescott *et al.* 1979). It has also shown beneficial effects in other settings of acute liver failure (Harrison *et al.* 1991), in chronic obstructive pulmonary disease (Grandjean *et al.* 2000; Stey *et al.* 2000), in human immunodeficiency virus infection (De Rosa *et al.* 2000), in radiocontrast-induced nephropathy (Tepel *et al.* 2000) and in cardiopulmonary by-pass surgery (Fischer *et al.* 2004; Tossios *et al.* 2003).

Its main actions are as a glutathione precursor and as a scavenger of reactive oxygen (ROS) and nitrogen species (Cotgreave 1997; Glantzounis *et al.* 2006; Yan *et al.* 1995). It has been shown in chapters 6 and 7 that NAC reduces warm liver ischaemia-reperfusion (I/R) injury in both normal (Glantzounis *et al.* 2004) and steatotic liver (Fusai *et al.* 2005) but the mechanism of action is not completely clear. The use of antioxidants for amelioration of liver I/R injury requires further study of the mechanism of action.

In vitro evidence suggests that low molecular weight thiols are implicated in nitric oxide (NO) metabolism by interacting with plasma S-nitrosothiols (RSNOs) (Marley *et al.* 2001). It has also been shown that production of RNS could have a negative effect on cellular energy production through inhibition of mitochondrial enzymes such as cytochrome oxidase (Cooper 2002; Koeck *et al.* 2004).

Recent experimental evidence shows that NO synthesis is markedly upregulated with liver I/R injury (Serracino-Inglott *et al.* 2003). It is postulated that following reperfusion NO

production is reduced but subsequently large amounts of NO are produced due to the expression of inducible NO synthase (iNOS) with potential deleterious effects. NO can produce RNS such as peroxynitrite after the reaction with superoxide anion, can form S-nitrosothiols or be metabolized to nitrite and nitrates. However the role of NO in liver I/R is still controversial (Glantzounis *et al*. 2005a).

The aim of this study was to look at the effects of NAC administration on liver function, microcirculation, cellular energy production, NO metabolism and oxidative stress. For this reason the experimental rabbit lobar liver I/R model, which has been validated and refined in chapter 4 was used. In this model, for continuous monitoring of arterial blood pressure and blood sampling, a cannula is placed in the right ear artery, instead of the femoral artery in order to avoid the systemic effects of limb ischaemia. Cellular energy production was assessed through the monitoring of changes in the redox state of hepatic cytochrome oxidase by NIRS and analysis of bile composition by proton nuclear magnetic spectroscopy (^{1}H NMR). NO activity was assessed by measurement of nitrite / nitrate plasma levels with capillary electrophoresis, plasma S-nitrosothiols with electron paramagnetic spectrometry (EPR) and liver tissue nitrotyrosine by mass spectrometry. The degree of oxidative stress post I/R was assessed through the oxidation of the dye dihydrorhodamine to rhodamine.

8.2 Materials and methods

8.2.1 Animal model.

The study was conducted under a license granted by the Home Office in accordance with

the Animals (Scientific Procedures) Act 1986. New Zealand white rabbits (3.4 ± 0.8 kg,

n=18) were used. Anaesthetic protocol and surgical procedure are described in section 3.1.

A radio-opaque catheter 20 gauge (GA) was inserted into the right marginal ear artery for

monitoring of arterial blood pressure and collection of blood samples. Ear marginal veins

were cannulated with radio-opaque catheters (22 GA) for the administration of anaesthetics,

fluids and medication. Laparotomy was performed through a roof top transverse incision.

Lobar ischaemia was induced by clamping the vascular pedicles of the median and left

lobes of the liver, using an atraumatic microvascular clip. After 60 min of ischaemia the

vascular clip was removed and reperfusion was allowed for 7 h. In all groups, 15 ml/kg/h of

0.9 % NaCl was administered through the ear marginal vein to compensate for

intraoperative fluid loss.

8.2.2 Experimental groups and protocol

Three groups of animals were used (n=6 each). In the NAC group 150 mg/kg NAC

(Parvolex[®]; Medeva Pharma, Ashton-under-Lyne, Lancashire, UK) in 20 ml 5% dextrose

was infused intravenously through the ear vein over the 15 min before reperfusion and

maintained at 10 mg/kg/h in 5 % dextrose (10 ml/h) during the 7 h reperfusion period. In

the I/R group 20 ml 5 % dextrose was infused intravenously 15 min before reperfusion and

continued at a dose of 10 ml/h during the reperfusion period. Animals in the sham operation

group underwent laparotomy but no liver ischaemia. Laparotomy and baseline

measurements were completed over 1 h. In the sham group monitoring continued for the

equivalent of the ischaemia and reperfusion periods in treated animals (total monitoring period 9 h).

Arterial blood samples (1 ml each) were taken before the induction of liver ischaemia (baseline), at the end of ischaemia (60 minutes) and two, five and seven hours after reperfusion for measurement of alanine aminotransferase (ALT) activity. Serum was separated from the samples and stored at $-20°C$ until assayed (see also section 3.5).

8.2.3 Hepatic microcirculation

Hepatic microcirculation was measured by a surface laser Doppler flowmeter (for details see section 3.2).

8.2.4 Measurement of cytochrome oxidase (cyt ox) activity

Changes in cytochrome oxidase redox state during liver I/R were measured by NIRS (section 3.3). Briefly, Cyt Ox is the terminal complex of the mitochondrial respiratory chain. It takes electrons from cytochrome c and catalyses the reduction of oxygen to water with the concomitant synthesis of ATP through the oxidative phosphorylation process (Capaldi 1990; Edwards et al. 1991). Cyt Ox has 4 redox active metal sites, which exhibit different absorption characteristics depending on their redox state. The copper centers are optically active in the NIR light in contrast with the heme centers that absorb visible light (Cooper et al. 1994; Jobsis FF 1992). Absorption of the NIR light occurs primarily at the CuA center within Cyt Ox. The contribution of the heme centers to absorption of NIR is less than 10 % of the total signal in the reduced oxidised spectrum (Cooper et al. 1994).

Thus, the signal measured by NIRS is almost entirely due to the Cu_A center. The signal intensity decreases on reduction of this center. The redox state of Cyt Ox Cu_A is dependant on cellular oxygen availability (Takashima *et al.* 1995). In the presence of oxygen electron transfer occurs and the center becomes oxidised, whereas lack of oxygen results in a decreased flow of electrons and the center becomes reduced. Thus, reduction to Cyt Ox levels measured by NIRS refers to accumulation of electrons in the Cu_A centre of Cyt Ox.

Data are presented as changes in Cyt Ox Cu_A redox state relative to baseline. Decrease (vs. baseline) in the redox state of Cyt Ox Cu_A indicates that the Cu_A center becomes reduced and electrons accumulate in the Cu_A center of Cyt Ox . Increase (towards baseline) in the redox state of Cyt Ox Cu_A indicates that the Cu_A center becomes oxidised and electron transfer through the respiratory chain occurs.

8.2.5 Measurement of bile flow and ^1H NMR bile spectroscopy

The common bile duct was cannulated with a polyethylene catheter (PE-50, 0.58 mm inner diameter, Portex, Kent, UK) for continuous collection of bile. Bile samples were taken at baseline, following 60 min ischaemia and hourly during reperfusion. Bile volume was expressed as $\mu L/min/100g$ of liver wet weight.

^1HNMR analysis was performed on an 11.7 Tesla (500 MHz for protons) spectrometer (Varian Unity +; Varian, Palo Alto, CA, USA) at 25°C (see also section7.2.4). Bile was thawed at room temperature and placed in 5mm NMR tube. For a field/frequency lock a coaxial capillary insert was used (Wilmad, Buena, NJ, USA). This capillary insert was filled with a deuterium oxide solution of sodium $[^2d_4]$- trimethysilylpropionate (TSP) that

acted both as a chemical shift reference and quantification standard. This capillary was calibrated by using a series of known concentration solutions of deoxycholate and a calibration curve was obtained. This was then used for quantification of bile components (lactate, acetate, pyruvate and phosphatidylcholine) which enabled an accurate comparison of bile levels between the groups. One dimensional NMR spectra were obtained at 500 MHz with a sweep width of 6 kHz. Presaturation of bile was carried out to attenuate the intensity of water signal. The spectra were analysed using software from MestRe-C version 3.1.1 (Universidade de Santiage de Compostela, Spain). All spectra were integrated using a fixed range for each peak and published peak assignments (Ellul *et al.* 1992; Melendez *et al.* 2001; Sequeira *et al.* 1994; Sequeira *et al.* 1995) shown in table 7.1.

8.2.6 EPR spectrometry

Blood samples were defrosted just before analysis by EPR spectrometry. The samples were analysed using a JEOL JES RE1X spectrometer (Jeol (UK) Ltd, Welwyn Garden City, UK) equipped with an ES-UCX2 cylindrical mode X-band cavity. Samples were analysed at room temperature in a WG-LC-11 quartz flat cell (Wilmad Glass, Buena, NJ, USA). The instrument parameters differed for each set of experiments.

S-nitrosothiols analysis

RSNOs were measured in plasma by EPR spectrometry (Rocks *et al.* 2005) . With this technique the RSNOs are degraded using an alkaline pH of $\geq$ 10.5 in the presence of the spin trap complex N-methyl-D-glutamine dithiocarbamate $(MGD)_2$ - Fe^{2+} as described in

section 3.9. Briefly, blood samples (1 mL) were taken from the ear arterial line at baseline, end of ischaemia, 2nd, 5th and 7th h post-reperfusion or the equivalent time in the sham group, into EDTA-coated tubes containing 10 μL of N-ethylmalemide (NEM). Blood also was taken at the end of the experiment from the right hepatic vein.

The samples were centrifuged immediately at 3000 rpm for 10 min. The supernatant was aliquoted into small centrifuge tubes and snap frozen in liquid nitrogen. During analysis plasma samples were placed in an evacuated glass vial, kept on ice and the concentration of RSNOs present was measured the same day, as previously described (Rocks *et al.* 2005). Briefly, 20 mM ammonium ferrous sulphate solution in degassed CAPS (pH 10.5) was added to MGD to form the $(MGD)_2$-Fe^{2+} complex (final concentrations of MGD and Fe^{2+} were 50mM and 10 mM respectively). This was then added in a ratio of 1:1 volume/volume to the plasma samples, incubated for 3 min and measured by EPR spectrometry. The samples were analysed using a JEOL JES RE1X spectrometer (Jeol Ltd, Welwyn Garden City, UK) equipped with an ES-UCX2 cylindrical mode X-band cavity. Samples were analysed at room temperature in a WG-LC-11 quartz flat cell (Wilmad Glass, Buena, NJ, USA). The instrument parameters differed for each set of experiments. The spectra were recorded using the SpecESR programme, version 1.0 and the mean peak area was determined. Water was taken through the same procedure to act as a control. The EPR spectrometer parameters were: microwave frequency 9.45GHz, microwave power 20mW, centre field 330 mT, sweep width ± 5 mT, sweep time 250 s, number of data points 8192, time constant 1 s, modulation frequency 100 kHz, and modulation width 0.4 mT. The spectra were taken as an average over 3 scans. The concentration of nitrosothiols present

was determined by comparison to a nitrosoglutathione calibration curve (0, 300, 600, 800, and 1000 nM).

8.2.7 Measurement of nitrite (NO2⁻) / nitrate (NO3⁻) by capillary electrophoresis

$NO^.$ metabolites, NO_2^- and NO_3^-, concentrations are determined using a capillary electrophoresis method (Davies *et al.* 1999) as described in section 3.8. Briefly, blood samples were taken from the ear arterial line at baseline, end of ischaemia, 2nd, 5th and 7th h post-reperfusion or the equivalent time in the sham group into EDTA-coated tubes. Blood also was taken at the end of the experiment from the right hepatic vein. The samples were centrifuged immediately at 3000 rpm for 10 min. The supernatant was aliquoted into small centrifuge tubes, snap frozen in liquid nitrogen and kept in -80 $^\circ$C until assayed.

Plasma samples were centrifuged at 13000rpm through a Vectrospin Micro 30kDa ultra-filtration device (Whatman International Limited, Kent, UK) for 30 min. A P/ACE System 5500 equipped with a P/ACE diode array detector (DAD) and automatic injector, a fluid-cooled column cartridge and a System Gold data station was used. Samples were passed through a 50 μm x 47 cm (40 cm to detector) fused-silica capillary with an electrolyte consisting of 150mM sodium chloride / 5 mM Tris-HCl at pH 7.4 with 2 mM TTAB. The analysis was performed at 25°C. The solutions were injected onto the capillary by hydrodynamic injection using a pressure injection of 0.5 psi for 15 s. The components in the samples were separated using a −10kV voltage separation. The DAD was set at 214 nM with a bandwidth of 4 nM. The capillary was rinsed before each injection with 0.1 M NaOH and electrolyte for 1 min and 2 min, respectively. Aqueous standard solutions were formed using concentrations of 10 to 50 μM sodium nitrate and 2 to 10 μM sodium nitrite.

8.2.8 Dihydrorhodamine 123 oxidation

Oxidation of DHR 123 to rhodamine (RH) was measured with the method described in section 3.10. Briefly, Seven hours after reperfusion in the I/R group or the equivalent time in the sham group animals were injected with DHR 123 (2 µmol/kg in 0.8 mL normal saline 0.9 %). Twenty minutes later plasma samples were collected for rhodamine fluorescence evaluation. Fluorescence measurements were performed at an excitation wavelength of 485 nm and emission wavelength of 538 nm. The concentration of RH formation was calculated using a standard curve obtained with authentic RH (1 to 40 nM) prepared in plasma obtained from untreated rabbits.

8.2.9 Histology

Formalin fixed paraffin embedded blocks of liver were cut at 4 microns and stained with haematoxylin and eosin (H&E) and reviewed by a pathologist, blinded to the treatment of the animals. The tissue was semiquantitatively scored for the number of polymorphs in a high powered field, using an Olympus BX50 microscope at x 400 magnification. A score was devised; a score of 0-3 was given to the number of single PMNs in sinusoids (1=1-5, 2=5-20, 3=>20); a score of 0-3 for aggregated PMNs (1=1-2 clusters, 2=2-5, 3=>5). This system was validated with a quantitative system where the total number of polymorphs counted in 50 hpfs and the average then given; this was compared to the semiquantitative system. A comment was also made if the aggregated PMNs were associated with hepatocyte necrosis. The presence or absence of apoptosis, judged by shrunken, darkly stained hepatocytes, was also made in a semiquantitative manner. Comment was made on presence or absence of Kupffer cell hyperplasia and if there were any other changes to

hepatocytes, taking particular note of any zones affected. Immediately subcapsular areas and foci around large hepatic veins were not counted, as these would have shown a disproportionate amount of PMN infiltrate.

8.2.10 Data collection and statistical analysis

Data from the pulse oximeter, blood pressure monitor, LDF and NIRS were collected continuously on a labtop computer. The data were averaged for 2 min before the induction of ischaemia (baseline), at the end of ischaemia and at the end of each h of a 7-h reperfusion period. Changes in Cytochrome oxidase activity at the end of each period were calculated relative to baseline. Bile spectroscopy values are expressed as a percentage of baseline values. Data were presented as mean (SD). Student's t test for paired samples and one-way ANOVA with Bonferroni adjustment for multiple comparisons were used for statistical analysis. $P < 0.050$ was considered statistically significant.

8.3 RESULTS

8.3.1 Systemic haemodynamics

In all three groups systemic mean arterial blood pressure fell during the experiment compared to baseline. There were no haemodynamic differences between NAC and I/R groups during the whole experiment. However MABP was significantly higher in the sham group compared to NAC and I/R after the 3rd h of reperfusion (Figure 8. 1).

Pulse rate remained stable in all groups throughout the experiment. There were no statistically significant differences between the groups (Figure 8.2).

Oxygen saturation (SaO_2) remained stable through the experiment in all groups.
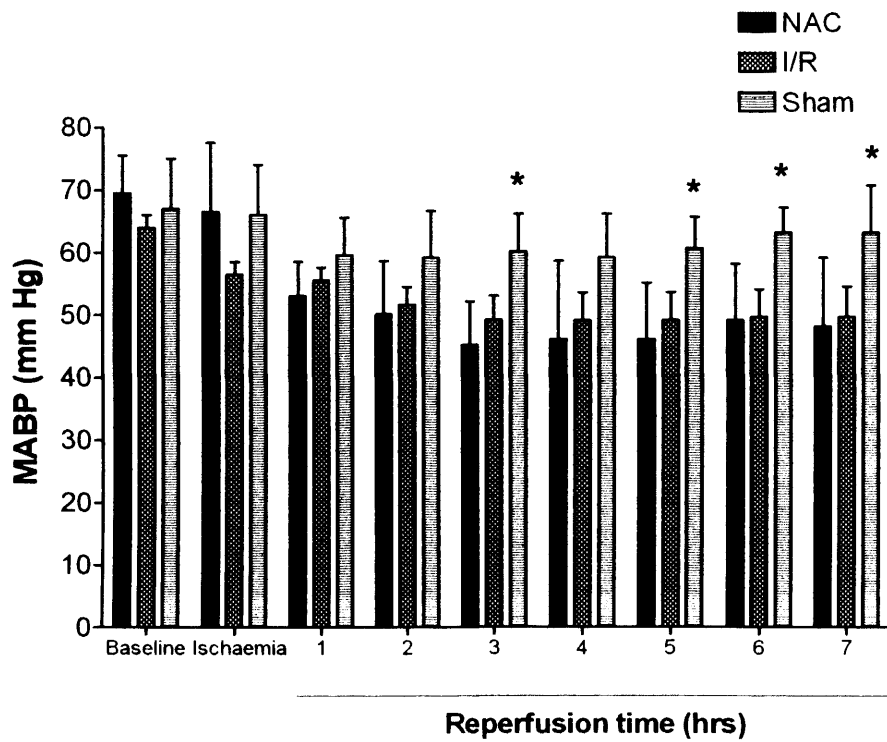
Figure 8.1 Mean arterial blood pressure (MABP) during ischaemia and reperfusion. Values are mean (SD). MABP fell during the experiment compared to baseline in all groups. This reduction was more obvious in the NAC and I/R groups. *p < 0.05 versus NAC and I/R group. NAC, N-acetylcysteine, I/R: ischaemia-reperfusion

NAC
I/R
Sham

Reperfusion time (hrs)
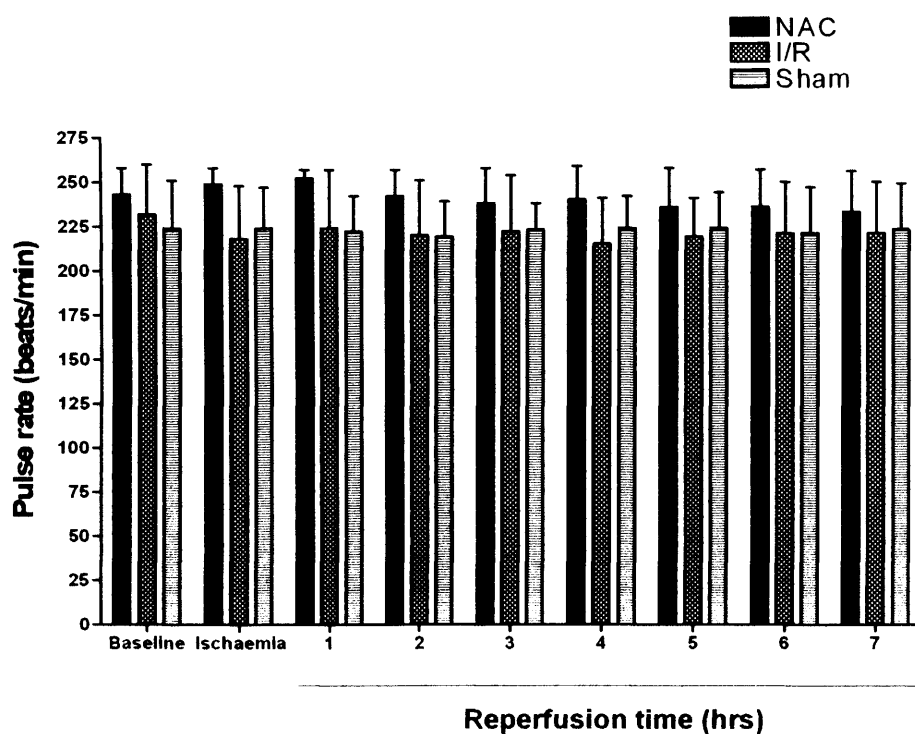
Figure 8.2 Pulse rate during ischaemia and reperfusion. Values are mean (SD). There were no significant differences between groups. NAC, N-acetylcysteine, I/R, ischaemia-reperfusion.

8.3.2 Liver function tests

Mean serum ALT activity at baseline was within the normal range, with no significant differences between the three groups (Figure 8.3). After reperfusion, the mean ALT activity in the NAC and I/R groups was significantly higher than that in the sham operated group. The serum ALT activity was lower in the NAC group compared to the I/R group at 2 h (p = 0.02), 5 h (p = 0.04) and 7 h (p = 0.01) post reperfusion (Figure 8.3).
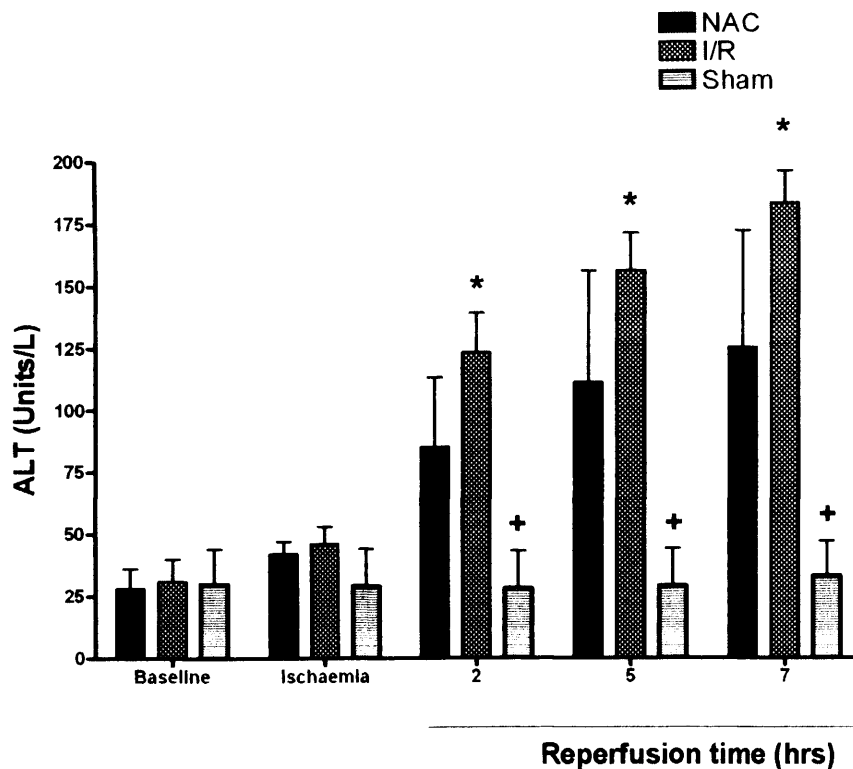
Figure 8.3 Serum alanine aminotransferase (ALT) activity during ischaemia and reperfusion. Values are mean (SD). *p < 0.050, I/R vs. NAC, *p < 0.05, Sham vs. NAC. NAC, N-acetylcysteine, I/R, ischaemia-reperfusion.

8.3.3 Hepatic microcirculation

There was no significant change in the hepatic microcirculation over the 9-h sham operation. Following I/R, flow in the microcirculation was reduced in the NAC and I/R groups. NAC treatment was associated with an increased flow in the hepatic microcirculation during reperfusion compared to the I/R group; this difference became significant after the 3rd h (p=0.04) (Figure 8.4).
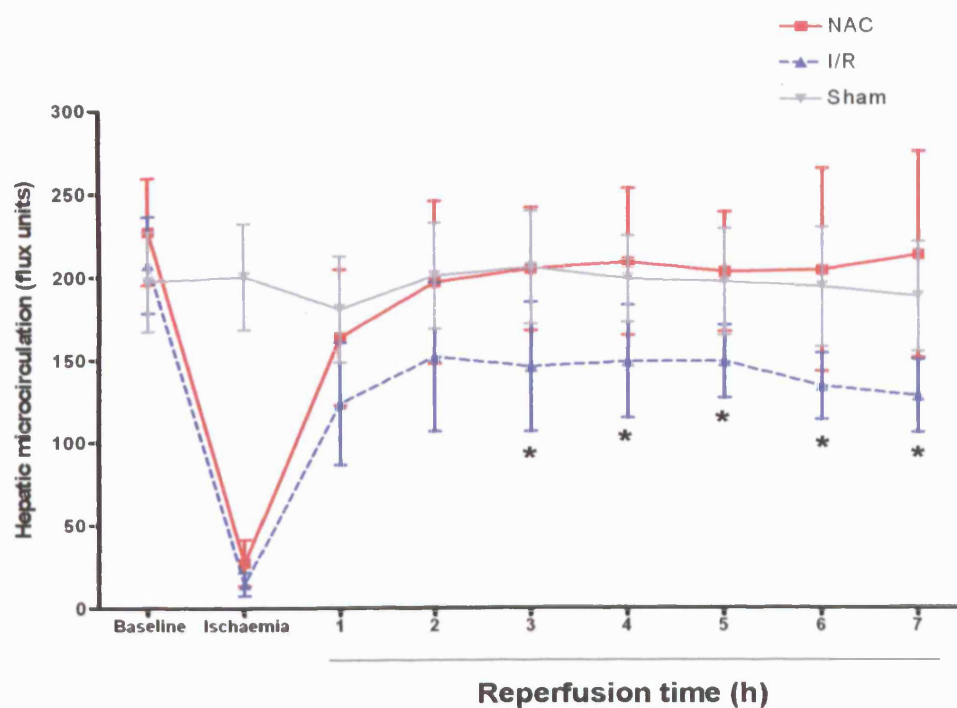
Figure 8.4 Hepatic microcirculation during ischaemia and reperfusion. Values are mean (SD). There were no significant differences in the hepatic microcirculation during the experiment in the sham–operated group. Microcirculation in the I/R group was significantly lower compared to the NAC group after the 3rd h of reperfusion *p<0.05 vs. NAC group. NAC, N-acetylcysteine, I/R, ischaemia-reperfusion.

8.3.4 Cyt Ox activity

During ischaemia Cyt Ox redox state decreased significantly compared to baseline and sham (Figure 8.5). During the early phase of reperfusion cytochrome oxidase redox state returned towards baseline in the I/R group, but subsequently decreased again. In the NAC group, cyt ox redox state was significant better compared to I/R group during the late phase

of reperfusion (5th h: p=0.04, 6th h: p=0.03, 7th h: p=0.03). Of note cyt ox redox state decreased also in the sham group during the experiment compared to baseline (figure 8.5).
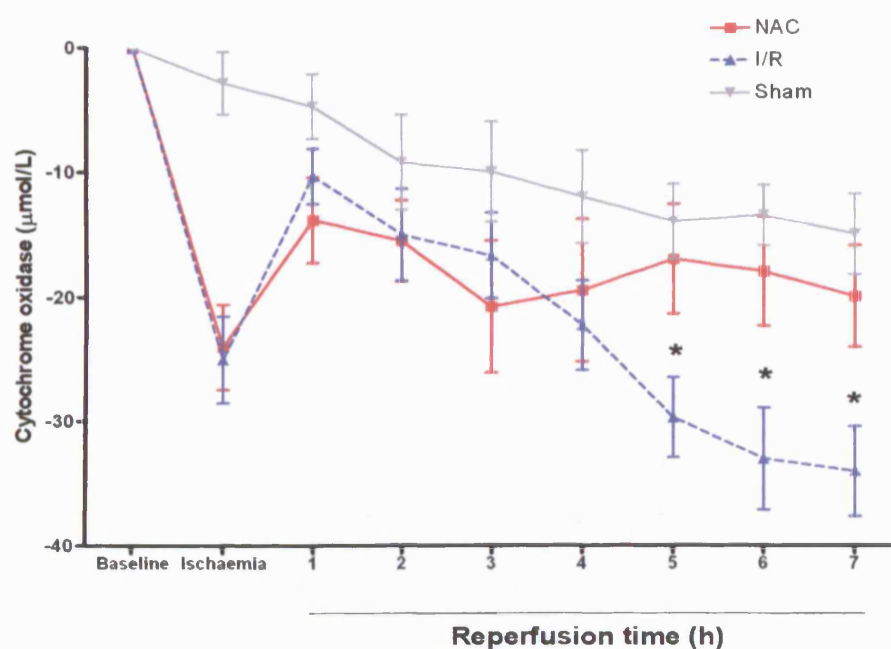
Figure 8.5 Changes in the redox state of cytochrome oxidase (cyt ox) during ischaemia and reperfusion. Values are mean (SD). There was a significant difference in the redox state of cyt ox between NAC and I/R group during the late phase of reperfusion (after the 5th h). *p < 0.05 vs. NAC group. NAC, N-acetylcysteine, I/R, ischaemia-reperfusion.

8.3.5 Bile flow and composition

Bile Flow: Bile flow remained stable in the sham group over the experiment. Bile flow in the I/R group was similar to sham at baseline but reduced significantly during ischaemia

(Figure 8.6). It increased again during reperfusion but did not return to baseline values. NAC administration ameliorated the decrease during reperfusion. However the difference compared to I/R group did not reach significance.

^{1}H NMR spectroscopy of bile:

^{1}H NMR gave a characteristic signal for analysis of bile composition (Figure 8.7). The results of the integration of the area under curve for the assigned peaks are shown in table 8.1. I/R produced significant alterations to bile composition, with an increase to lactate levels *(Table 8.1)* and reduction in acetate (Table 8.1) during reperfusion. NAC administration ameliorated the increase in bile lactate and the decrease in bile acetate levels (Table 8.1). The differences were significant at 5 h and 7 h post reperfusion respectively.

During ischaemia there was an increase in phosphatidylcholine (PC) levels in both groups. PC levels continued to rise in controls for 5 h post-reperfusion. In the NAC group, PC levels rose to peak at 2 h post-reperfusion then fell to less than baseline values at 7 h post-reperfusion. PC levels were consistently lower in the NAC group than in I/R (P=0.0055).
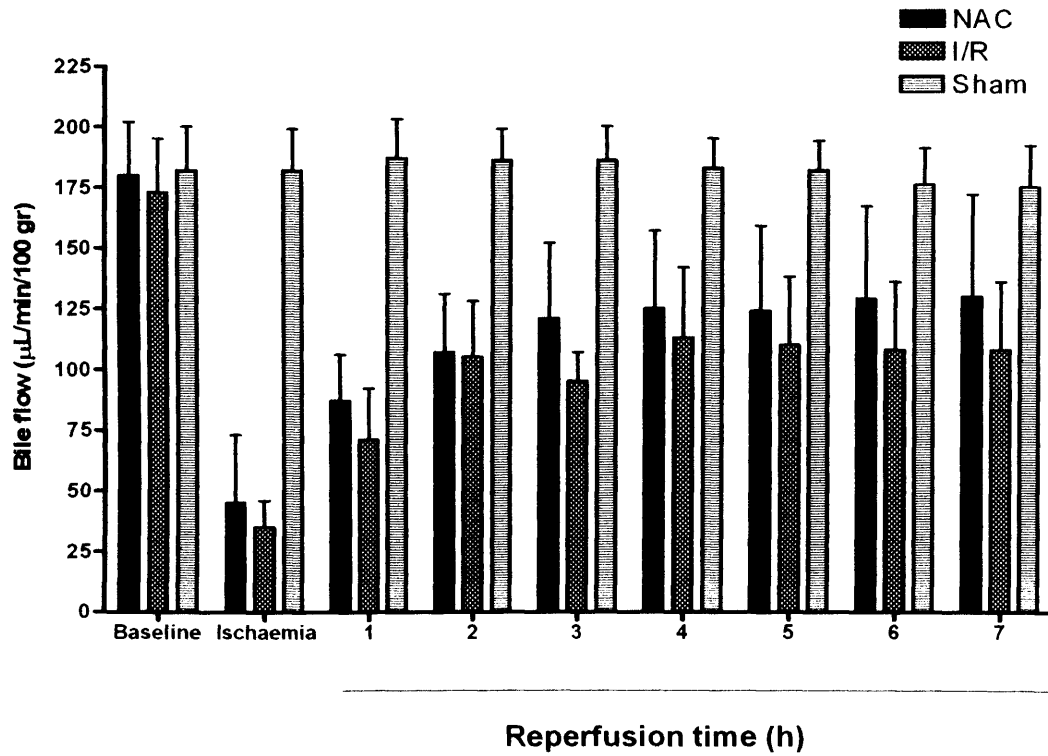
Figure 8.6 Bile flow (μL/min/100g) during ischaemia and reperfusion. Values are mean (SD). Bile flow remained stable through the experiment in the sham group. It decreased significantly from baseline in the I/R and NAC groups. Although NAC administration was associated with better flow during reperfusion compared to I/R group; this difference did not reach significance.
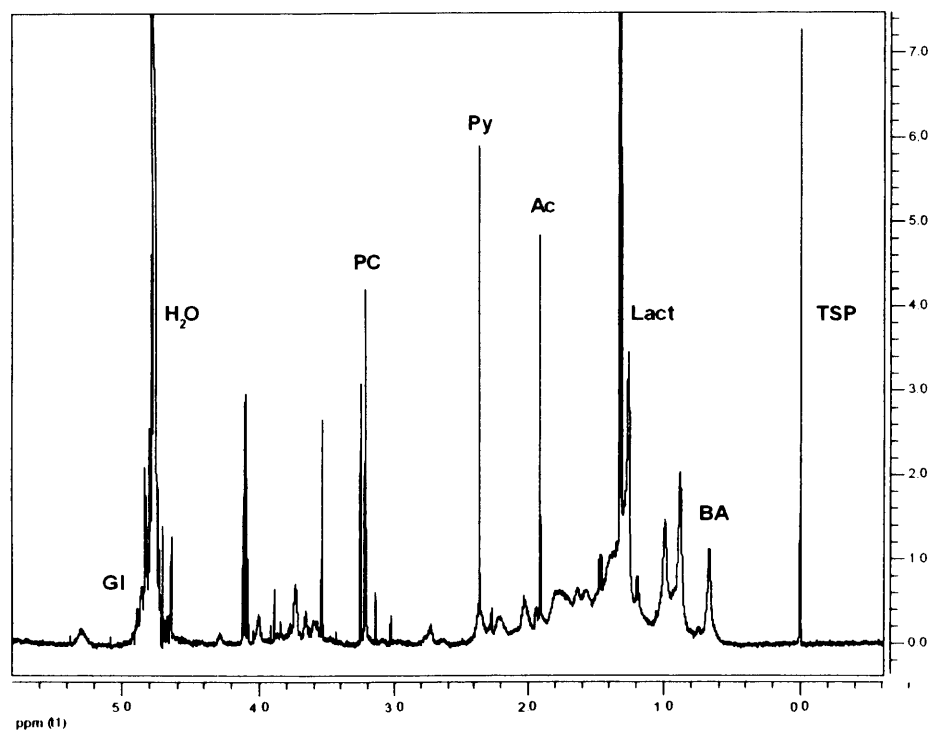
Figure 8.7 ¹H NMR spectrum of baseline control bile. TSP=sodium trimethylsilyl-[²H₄]propionate standard, BA=bile acid, Lact=lactate, Ac=acetate, Py=pyruvate, PC=phosphatidylcholine head group, H₂O=water, Gl=glucose

Table 8.1: Results of peak integration of ^{1}HNMR bile spectra

	Group	Baseline	Ischaemia	2 hr Reperf.	5 hr Reperf.	7 hrs Reperf.
PC head group	I/R	1.88±0.85	2.18±1.18	2.90±1.13	2.97±0.54	1.62±0.57
	NAC	1.04±0.21	1.55±0.17	2.36±0.29	1.32±0.24	0.89±0.09
	P	0.0914	0.4048	0.3842	**0.0005**	**0.0417**
Lactate	I/R	5.28±1.74	10.05±4.64	9.40±3.09	16.08±8.41	26.28±7.883
	NAC	6.85±0.20	7.27±0.79	8.46±1.19	8.21±0.91	8.60±0.35
	P	0.1187	0.3530	0.5881	0.1625	**0.0069**
Acetate	I/R	2.32±1.87	5.38±6.48	0.97±0.13	1.78±1.61	3.65±3.03
	NAC	4.53±0.37	4.35±0.16	2.64±1.22	5.24±1.59	7.89±1.18
	P	0.0509	0.7990	**0.0341**	**0.0186**	0.0575
Pyruvate	I/R	0.63±0.30	0.68±0.43	0.69±0.11	0.84±0.21	0.80±0.55
	NAC	0.96±0.18	1.10±0.11	1.09±0.13	1.52±0.32	1.61±0.09
	P	0.1070	0.1652	**0.0040**	**0.0176**	0.0575
Glucose	I/R	0.54±0.44	1.47±0.12	2.67±1.71	2.81±1.24	0.79±0.36
	NAC	0.77±0.51	0.54±0.26	2.63±1.91	0.98±0.45	1.05±0.45
	P	0.4607	0.2140	0.9791	**0.0471**	0.3639

NAC: N-acetylcysteine, PC: phosphatidylcholine

8.3. 10 S-Nitrosothiols

The $(MGD)_2$-Fe^{2+} nitrosothiol adduct gave a three line signal (a_N=1.28mT, g=2.04) when analysed by EPR spectrometry (Figure 8.8). A signal was observed in the majority of samples. The mean peak area of the signal was determined from the last two peaks. The RSNOs concentration was calculated using the equation of the nitrosoglutathione standard curve. The plasma RSNOs concentrations were plotted as mean concentration and standard deviation (SD) (Figure 8.9). There was no significant difference in plasma S-nitrosothiol levels during the experiment in the sham group. The RSNOs levels increased significantly in the I/R group during reperfusion compared to baseline, and this increase reached

significance at the 5th hour post reperfusion and at the 7th hour post reperfusion (hepatic

vein sample). NAC administration prevented the increase in RSNOs. The difference (NAC

vs I/R) was significant at 5h post reperfusion in plasma samples taken from the ear arterial

line and at 7 h post reperfusion in samples taken from the the right hepatic vein (p < 0.05).

ImT
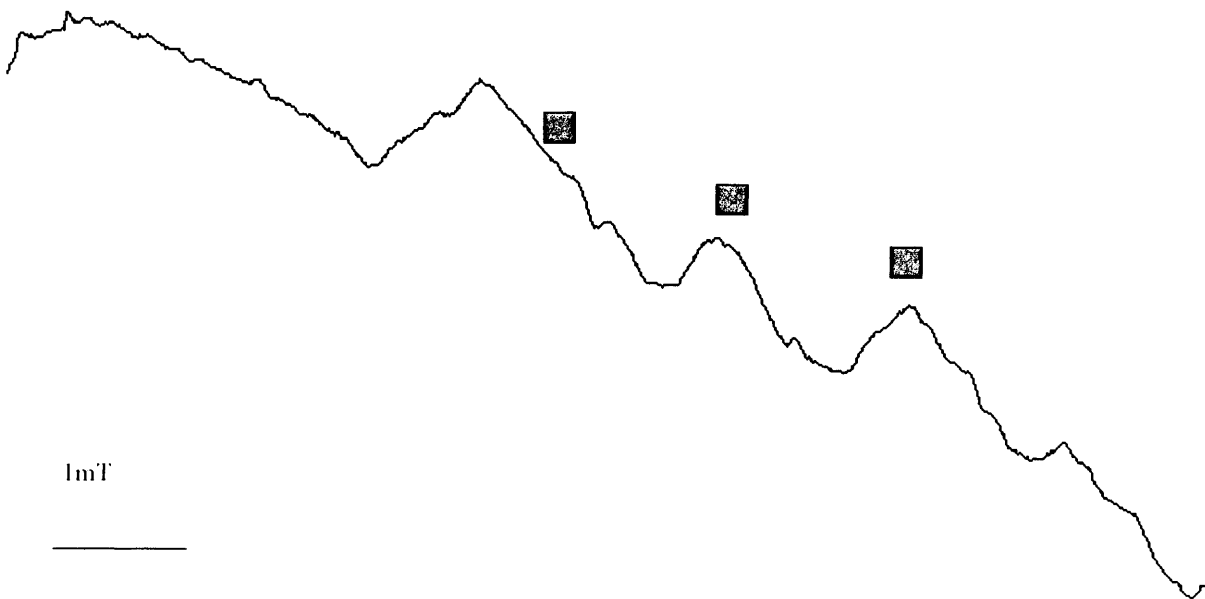
Figure 8.8. A typical nitrosothiol EPR spectrum of in a plasma sample from a sham rabbit 5 hours after reperfusion. After the plasma sample has been added to the spin trap complex, $(MDG)_2$-Fe^{2+}, a three-line signal can be seen when analyzed by EPR spectrometry, indicated by shaded boxes, with a_N=1.28 mT and g=2.04.
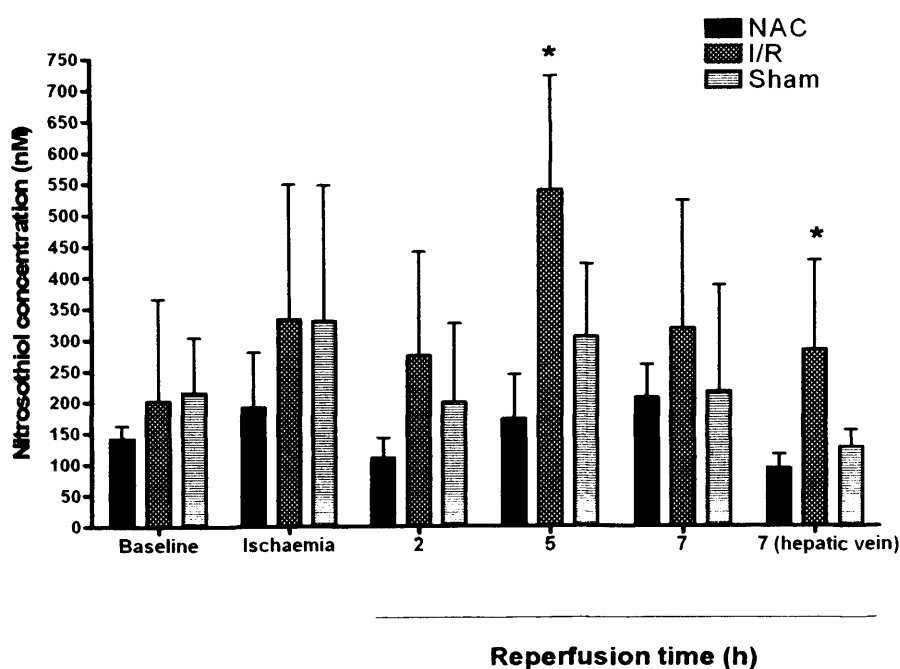
Reperfusion time (h)

Figure 8.9 Plasma nitrosothiols (RSNOs) concentration (nM). Results are presented as mean ± SD. The RSNOs levels increased significantly in the I/R group during reperfusion compared to baseline, and this increase reached significance at the 5th h post reperfusion. NAC administration prevented the increase in RSNOs. The difference (NAC vs I/R) was significant at 5h post reperfusion in plasma samples taken from the ear arterial line and at 7 h post reperfusion in samples taken from the the right hepatic vein. (*p<0.05: I/R vs NAC and sham).

8.3.11 Nitrite, nitrate, NOx activity

NO_3^- and NO_2^- were measured using a capillary electrophoresis method, as described above, in all samples. NO_3^- was eluted at 3.9 min, whilst NO_2^- was eluted at 3.7 min, as shown in Figure 8.10. The mean and SD were plotted for NO_2^- and NO_3^- as well as the NO_x concentrations for each group (as shown in Figures 8.11, 8.12 and 8.13, respectively). There was no significant difference in nitrite levels, during the experiment in the sham group. Nitrites increased significantly during ischaemia and reperfusion in the I/R group.

NAC administration prevented this increase and this difference reached significance at 2 h post reperfusion and at 7 h post reperfusion (hepatic vein). Nitrate levels decreased during the experiment in the sham group. They decreased also significantly during reperfusion in the I/R group. In the NAC group they increased significantly from baseline at 2 h post-reperfusion and then decreased.
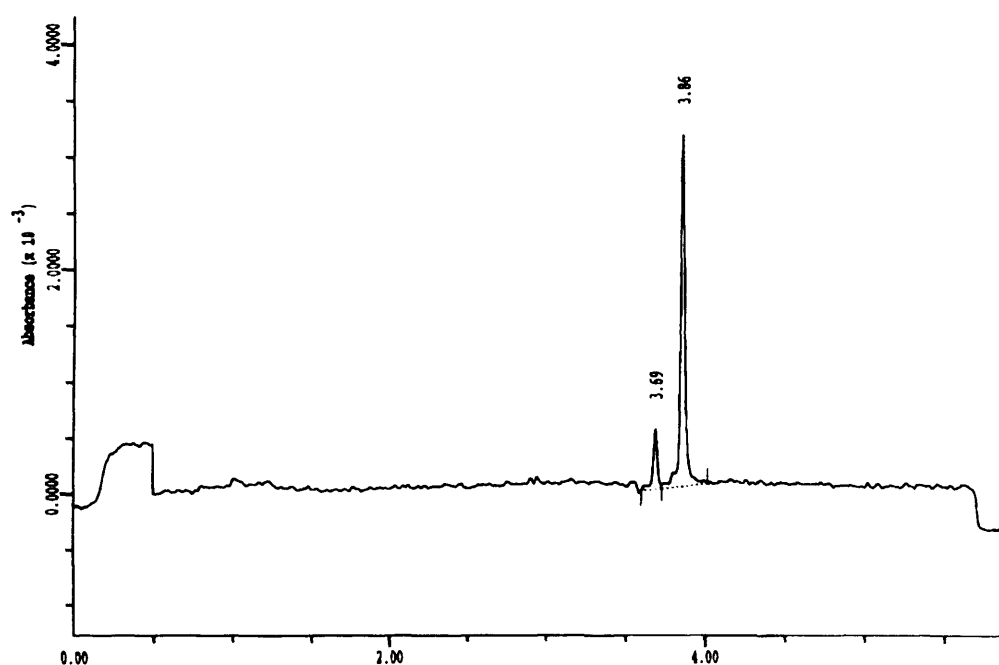
Figure 8.10 A typical electropherograph showing the elution of NO_3^- and NO_2^- from a sham rabbit plasma sample at end of ischaemia. NO_3^- is shown to elute at 3.9 min, whilst NO_2^- elutes at 3.7 min.
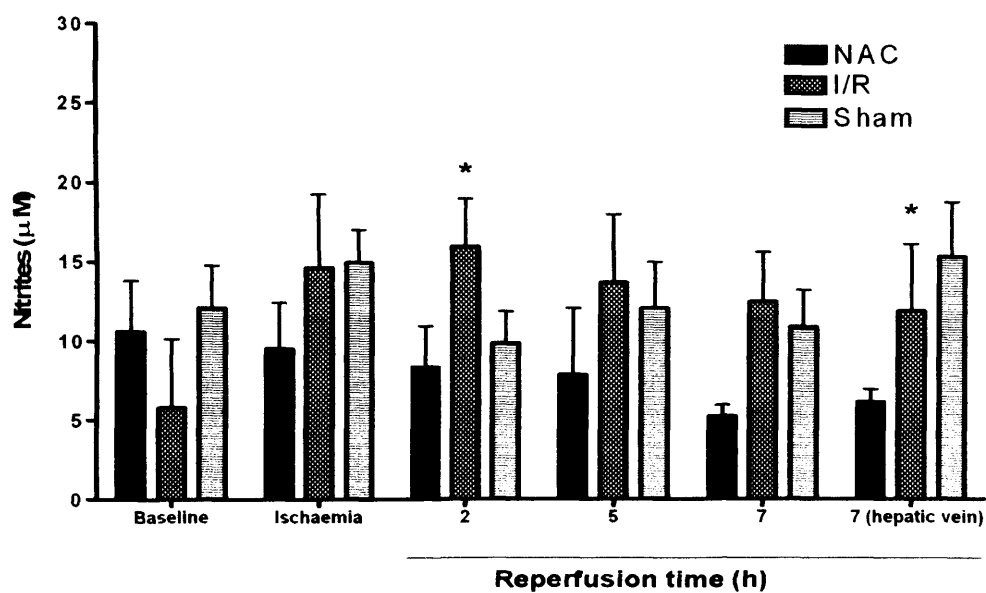
Figure 8.11 Plasma nitrite (NO_2^-) concentration (μM). Results are presented as mean ± SD. Nitrite levels increased significantly during ischaemia and reperfusion in the I/R group. NAC administration prevented the increase in nitrites. *p<0.050: I/R vs NAC.
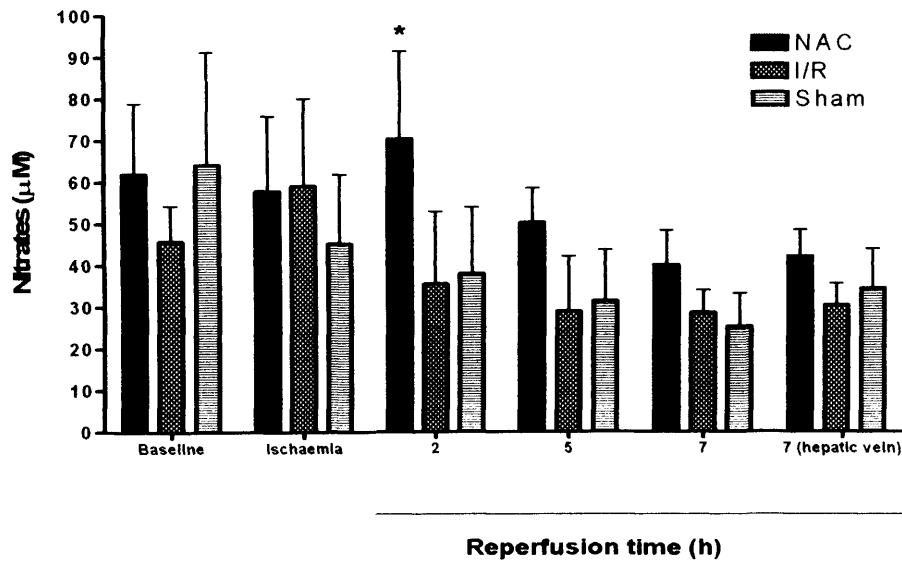
Figure 8.12 Plasma nitrate (NO_3^-) concentration (µM). Results are presented as mean ± SD. Nitrate levels decreased significantly during reperfusion in the I/R group. *p<0.050 vs. baseline.
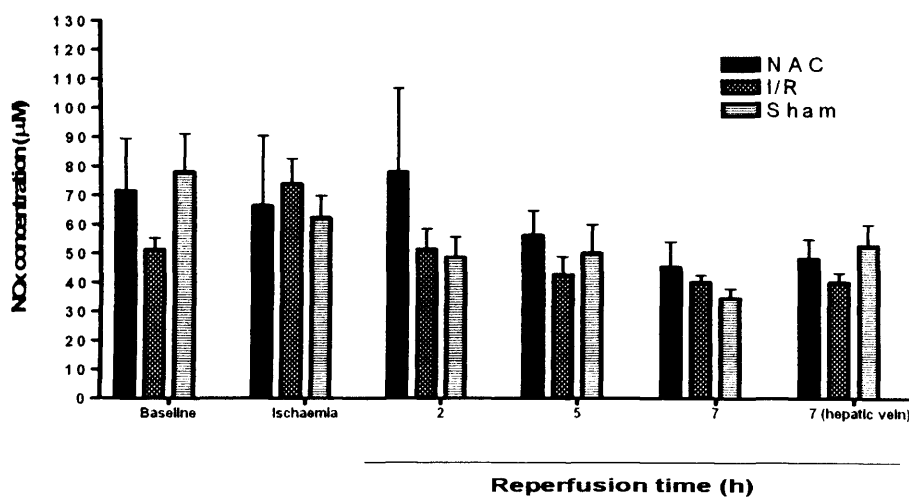
Figure 8.13 The mean NOx (sum of NO_3^- and NO_2^-) concentration in plasma samples. Results are presented as mean ± (SD). NOx concentration did not differ significantly from baseline at any time point in either of the three groups. There were also no differences between the three groups during the experiment.

8.3.12 Dihydrorhodamine 123 oxidation

Liver ischaemia caused a significant increase in the RH fluorescence of plasma, consistent

with the oxidation of DHR 123 by RNS and ROS during the reperfusion phase. RH values

were significantly higher in the I/R group compared to the sham group (Figure 8.14).
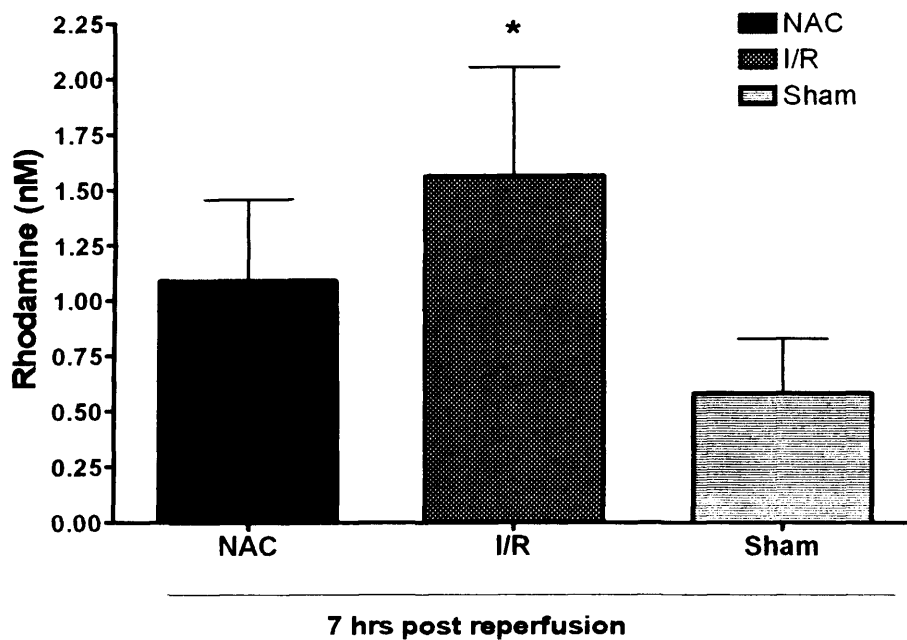
Figure 8.14 Plasma reactive nitrogen species production as assessed by oxidation of dihydrorhodamine 123 to rhodamine (mean ± SD). Liver I/R caused a significant increase in the rhodamine fluorescence of plasma, 7 h post reperfusion compared to sham group, while NAC administration ameliorated the increase (*p<0.05: control vs NAC and sham).

8.3.13 Liver tissue nitrotyrosine

Liver I/R caused a decrease in liver tissue nitrotyrosine levels. This decrease was not significant compared to baseline. However if we look at the nitrotyrosine / tyrosine (NT/T) there is a significant decrease at 7 h post reperfusion compared to baseline (Figure 8.15). NAC administration ameliorated this increase (Figure 8.15) (*p<0.05 vs baseline).
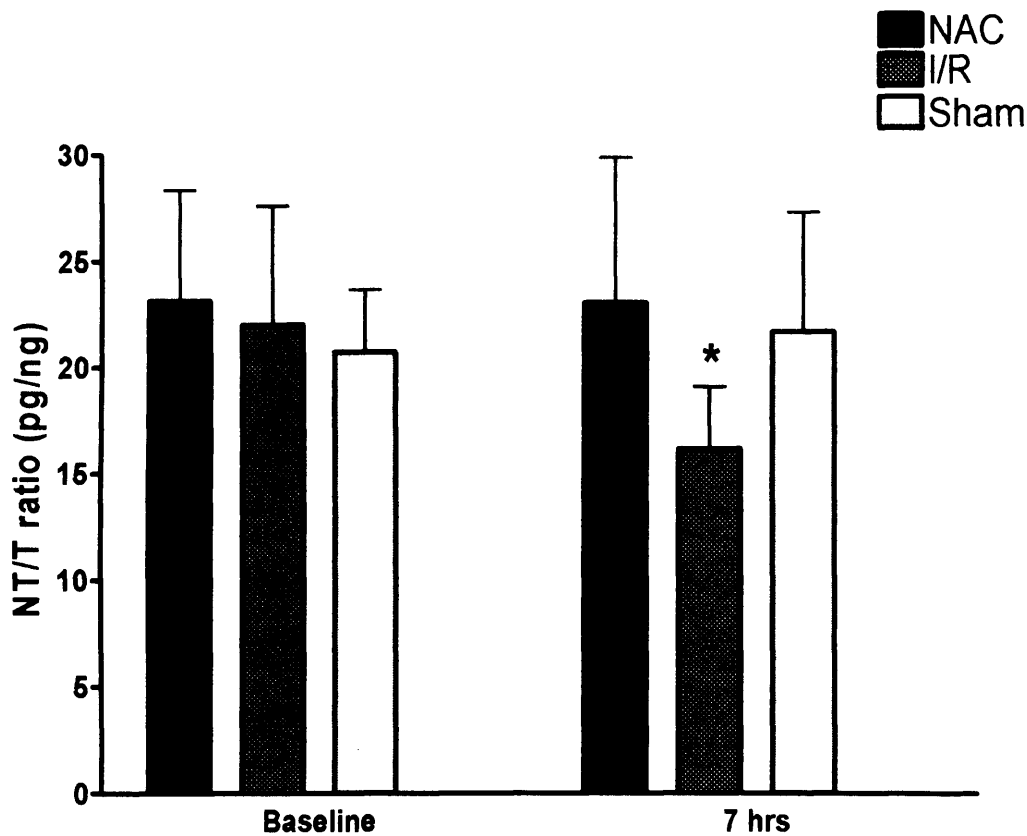
Figure 8.15 Liver tissue nitrotyrosine / tyrosine (NT/T) ratio (pg/ng) measured by mass spectrometry. Results are presented as mean ± SEM. Liver I/R caused a significant decrease in NT/T ratio at 7 h post reperfusion compared to baseline. NAC administration ameliorated this increase (*p<0.05 vs baseline).

8.3.14 Histopathology

The histological semiquantitative score regarding the number of single PMNs in sinusoids was 9 for the sham group, 14 for the I/R group and 12 for the NAC group. The score regarding the aggregated PMNs was 2 for the sham group, 11 for the I/R group and 9 for the NAC group.

There was no evidence of necrosis, Kupffer cell hyperplasia or apoptosis in the sham group.

Hepatocellular necrosis, apoptosis and Kupffer cell hyperplasia was more severe in the I/R group compared to the NAC group (Figures 8.16 and 8.17). The average number of PMNs / hpf (quantitative score) was: 11 ± 7 for the sham group, 38 ± 33 for the I/R group and 18 ± 9 for the NAC group.
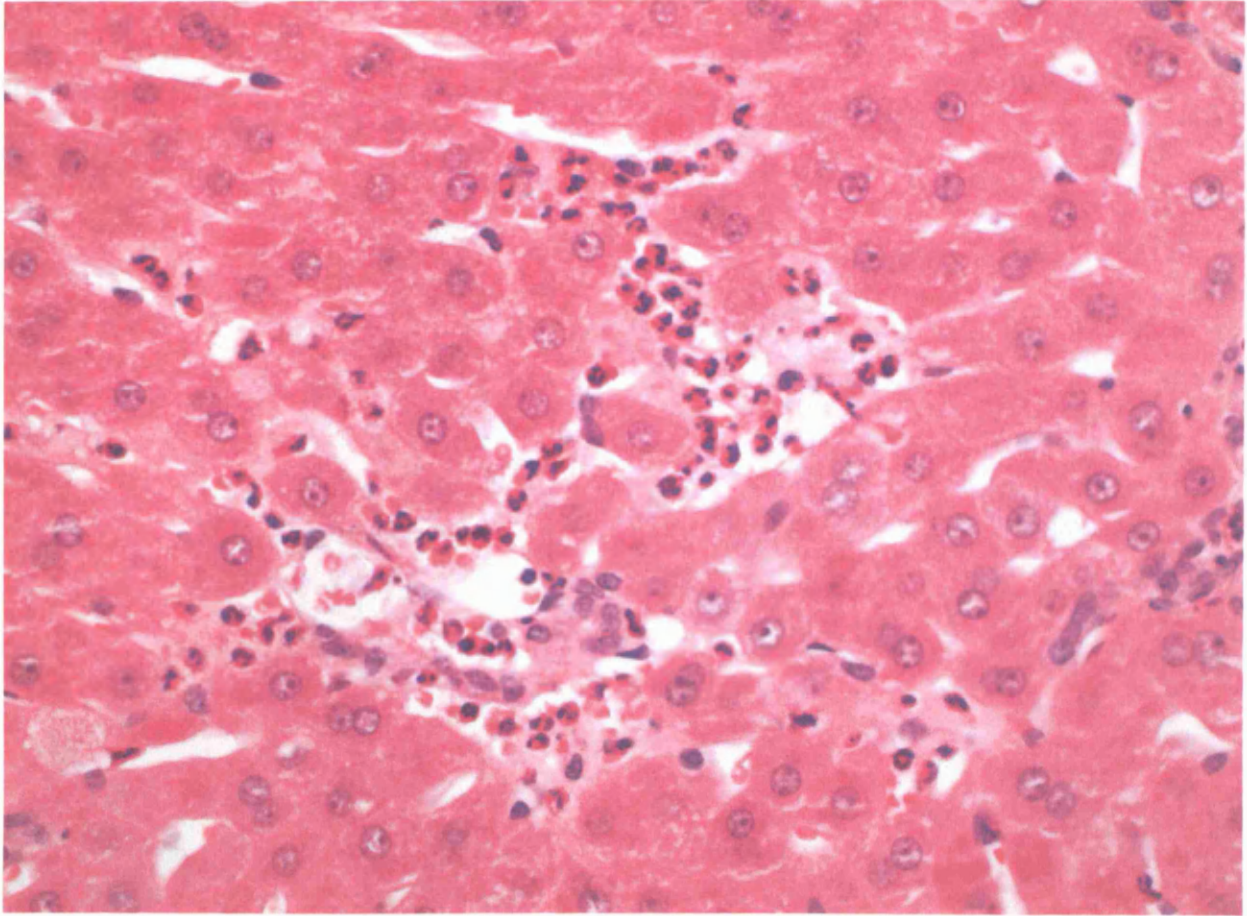
Figure 8.16 Representative photomicrographs of liver histology at 7 h post- reperfusion in the I/R group. It shows prominent numbers of aggregated neutrophils within sinusoids and associated degenerate hepatocytes (H&E x40).
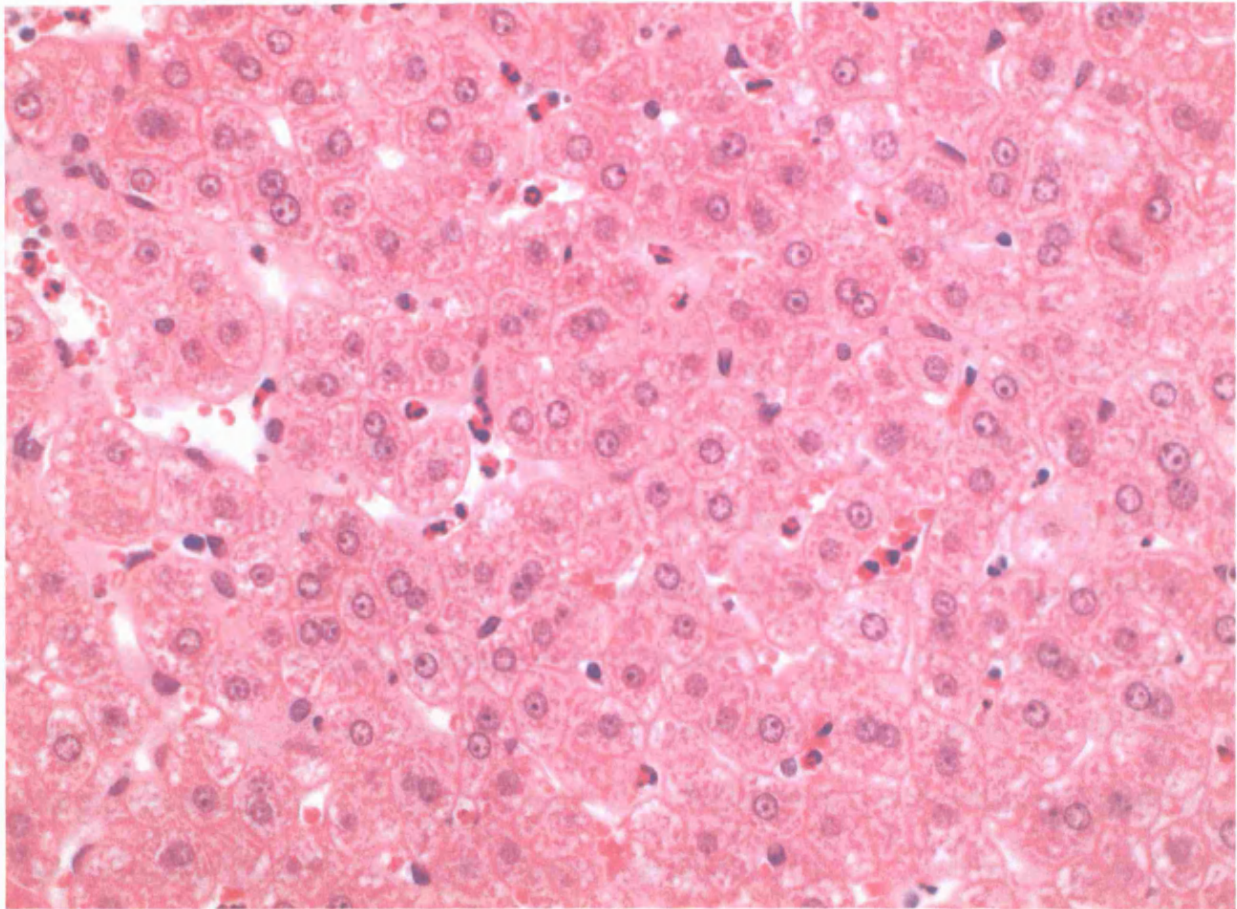
Figure 8.17 Representative photomicrographs of liver histology at 7 h post- reperfusion in the NAC group. It shows moderate numbers of neutrophils within sinusoids, some of which are aggregated. Little hepatocyte damage is apparent (H&E x40).

8.4 Discussion

The present study investigated the effect of NAC administration on cellular energy production and NO activity in a liver lobar I/R model. Our previous experimental studies in rabbits with normal (chapter 6) and steatotic livers (chapter 7) undergoing lobar I/R injury (Glantzounis *et al.* 2004; Fusai *et al.* 2005) have shown that continuous NAC administration improves liver function and reduces liver injury. The beneficial effects were more obvious during the late phase of reperfusion injury. However, the mechanism of this improvement was not established. It is well known that NAC is a precursor of glutathione, and also can act as a direct scavenger of free radicals (Cotgreave 1997; Yan *et al.* 1995). Previous in vitro studies have shown that NAC is also involved in NO metabolism and undergoes transnitrosation reactions with S-nitrosothiols (RSNOs) leading to RSNOs decomposition (Jourd'heuil *et al.* 2000;Marley *et al.* 2001). There is also evidence that GSH can inhibit peroxynitrite formation (Nakamura *et al.* 2000).

This study aimed to evaluate RNS formation following I/R and the in vivo effects of NAC on cellular energetics and NO metabolism. A rabbit model of liver lobar I/R was used. The time period chosen has allowed haemodynamics, hepatic function and changes in bioenergetics and NO metabolism to be evaluated in both the early and initial late phase of liver I/R injury. The duration of general anaesthesia was similar to that for major human liver surgery (liver resection and transplantation).

During reperfusion significant changes occurred in bile production and composition. Bile formation is an active secretory process involving bile salt-dependent and –independent mechanisms (Bowers *et al.* 1987). Bile level may reflect the degree of ATP degradation

during the ischaemia period. Bile excretion is used as an indicator of liver function and recovery following ischaemia in experimental studies (Bowers *et al.* 1987; Jamieson *et al.* 1988; Nakano *et al.* 1997; Terajima *et al.* 2000) and clinical liver transplantation (Adam *et al.* 1992; Anthuber *et al.* 1996; Fisher *et al.* 1999; Sumimoto *et al.* 1988).

However, little is known about changes in bile composition during liver ischaemia and following reperfusion and less is known about the effect of therapeutic strategies to ameliorate I/R injury. This is the first study utilizing ^{1}H NMR analysis of bile to study the effect of NAC on normal rabbit livers in a controlled experimental model of warm liver I/R. The use of spin echo spectra allowed the interference from broad lipid signals to be reduced and more clearly identify and comparatively quantify bile constituents. Continuous bile flow was recorded in both the I/R and NAC groups, and ^{1}H NMR spectroscopy revealed fluctuations in the concentration of several bile constituents despite there being no significant differences in bile flow at during ischaemia and reperfusion between I/R and NAC group. More specifically, NAC administration enhanced the biliary excretion of acetate and pyruvate and reduced the excretion of PC.

Biliary lactate was higher in the I/R group during reperfusion compared to the I/R group. This difference was more prominent during the late phase of reperfusion which corresponds to a large burst of neutrophil activity (Clavien 1998; Jaeschke *et al.* 1990). It has been observed that the injury produced during the initial part of the late phase is a more extensive hepatocellular injury in comparison to the early phase (Clavien 1998; Harbrecht *et al.* 1993; Jaeschke *et al.* 1990). Moreover, an element of neutrophil sequestration, sinusoidal narrowing (Vollmar *et al.* 1994) and vasoconstriction (Pannen 2002) combine to

form a 'no-reflow paradox' where by hepatocytes are subjected to a further perfusion deficit and persistent ischaemia (Menger *et al.* 1992). This may explain the observed peak in lactate at this late time-point especially since lactate levels have been shown to become elevated in bile even when they are not elevated in serum (Nishijima *et al.* 1997). Acetate is produced as a result of fatty acid β-oxidation in the liver (Ambrose and Easty 1977). This reaction requires ATP at its initiation. Following I/R injury ATP is consumed to clear excess lactate and pyruvate and may not be readily available for fatty acid oxidation. The I/R–induced reduction or depletion of ATP may result in a decreased ability to utilize β-oxidation causing acetate production to falter. The higher bile acetate levels in the NAC group would suggest improved ATP production and energetics. The increased excretion of pyruvate in the bile of NAC treated livers is difficult to interpret although this has been reported also in our study with steatotic liver (chapter 7) (Fusai *et al.* 2005). Pyruvate is transported in the mitochondria, and after an oxidative decarboxylation, it is transformed into acetyl coenzyme A and used for the citric acid cycle. There could be a relationship between the increased of pyruvate and acetate in the NAC group and better mitochondrial preservation.

The main source of PC in the hepatocytes is the cell membrane. The increase following ischaemia and again at 2 and 5 h post-reperfusion correlates well with the theory of cellular breakdown and membrane lipid peroxidation (Suzuki *et al.* 2000). This rise coincides with hepatocellular injury during ischaemia with the 'no-reflow period'. PC levels at these time-points were reduced in the NAC treated group. Increased PC has also been observed in association with poor graft function (El Desoky *et al.* 1999; Fusai *et al.* 2005). During I/R injury membrane phospholipids are degraded and glycogen is broken down. This study

demonstrates increasing loss of these compounds in bile with increasing severity of injury. The presence of biliary glucose and its increase following I/R has been noted recently (Habib *et al.* 2004).

In this study Cyt Ox redox state decreased significantly during ischaemia, subsequently recovered partially during the early phase and decreased further during the late phase to levels below the ischaemic values. NAC prevented the reduction in Cyt Ox during reperfusion and this could explain the beneficial effects to the energy dependent process of bile flow.

The current study showed that liver I/R decreased significantly the redox state of Cyt Ox, increased the levels of RSNOs and decreased liver tissue nitrotyrosine, markers of NO activity. NO can regulate mitochondrial respiration by inhibiting Cyt Ox (Cooper 2002). This inhibition is reversible at low concentrations of NO. However, Cyt Ox inhibition at high NO levels leads to increased formation of superoxide, which then reacts with NO leading to the formation of other RNS such as peroxynitrite. Peroxynitrite at high levels can cause irreversible damage to Cyt Ox. This probably occurs via the combination of tyrosine nitration and damage to prosthetic groups (Cooper 2002; Aulak *et al.* 2004; Koeck *et al.* 2004). Furthemore, formation of ROS and RNS is enhanced under conditions of hypoxia (Turrens 2003).

In the present study significant oxidation of DHR 123 to RH occurred in the I/R group 7 h post reperfusion. At this time point a significant increase in RSNOs formation was noted while the redox state of Cyt-ox Cu_A was significantly decreased and hepatic microcirculation was also reduced. The underlying mechanism could be that NO produced by iNOS induction and superoxide anion produced by activated neutrophils during the late phase of reperfusion lead to production of peroxynitrite. Peroxynitrite and hypoxia generated by low flow cause reduction in Cyt Ox, and mitochondrial dysfunction which results in increased mitochondrial RNS and ROS formation. These oxidants possibly contribute to further deterioration in microcirculation and liver injury. The decrease in liver nitrotyrosine post-reperfusion, found in the present study, is not what we would expect, since nitrotyrosine formation is regarded as a marker of peroxynitrite. It seems that the reactions between NO, RNS and proteins are complex and probably increased proteolytic activity occurs during I/R. NO mediated hepatotoxicity has already been observed in chronic liver inflammatory conditions such as ethanol induced hepatotoxicity, where induction of iNOS seems to play a major pathogenetic role (McKim *et al.* 2003; Venkatraman *et al.* 2003).

NAC administration prevented the decrease in the redox state of Cyt Ox and liver tissue nitrotyrosine, the increase in RSNOs and ameliorated the oxidation of DHR 123 to RH. This is the first in vivo study in liver I/R showing the interaction between NAC and plasma RSNOs. In vitro evidence suggest that there is a dynamic interaction between S-nitroso-albumin and low molecular weight thiols in plasma in controlling vascular tone and function (Marley *et al.* 2001). Both high and low molecular weight thiols can be S-

nitrosated in plasma by reaction with NO or inter-converted by trans-nitrosation reactions. Peroxynitrite can also generate S-nitrosothiols through an oxygen independent reaction (Marley *et al.* 2001).

Low molecular weight thiols enhance the decomposition of S-nitrosoalbumin which is the main high molecular weight thiol in plasma. The exact reaction mechanisms by which thiols promotes rapid metabolism of plasma RSNOs is not clear, although both transnitrosation and reductive reactions have been reported (Jourd'heuil *et al.* 2000; Marley *et al.* 2001).

This study would suggest that NAC exerts its beneficial effects on liver I/R, through inhibition of formation of RNS such as peroxynitrite and the continuous maintenance of mitochondrial activity. Modulation of NO metabolism could be a potential target for ameliorating I/R injury.

8.5 Chapter summary

N-acetylcysteine (NAC) reduces warm liver ischaemia-reperfusion (I/R) injury but the mechanism of action is not clear. The present study has investigated the effect of NAC administration on cellular energy production and nitric oxide metabolism in the rabbit lobar I/R model.

Lobar liver ischaemia was induced for 60 min, followed by 7 h of reperfusion. In the NAC group (n=6) 150 mg/kg/h of NAC was administered iv, over the 15 min before reperfusion and maintained by continuous infusion at a dose of 10 mg/kg/h during the reperfusion period. Control groups were I/R alone (I/R group, n=6) and sham laparotomy (Sham group, n=6). Cellular energy production was assessed through the measurement of liver cytochrome oxidase redox state (Cyt Ox) by near-infrared spectroscopy, and by analysis of bile composition using proton nuclear magnetic resonance (^{1}H NMR) spectroscopy. Nitric oxide metabolism was assessed through measurement of plasma S-nitrosothiol levels by electron paramagnetic resonance spectrometry, nitrite and nitrates through capillary electrophoresis. Reactive nitrogen species formation was studied by measuring liver tissue nitrotyrosine with mass spectrometry and through the oxidation of the fluorescent dye dihydrorhodamine (DHR) 123.

In the I/R group Cyt Ox redox state decreased significantly during reperfusion compared to baseline and sham, while bile lactate levels were significantly increased and bile acetate levels were decreased.. Plasma S-nitrosothiol levels were significantly increased at 5 h post reperfusion ($p<0.05$, I/R vs sham) and oxidation of DHR 123 was significantly increased 7 h post reperfusion ($p<0.05$, I/R vs sham,). NAC administration ameliorated the decrease in tissue Cyt Ox redox state, decreased bile lactates and plasma nitrosothiol levels and increased bile acetate levels (all $p<0.05$ at 5 h post reperfusion, NAC vs I/R). Furthermore NAC decreased the oxidation of DHR 123 and prevented the reduction in liver nitrotyrosine ($p<0.05$, NAC vs. I/R).

In conclusion, the protective effect displayed by NAC in warm liver I/R involves inhibition of reactive nitrogen species formation and maintenance of mitochondrial activity.

CHAPTER 9

THESIS DISCUSSION,

CONCLUSIONS AND FUTURE WORK

9.1 Methodological considerations

9.1.1 The experimental model

Liver I/R injury has major clinical importance in different clinical settings such as liver resections for liver tumours, liver transplantation, haemorrhagic shock with fluid resuscitation, cardiac by-pass surgery, laparoscopic surgery. The study and the clarification of the pathophysiology will facilitate the understanding of the complicated mechanisms involved in the pathogenesis of reperfusion injury. Then, the effect of therapeutic strategies to ameliorate liver I/R should be assessed. Experimental models are very useful for the study of the mechanisms of liver I/R and the assessment of the effectiveness of therapeutic strategies, since they provide controlled conditions which allow the researchers to study the effects of an intervention on a single homogenous population.

The experimental model used in this study offered the opportunity to assess both haemodynamic and functional parameters in the rabbit liver, in sham operated animals and in animals submitted to liver I/R (control and treated groups). For maintenance of anaesthesia an inhaled agent (Isoflurane) was used which is mainly metabolised in the lungs rather than the liver. This avoids cumulative effects and is also less hepatotoxic compared to other inhaled agents such as halothane (Stachnik 2006). The animal selected for the experimental procedures was rabbit. This was done mainly due to the size, which allows application of a

proper anaesthetic protocol and maintenance of metabolic stability with long anaesthesia. It allows also the easy placement of probes, continuous measurement of bile flow by cannulation of the common bile duct and blood withdrawal at different time points with maintenance of the haemodynamic stability. The anatomy of the rabbit liver with separate lobes makes easier the identification of vascular elements and selective interruption of the blood supply.

The sham group allowed the effects of prolonged anaesthesia and surgical trauma alone to be evaluated. The duration of general anaesthesia was similar to that for major human liver surgery (liver resection and transplantation).

One of the main criticism of the liver lobar I/R model is that it does not simulate the clinical conditions of total liver ischaemia, which occur in liver transplantation or in liver resections when Pringle manoeuvre is applied. However, work from our group showed that 30 min of total hepatic ischaemia is poorly tolerated by the rabbits, resulting in cardiac arrest due to severe reduction in MABP and metabolic acidosis (Kanoria et al. 2004). Also, acute portal venous occlusion causes venous congestion in the mesenteric bed and compromise of the intestinal mucosa, resulting in bacterial translocation and onset of systemic inflammatory response syndrome (Koo et al. 1992). In the present study blood flow to the medial and left lateral lobes is interrupted leaving the right and caudate lobes with normal circulation. Lobar ischaemia maintains splanchnic blood flow and prevents the haemodynamic instability

associated with mesenteric congestion and portal bacteraemia found with total inflow occlusion (Koo *et al.* 1992). Ischaemic time is similar to time used in liver resections.

The current model may be preferable also to a liver transplant model for the study of liver I/R, because in contrast with preservation of organs at low temperatures, normothermic ischaemia produces rapid liver injury, with the option of in-vivo study without the systemic effects of transplantation. The technical difficulty of re-establishing portal and arterial blood flow, the denervation of the organ, artefacts due to organ cooling and the possibility of immunological involvement make experimental liver transplantation a complex experimental model. Normothermic ischaemia of the liver is to some extent a simulation of the circulatory and metabolic conditions of transplantation, being nevertheless a simple, reproducible and efficient experimental method with low mortality and morbidity.

This model was further refined with the cannulation of the ear artery instead of the femoral artery for monitoring of arterial blood pressure and blood withdrawal. A set of experiments showed that the cannulation of the femoral artery causes remote hepatic injury. The mechanism for this injury was not investigated, but it is possible that low flow reperfusion injury and the release of mediators such as cytokines could be the cause. Although the remote effect of limb I/R injury was studied only on liver function, it is plausible to assume that prolonged femoral artery cannulation causes remote injury to other organs such as lung, heart, kidneys. Experimental studies focusing on the application of an intervention and study of specific organ function should avoid femoral artery cannulation.

One of the limitations of the model is that it is not appropriate for recovery and thus can not

assess the effect of NAC in later time points where organ failure, morbidity and mortality

could be evaluated.

In the present study a model of steatosis induced by high cholesterol diet was used for the

study of the effect of NAC in fatty livers. High-cholesterol feeding induces the formation of

steatotic livers in which there is accumulation of triacylglycerol (triglyceride) and cholesterol

in the liver (Lee and Ho 1975; Mastai et al. 1996; Wanless et al. 1996). Animals models

used for the study of non alcoholic fatty liver disease (NAFLD) are divided into two broad

categories: those caused by genetic mutation and those with an acquired phenotype produced

by dietary or pharmacological manipulation. The genetic leptin-deficient or leptin resistant is

the most popular model and the dietary methionine / choline deficient model is the most

commonly used in the second category (Anstee and Goldin 2006). However, neither these

two models has reproduced either the phenotype or the pathogenic mechanism of human

steatosis-steatohepatitis (Anstee and Goldin 2006).

The high cholesterol diet model produces a central lobular deposition of fat, similar to that

found in the majority of human steatotic livers. The liver weight and the liver weight (LW) /

body weight (BW) ratio are increased significantly compared to rabbits with normal liver.

Macroscopically the liver is enlarged, yellowish in colour with rounded edge and firm

consistency. Microscopically there is moderate steatosis with macrovesicular fat

accumulation and inflammatory cell infiltrate. Biochemically there is elevated bilirubin,

elevated transaminases, low albumin, high cholesterol and slightly high triglycerides. Although it has characteristics similar to the human steatosis, it is not the ideal model, since the steatosis is produced by high cholesterol diet and mainly leads to significant hypercholesterolemia and mild hypertriglyceridemia. Probably the development of combined models of genetically modification along with high fat diet will produce a situation similar to the human steatosis.

9.1.2 Laser Doppler Flowmetry (LDF)

The measurement of microcirculatory blood flow is of major importance in the study of the pathophysiology of liver I/R injury and its modulation by therapeutic strategies. There is experimental and clinical evidence that changes in microcirculation during the immediate reperfusion period in liver transplantation correspond directly with the maximum postoperative enzyme release from the liver (Klar *et al.* 1997), and are of value in assessing and predicting the degree of liver injury (Pannen 2002). The sinusoidal flow is actively regulated and redistributed at the level of the microcirculation through a balance between vasoconstrictive substances such as endothelin and vasodilatory agents such as NO and carbon monoxide.

In the present study, laser doppler flowmetry was used to measure blood in capillaries and sinusoids rather than in major vessels running deeply inside the liver substance, thus the described range of penetration was considered sufficient to study blood flow in a liver lobe with a maximum thickness of less than 10 mm. This method does not measure the microcirculatory blood flow of the liver as a whole, which would be ideal. However, the

location of the probe in a fixed point of the liver surface, throughout the experiment, allows the continuous record of the flow signal. This can be considered representative of the microcirculatory changes on that point during the different stages of the procedure, using the same place as control.

During the application of LDF in the experiments a few problems were encountered. One being motion artefact. The laser Doppler monitor can produce artefact signals due to vibration or other movements of the fiberoptic probe itself and due to relative movements between the probe and the explored tissue. With the rabbit liver there is considerable amount of respiratory movement. In the current experiments it was possible to reduce significantly the artefact signals with a good level of anaesthesia, good mobilisation of the liver to avoid diaphragmatic movements and placement of the probe loosely in contact with the liver allowing it to move with it.

A baseline recording of flow as a standard (100%) was used in each individual experiment and expressed in arbitrary "Perfusion Units". This is because the backscattered light varies from one organ to another depending on factors including light absorption and red cell fraction on different tissues and it is not possible to translate the values into a unit of flux that can be utilised for different organs; nevertheless, when applied to the same organ the signal is reproducible within a narrow range of variation. Another factor which affects the baseline measurements is the type of probe used in the experiments. This could explain the difference

in baseline found in two different experimental procedures (femoral artery group and ear artery group, normal liver) where different probes were used in each set of experiments.

9.1.3 Near infrared spectroscopy (NIRS)

During NIRS application in the experiments a few problems were noticed. The interference by the effect of operating lights on the measurement requires covering of the probes with lightproof back cloth. Variation in measurements were found with the re-application of the probes even on the same site of measurement which may be caused by variation of the optical properties of the area under investigation which are tested and accounted for by the spectroscopy with the initial setting. This problem restricts the NIRS application as the probes must be applied and maintained in the same site without movement during the whole procedure, which may be impractical in clinical situation. However, in the present study recording of cytochrome oxidase (Cyt Ox) data was not affected from the re-application of probes and accurately represented the redox state of the enzyme which correlated with the changes in microcirculation and transaminases, as well as the changes in bile composition. The measurement of cytox with NIRS has been validated against other techniques, by our group, such as nuclear magnetic resonance spectroscopy (Seifalian et al. 2001b) and partial oxygen pressure (El Desoky et al. 2000). NIRS is a powerful method of obtaining information about mitochondrial activity and cellular energy production. This has major importance as clinical evidence shows that the degree in impairment of the hepatic mitochondrial redox state determines the survival rate of patients after haemorrhagic shock and resuscitation (Nakatani et al. 1995).

9.1.4 Histology

The semiquantative histological score used in this study provides enough information in order to detect differences between two experimental groups. However, when the differences are not very significant then the use of a quantitative score will detect differences which the semi quantitative score is not able to detect.

9.1.5 Proton nuclear magnetic resonance (^{1}HNMR) spectroscopy and electron paramagnetic resonance (EPR) spectrometry

Proton NMR spectroscopy demonstrated significant changes in bile composition during liver ischaemia-reperfusion. It can be used also as a marker for the assessment of the effectiveness of different therapeutic strategies such as antioxidants. The quantitative measurement provides more precise information compared to the measurement of the changes relative to baseline. The further development of bile analysis with proton NMR spectroscopy could have clinical application in the postoperative assessment of liver graft function and early diagnosis of dysfunction.

EPR provides quantitative measurement of stable free radicals such as ascorbyl radical, but also of bio molecules such as S-nitrosothiols (RSNOs) which come from reaction of free radicals with thiols. Measurement of RSNOs by EPR has previously validated against an established chemiluminenescence method (Rocks *et al.* 2005). This is a quick and sensitive technique that, due to the nature of EPR spectrometry, is not directly affected by other NO

metabolites. The samples do not require pre-treatment (e.g. to be deproteinised). This allows both high and low molecular weight RSNOs to be analyzed, which includes S-nitrosoalbumin. Our results suggest that S-nitrosothiols plasma levels are a good marker of nitrosative stress. EPR could have potential clinical applications in the measurement of oxidative stress in biological fluids or tissues and also assessment of the effectiveness of therapeutic strategies against oxidative stress.

9.2 Role of nitric oxide in liver I/R

The role of NO in liver I/R is still ambivalent. Under physiological conditions only constitute endothelial NOS is present in the liver. Low levels of NO regulate hepatic perfusion by maintaining a balance with vasoconstrictors such as endothelin. NO also prevents platelet adhesion, thrombosis, polymorphonuclear cell accumulation and secretion of inflammatory mediators (Gauthier *et al.* 1994; Mittal *et al.* 1994). During reperfusion initially NO levels decrease, but afterwards large amounts are produced through induction of iNOS. There is experimental evidence that iNOS messenger RNA expression starts 1 h post reperfusion with increased iNOS activity at 5 h post –reperfusion (Hur *et al.* 1999). Induction of iNOS may have either toxic or protective effects. Different studies have shown inducible nitric oxide synthase inhibition to be either beneficial or detrimental. Furthermore, the use of a selective iNOS inhibitor showed beneficial effects in experimental models with endotoxaemia (Thiemermann *et al.* 1995; Wang *et al.* 1998), but not with liver I/R (Majano *et al.* 2005).

The toxic effects of NO are linked with the production of peroxynitrite, which is the product of O_2^- and NO. Peroxynitrite can cause cell injury through multiple pathways, such as initiation of lipid peroxidation, direct inhibition of mitochondrial respiratory chain enzymes, inhibition of membrane Na^+/K^+ ATPase activity, or oxidative protein modification such as formation of nitrotyrosine (Doulias et al. 2001; Hon et al. 2002; Szabo 2003). Whether NO will act as a cytoprotective or cytotoxic agent depends on a number of factors, such as NO/ superoxide anion ratio, hepatic stores of reduced glutathione and duration of ischaemia.

The present study has shown that during the late phase of reperfusion large amounts of nitric oxide are produced, as indicated by the rise in nitrites and RSNOs. However at this point significant liver injury occured as indicated by the increase in ALT activity, the reduction in bile flow, in microcirculation, the changes in Cyt Ox redox state and histology. There was also evidence of significant oxidative stress during that period, as shown by the increased oxidation of dihydrorhodamine to rhodamine which is partially peroxynitrite dependent. The major factor which can contribute to this injury could be RNS and ROS. Peroxynitrite could be one of the major factors responsible for this increase. The current study showed also that increased NO levels in the late reperfusion period is associated with a reduction in the parenchymal perfusion. The reduced flow could be secondary to the detrimental effects of RNS in microcirculation.

Experimental evidence suggests that NO controls oxygen consumption by inhibiting cyt ox. Although in small concentrations of NO this inhibition has a regulatory role in cell function,

in large concentrations of NO can be detrimental. Further studies are required to elucidate the role of NO and mitochondrial activity in liver I/R.

9.3 Role of NAC in liver I/R

N-acetylcysteine is an agent with a wide spectrum of effects and minor side effects such as anaphylactic reaction (skin rash). It can be administered instead of L-cysteine which can be toxic. Due to its small size it can overcome the problems associated with the administration of the endogenous anti-oxidant glutathione.

Its beneficial role has been well established in the fulminant liver failure due to paracetamol overdose. In this field there are several randomised controlled clinical trials which have shown a clear benefit (Chyka *et al.* 2000). However, there is controversy about its role in fulminant liver failure due to other causes. The mechanism postulated for the protective effect in liver failure due to paracetamol overdose is inhibition of peroxynitrite. However, we should bear in mind that the mechanisms of injury are different between liver I/R injury and other forms of hepatic injury. Acute adenosine triphosphate depletion occurs with ischaemia but not in chronic liver disease. Also the signalling pathways and the mechanisms of cell death are different in settings of acute liver injury and chronic disease.

The therapeutic role of NAC in human diseases has been reviewed recently. Although beneficial effects were demonstrated in conditions associated with oxidative stress such as AIDS, cancer and I/R injury; the current evidence does not support routine application (Aitio 2006).

The studies on NAC in this thesis were planned due to the lack of previously controlled experimental data, on the role of continuous NAC infusion on liver function during the early and late phase of hepatic I/R injury. The effect of NAC was evaluated using a controlled model where both phases of liver I/R could be studied. The dose of NAC used was similar to that used for patients with fulminant liver failure due to paracetamol overdose. This thesis has provided significant information on the timing of the maximum protective effect of NAC and also on the mechanism of action. It seems that the protective effect becomes apparent when the oxidative stress reaches a maximum. This seems to occur after the 5th h of reperfusion. In addition, the modulation of NO metabolism with NAC administration provides further new information. We have showed that large amount of NO is produced during the late phase of reperfusion and associated with significant liver injury. NAC administration reduced NO production ameliorated liver injury and maintained mitochondrial activity and cellular bioenergetics.

9.4 Overall conclusions arising from the thesis and suggestions for future work

The major findings of the study were:

1. Cannulation of the femoral artery induces remote liver injury and should be avoided in studies focusing on liver function. The use of the ear artery instead of the femoral avoided the remote hepatic injury. Studies focusing on the study of specific organ function after application of an intervention should avoid femoral artery cannulation.

2. Lobar liver I/R caused significant decrease in hepatic microcirculation, liver tissue oxygenation and energy metabolism as was expressed by changes in the redox state of cytochrome oxidase and bile composition. It also caused significant alterations in NO metabolism during the late phase, as indicated by the increase in plasma nitrates, plasma S-nitrosothiols and decrease in liver tissue nitrotyrosine. Further studies should clarify further the relationship between mitochondrial activity, NO metabolism and liver I/R injury. Also, the use of proton NMR spectroscopy for bile analysis and of EPR for measurement of S-nitrosothiols and free radicals could have applications in clinical studies.

3. Continuous infusion of N-acetylcysteine in rabbits with normal liver significantly improved hepatic microcirculation, intracellular oxygenation and reduced liver transaminases. The main protective effect of NAC was more obvious during the late phase of reperfusion (after the 5th h). Similar results were obtained in rabbits with moderate hepatic steatosis.

4. The protective effect of NAC was associated with improved cellular energy metabolism (maintenance of mitochondrial activity, decreased lactates in bile) and with significant modification of nitric oxide metabolism (reduction in plasma nitrites, S-nitrosothiols and RNS).

The above conclusions suggest a protective role for N-acetylcysteine in liver I/R injury. This protective effect should be further investigated in recovery models, where NAC could be administered continuously through an implantable pump and assess outcome in terms of morbidity, mortality and organ failure. The administration of more powerful thiols, such as bucillamine, should also be investigated as they may have a major role in I/R injury (Amersi *et al*. 2002).

However, we should bear in mind that the body response to liver resection is very complex and that the oxidative stress produced by I/R injury is only one part of the pathway. Experimental evidence suggests that Kupffer cell activation and the subsequent release of cytokines such as tumour necrosis factor-α (TNF-α) and intereukin-6 (IL-6) play a pivotal role in liver regeneration and prevention of liver failure (Shiratori *et al*. 1996). Also induction of iNOS may have a role in liver regeneration (Garcia-Trevijano *et al*. 2002;Rai *et al*. 1998;Ronco *et al*. 2004). In order these areas to be further clarified, it is important recovery models to be established; where both modulation of liver I/R injury and the influence on liver regeneration can be studied.

In clinical practise evidence suggests that patient age and underlying liver disease (cirrhosis, steatosis) increase significantly the risks for postoperative liver failure leading to increased morbidity and mortality (Helling 2006). Clinical studies have shown a significant increase in mortality after the age of 65, mainly with extended liver resections (Koperna, Kisser, and Schulz 1998). One of the main factors seems to be susceptibility to liver ischaemia and oxidative stress.

Also, hepatic steatosis and steatohepatis have been reported worldwide and are increasingly recognised as the leading cause for liver dysfunction and cirrhosis in the non-alcoholic, viral hepatitis negative population in Europe and North America. Steatotic livers are very susceptible to oxidative stress leading to high rates of liver failure after liver resection and in primary non-function or dysfunction following liver transplantation both in cadaveric (Selzner and Clavien 2001) and living donor liver transplantation (Soejima et al. 2003). Hence there may be an increased role for antioxidant therapies.

Clinical trials have produced conflicting results regarding the use of antioxidants in liver transplantation. One of the reasons for this inconsistency in the results is that small groups of relatively young patients with normal underlying liver function or good graft function were studied. The main protective effect would be anticipated in older patients with diseased livers (post chemotherapy, steatosis) undergoing liver resection and in patients undergoing liver transplantation with marginal grafts. Clinical trials need to be designed accordingly.

Appendix

Publications arising out of work described in this thesis

Papers

1. Continuous infusion of N-acetylcysteine reduces liver warm ischaemia-reperfusion injury.
   Glantzounis GK, Yang W, Koti R, Mikhailidis DP, Seifalian AM, Davidson BR.
   Br J Surg 2004; 91 (10):1330-39.

2. The contemporary role of antioxidant therapy in attenuating liver ischaemia-reperfusion injury: A review.
   Glantzounis GK, Salacinski H, Yang W, Seifalian AM, Davidson BR.
   Liver Transpl 2005; 11:1031-1047.

3. N-acetylcysteine ameliorates the late phase of liver ischaemia-reperfusion injury in an experimental model with hepatic steatosis.
   Fusai G, Glantzounis GK, Hafez T, Yang W, Sheth H, Kanoria S, Parks HG, Seifalian AM, Davidson BR.
   Clin Sci (Lond) 2005; 109 (5):465-473.

4. Response to Antioxidant Therapy in Liver Ischemia-reperfusion Injury.
   Glantzounis GK, Davidson BR.
   Liver Transpl 2006; 12 (5): 892-893.

5. Pharmacological modulation of liver ischemia-reperfusion injury.
   Glantzounis GK, Mikhailidis DP, Seifalian AM, Davidson BR.
   Curr Pharm Des 2006; 12(23):2863-2865.

6. The role of thiols in ischemia-reperfusion injury.

   Glantzounis GK, Yang W, Koti R, Mikhailidis DP, Seifalian AM, Davidson BR.

   Curr Pharm Des 2006; 12(23): 2891-2901.

7. Formation and role of plasma s-nitrosothiols in liver ischemia-reperfusion injury.

   Glantzounis GK, Rocks SA, Salacinski HJ, Davidson BR, Winyard PG, Seifalian AM.

   Free Radic. Biol. Med. 2007; 42(6):882-892.

8. Femoral arterial line induces remote liver injury in a rabbit model of liver ischemia/reperfusion injury.

   Glantzounis GK, Sheth H, Thompson C, Kanoria S, Hafez TS, Davies S,

   Mikhailidis DP, Seifalian AM, Davidson BR.

   Surgery (submitted).

9. Changes in bile composition following hepatic warm ischemia / reperfusion: an experimental evaluation using proton magnetic resonance spectroscopy.

   Hafez TS, Glantzounis GK, Cox J, Sheth H, Yang W, Seifalian AM, Fuller B, Davidson BR.

   Clinical Science (submitted).

10. N-acetylcysteine reduces liver ischemia-reperfusion injury by modulation of nitric oxide metabolism and maintenance of mitochondrial activity.

    Glantzounis GK, Hafez TS, Sheth H, Pamecha V, Rocks SA, Winyard PG,

    Seifalian AM, Davidson BR

    Hepatology (submitted)

Abstracts

1. GLANTZOUNIS G, YANG W, KOTI R, SEIFALIAN A, DAVIDSON BR:
   N-Acetylcysteine modulates the late phase of liver ischemia-reperfusion injury.
   Biliary Association, Amsterdam, The Netherlands, May 27-30, 2001,
   HPB 2001, Vol. 3(1), P. 30.

2. GLANTZOUNIS GK, YANG W, SHETH H, SEIFALIAN AM, DAVIDSON BR:
   The effect of n-acetylcysteine on nitric oxide activity and   liver tissue
   oxygenation following warm ischemia-reperfusion injury. 4th Congress of the
   European Chapter of the International Hepato-Pancreato- Biliary Association,
   Istanbul, Turkey, May 2003, **HPB 2003, Suppl 1, Vol. 5, p. 57-58.**

3. GLANTZOUNIS GK, ROCKS SA, SHETH H, SALACINSKI H, DAVIDSON BR,
   WINYARD PG, SEIFALIAN IS. Direct measurement of nitrosothiols with electron
   paramagnetic spectroscopy in an experimental lobar liver ischemia-reperfusion
   model. European Congress of Free Radicals and Oxidative Stress, Ioannina, Greece,
   June 2003, **Free Radical Res 2003, Suppl 1, Vol. 37, p. 88.**

4. HAFEZ TS, GLANTZOUNIS GK, COX IJ, SHETH H, YANG W, SEIFALIAN
   AM, FULLER B, DAVIDSON BR. N-acetylcysteine and liver warm ischemia
   reperfusion injury: alterations to bile composition demonstrated by proton nuclear
   magnetic resonance spectroscopy. Brit J Surg 2003, Vol. 90 (5), p. 604.

5. HAFEZ TS, GLANTZOUNIS GK, COX IJ, SHETH H, YANG W, SEIFALIAN
   AM, FULLER B, DAVIDSON BR. N-acetylcysteine and liver ischemia reperfusion
   injury: alterations to bile composition demonstrated by proton nuclear magnetic
   resonance spectroscopy. 38th Annual Meeting of the European Association for the
   Study of the liver (EASL), Geneva July 2003. **J Hepatol 2003, Suppl. 2, Vol. 38, p.
   39.**

6. HAFEZ T, FUSAI G, GLANTZOUNIS G, YANG W, SHETH H, KANORIA S,
   PARKS HG, SEIFALIAN AM, DAVIDSON BR: N-acetylcysteine modulates bile
   composition following liver ischemia-reperfusion injury in an experimental model
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   BR, WINYARD PG, SEIFALIAN AM. Increased formation of nitrosothiols
   following warm liver ischaemia-reperfusion injury. British Society of
   Gastroenterology Annual Meeting, 21-24 March 2004, **GUT 2004, Suppl. 3, Vol.
   53, p. A92.**

8. GLANTZOUNIS GK, ROCKS SA, SHETH H, SALACINSKI H, DAVIDSON BR, WINYARD PG, SEIFALIAN AM. The role of S-nitrosothiols plasma levels as a marker of nitrosative stress in liver ischemia-reperfusion injury. 6th World Congress of the IHPBA, Washington DC, USA, 2-6 June 2004, **HPB 2004, Suppl 1, Vol. 6, p. 76.**

9. GLANTZOUNIS GK, SHETH H, YANG W, THOMPSON C, MIKHAILIDIS DP, SEIFALIAN AM, DAVIDSON BR. Limb ischaemia induces remote liver injury in a rabbit model of lobar liver ischemia / reperfusion. 6th World Congress of the IHPBA, Washington DC, USA, 2-6 June 2004, **HPB 2004, Suppl 1, Vol. 6, p. 81.**

10. HAFEZ T, FUSAI G, GLANTZOUNIS G, YANG W, SHETH H, KANORIA S, PARKS HG, SEIFALIAN AM, DAVIDSON BR: N-acetylcysteine modulates bile composition following liver ischemia-reperfusion injury in an experimental model with fatty liver. 6th World Congress of the IHPBA, Washington DC, USA, 2-6 June 2004, **HPB 2004, Suppl 1,Vol. 6, p. 123.**

11. GLANTZOUNIS GK, HAFEZ T, WANG WX, ROCKS S, SALACINSKI H, SHETH H, PARKES H, FULLER B, WINYARD P, SEIFALIAN A, DAVIDSON BR. Continuous infusion of N-acetylcysteine improves cellular energy production and decreases plasma S-nitrosothiols following liver ischemia-reperfusion injury. **Hepatology 2004, Suppl. 1, Vol. 40 (4), p. 378A-379A.**

12. GLANTZOUNIS GK, HAFEZ T, SHETH H, ROCKS S, FULLER B, WINYARD P, SEIFALIAN A, DAVIDSON BR. N-acetylcysteine reduces liver ischemia-reperfusion injury by modulation of nitric oxide metabolism and maintenance of mitochondrial activity. 7th World Congress of the IHPBA, Edinburgh, UK, 3-7 September 2006. **HPB 2006, Suppl 2, Vol 8, p. 164.**

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