

Therapeutic Modulation of Liver Ischaemia Reperfusion Injury

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

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Thesis Description

Chapter 1 is an overview of the pathophysiology of liver ischaemia reperfusion injury and the various molecular pathways involved in the manifestation of this injury.

Chapter 2 is a review of the various therapeutic modalities used to ameliorate liver ischaemia reperfusion injury with a special emphasis on antioxidant therapy and N-acetylcysteine

Chapter 3 reviews the role of glycine a nonessential amino acid and its application to liver ischaemia reperfusion.

Chapter 4 describes the various animal groups and materials and methods used in the various studies in this thesis.

Chapter 5 summarizes the experimental findings and effects of intravenous glycine on hepatic haemodynamics, liver microcirculation and portal blood flow following liver IRI.

Chapter 6 summarizes the experimental findings and effects of intravenous glycine on Cytokine expression and liver function tests following liver IRI

Chapter 7 describes the effect of intravenous glycine on bile flow and the application of proton nuclear magnetic resonance (¹HNMR) spectroscopy in analysing the changes in bile composition following liver IRI

Chapter 8 investigates the effect of prophylactically administered N-acetylcysteine on liver IRI in patients undergoing elective liver resection within a randomised controlled trial

Chapter 9 is a general discussion of the thesis and conclusions with plans for further research based upon the findings of this thesis

Abstract

Liver Ischaemia Reperfusion Injury (IRI) leads to production of reactive oxygen species and cytokines, which affects hepatocellular function following liver resection and transplantation.

This thesis examines 2 hypotheses:

- 1) The role of intravenous glycine in amelioration of liver IRI in a in vivo animal model of partial lobar liver IRI.
- 2) Does prophylactically administered N-acetylcysteine prevent liver IRI in patients undergoing elective liver resection.

Materials and Methods

- 1) A rabbit model of hepatic lobar IRI was used to evaluate glycine. 3 groups (n=6) Sham group (laparotomy alone), ischaemia reperfusion (I/R) group (1 hour ischaemia and 6 hours of reperfusion), and glycine I/R group (IV glycine 5 mg/kg prior to the I/R protocol) were used. Portal blood flow, bile flow and bile was analysed by ^1H NMR spectroscopy. Hepatic microcirculation, intracellular tissue oxygenation, serum $\text{TNF}\alpha$, IL-8, ALT, AST were measured at 1, 2, 4 and 6 hours following reperfusion.
- 2) A randomised double blind clinical trial was conducted to assess the effect of NAC on liver IRI following liver resections. The main outcomes were: morbidity and mortality, ICAM-1 expression in liver tissue, liver function tests. Patients were randomised to receive NAC as IV infusion (NACG) or a placebo group (PG) which received 5% dextrose only. Immunohistochemistry for ICAM-1 was carried out on perioperative liver biopsies.

Results

- 1) Glycine normalised the bile flow, reduced phosphatidylcholine shedding, lactate surge, and stimulated bile acid, pyruvate, glucose and acetoacetate release. Glycine improved portal blood flow, hepatic microcirculation by the 2nd hour, and hepatic intracellular tissue oxygenation by the 4th hour of reperfusion. Glycine ameliorated serum TNF α at 1, 2 and 4 hours and serum IL-8, AST and ALT up to 6 hours post reperfusion as compared to the I/R alone group.
- 2) Of the 43 patients, 15 received NAC, 16 were randomised to the PG, 12 were excluded due to inoperable tumours. Serum ALT was reduced in NACG (p=0.001), while serum ALP was higher in the NACG (p=0.003). ICAM-1 expression was up-regulated in 6/16 patients in the PG and in 3/15 patients in NACG. ICAM-1 was down-regulated in 1/15 patients in the NACG and none in the PG, the difference was not significant.

Conclusions

- 1) Glycine ameliorated liver IRI, improved bile flow and composition.
- 2) NAC ameliorated parenchymal liver injury and enhanced liver regeneration in patients undergoing elective liver resection.

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List of abbreviations

¹H NMR: Nuclear magnetic resonance

A

ADP: Adenosine diphosphate

ALT: alanine transferase

AP-1: activator protein 1

AST: aspartate transaminase

ATP: Adenosine Triphosphate

B

BAG-1: BCL2-associated athanogene

BCL-2: B-cell lymphoma 2

Bcl-xL: B-cell leukaemia XL

C

C3a: activated complement factor 3

C3b: complement factor 3 b

C5a: activated complement factor 5

CAM: Cellular adhesion molecule

cGMP: cyclic guanosine 3',5'-monophosphate

CINC: cytokine-induced neutrophil chemoattractant

Cl⁻ : chloride ion

COX: Cyclooxygenase

CRS: Carolina Rinse Solution

CytOx : Cytochrome oxidase

E

EC: Endothelial cell

ELISA: Enzyme linked immunosorbent assay

eNOS: endothelial nitric oxide synthase

ET-1: Endothelin-1

F

FK-506: Tacrolimus

G

GlyR: Glycine receptor

GSH: Glutathione

H

H₂O₂: Hydrogen peroxide

HO-1: Heme oxygenase-1

I

I/R: Ischaemia reperfusion

iC3b: inactivated complement factor 3b

ICAM-1: Inter Cellular Adhesion Molecule-1

IL-1: Interleukin-1

IL-10: Interleukin-10

IL-13: Interleukin-13
IL-6: Interleukin-6
IL-8: Interleukin-8
iNOS: inducible nitric oxide synthase
IPC: Ischaemic preconditioning
IRI: Ischaemia reperfusion injury
IκB: inhibitory protein Kappa B

J

JNK: Jun N terminal kinase

K

KC: Kupffer cell
KCN: potassium cyanide
KHB: Krebs-Henseliet buffer

L

LDF: Laser Doppler Flow
LFA: Lymphocyte function associated antigen-1
LPS: lipopolysaccharides

M

MAC: membrane attack complex
MAPKs: Mitogen activated protein kinases
MCP 1: Monocyte chemoattractant protein 1
MIP-2: Macrophage inflammatory protein 2
MnSOD: mitochondrial Superoxide dismutase
MPT: Mitochondrial permeability transition

N

NAC: N-acetylcysteine
NADPH: nicotinamide adenine dinucleotide phosphate
NFκβ : Nuclear factor kappa beta
NIR: Near infra red
NIRS: Near infra red spectroscopy
NO: Nitric oxide
NOS: Nitric oxide synthase
NOS-3: Nitric oxide synthase 3

O

O₂⁻: Superoxide anion radicals
ODFR: Oxygen derived free radical
OH[·]: hydroxyl radical
OLT: Orthotopic liver transplantation
ONOO⁻: peroxynitrite

P

PAF: Platelet activating factor
PC: Post ischaemic conditioning
PECAM-1: Platelet endothelial cell adhesion molecule-1
PGE₂: Prostaglandin E-2

PGI₂: Prostaglandin I-2
PGs: Prostaglandins
PMN: Polymorphonuclear lymphocyte/neutrophils
PNF: Primary non function

R

Redox: reduction/oxidation
RIPC: Remote ischaemic preconditioning
RISK: reperfusion injury salvage kinase
ROS: Reactive Oxygen species

S

SEC: Sinusoidal endothelial cell
SOD: Superoxide dismutase
STAT 3: signal transducer and activator of transcription 3
STAT 6: signal transducer and activator of transcription 6

T

TGF- β : transforming growth factor beta
TLR-4: Toll like receptor 4
TNF-receptor 1: Tumour necrosis factor receptor 1
TNF- α : Tumour necrosis factor alpha
TRAIL: TNF-related apoptosis inducing ligand

U

UW: University of Wisconsin

V

VCAM-1: Vascular endothelial cell adhesion molecule-1
VLA 4: Very late antigen 4

X

XO: Xanthine Oxidase

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Chapter 1

Ischaemia reperfusion injury in the liver: literature review and current concepts

1.1 Introduction

Restoration of blood flow to an ischemic organ is essential to prevent irreversible cellular injury. However, reperfusion itself augments tissue injury already caused by ischaemia alone(68;386), which if severe enough may result in systemic inflammatory response syndrome or multiple organ dysfunction syndrome(280). Cellular damage after reperfusion of previously viable ischaemic tissues is thus defined as ischaemia reperfusion injury (IRI)(68).

1.2 The clinical relevance of ischaemia reperfusion injury

IRI is a major determinant in affecting the outcome in a variety of human disease processes including cerebrovascular accidents (stroke), myocardial infarction, coronary artery bypass surgery, organ transplantation, liver resection, haemorrhagic shock with fluid resuscitation following major trauma, and limb revascularization (374). IRI is also associated with chronic rejection due to arteriosclerosis caused by the injury (118). It is also one of the important factors for nonfunction of microvascular flaps as well as necrosis of adipocutaneous and musculoskeletal flaps.

Acute organ failure following IRI is a well recognised complication in a number of organs including brain(43) ,heart(126), kidney(296), gastrointestinal tract(298), and liver(250).

In the liver this form of injury is a common sequel following complex liver resection and liver transplantation(32;168). In general, three types of ischaemic liver injury is clinically seen:

- 1) Cold ischaemia, seen in context of organ transplantation and graft preservation.
- 2) Warm ischaemia seen during liver transplantation, shock and liver resectional surgery

3) Rewarming ischaemia typically seen during and following graft implantation

Although liver transplantation provides effective therapy for most forms of acute and chronic liver failure, IRI, inherent to every transplantation is the main cause of both initial poor function (10%-30%) and primary non function(<5%) of the liver allograft.(66; 374). Organ shortage has led many centres to expand their criteria for acceptance of marginal donors like steatotic livers, small for size grafts, nonheart beating donors and aged donors (>70 years). Liver IRI is the primary factor affecting graft dysfunction seen in these marginal donors(56).

Liver resection is the gold standard for treatment of colorectal liver metastases(125;325). Newer chemotherapeutic agents have provided the option of curative liver resection in patients previously deemed unresectable(244). Thus increasing number of patients are now eligible for liver surgery despite advanced age, fatty liver disease, large tumours and preoperative chemotherapy.Hepatic steatosis affects 25% of the population and is associated with impaired microcirculation and poor liver function. Liver IRI in this group of patients further increases the morbidity associated with major liver surgery(65;66).

Liver IRI has been known to affect liver regeneration after hepatectomy(127). Following major liver resection or living related/ marginal graft liver transplantation, the ability of the liver to regenerate is crucial to maintain liver function. The failure of a partial liver to regenerate is considered a critical contributing factor in post surgical primary liver dysfunction and liver failure(66). Impaired liver regeneration and liver dysfunction has been directly linked to the extent and severity of liver IRI(65;66).

Thus limiting the extent of liver IRI may provide the key to make liver surgery safer especially for these high risk patients and improve the outcome of non heart beating, older and fatty donors in liver transplantation.

1.3 Pathophysiology of ischaemia reperfusion injury

Cellular effects of ischaemia are summarized in table 1.1.

The process of cell death by ischaemia has been studied for many years and various sequences of events that can lead to cell death have been suggested (320) (51). In aerobic cells the energy necessary to maintain cell integrity is supplied by the mitochondrial system through complete reduction of oxygen to water with the concomitant production of ATP through oxidative phosphorylation (236;285).

When oxygen supply to cells becomes insufficient by ischaemia or hypoxia, mitochondrial respiratory chain function alters because there is no final acceptor of electrons and the reduction/oxidation (redox) state of the mitochondrial enzymes becomes reduced(148). This causes inhibition of the mitochondrial ATP synthase with the subsequent reduction of oxidative phosphorylation(148). As a result a swift decrease of the stored energy-rich phosphates (ATP) occurs in the tissues as metabolic processes continue while oxidative phosphorylation ceases(266;351). Reduction of cellular ATP causes disturbances of membrane ion translocation by inhibition of the ATP-dependent sodium (Na^+)/potassium (K^+) ATPase, resulting in sodium influx and intracellular sodium accumulation with cell swelling and death(265).

Intracellular calcium accumulation is strongly implicated in the development of ischaemic injury and is thought to be a crucial step in the transition to irreversible damage(324;392). It occurs secondary to calcium release from the intracellular stores and inhibition of the ATP-dependent calcium pumps in the plasma membrane and

endoplasmic reticulum(324;392). The increased cytosolic calcium causes activation of cell membrane phospholipases resulting in phospholipid degradation and cell membrane disruption(28) (269). Calcium also activates tissue proteases such as xanthine dehydrogenase enhancing its conversion to xanthine oxidase (XO) which play a significant role in oxygen free radical production and reperfusion injury (83;184).

Table 1.1: Cellular effects of ischaemia

Altered Membrane Potential
Altered Ion Distribution (\uparrow intracellular Ca^{+2} / Na^{+2})
Cellular Swelling
Cytoskeletal Disorganization
\uparrow Hypoxanthine
\downarrow Adenosine 5 –Triphosphate (ATP)
\downarrow Phosphocreatinine
\downarrow Glutathione
Cellular Acidosis

Key: \uparrow =increased, \downarrow = decreased, Ca^{+2} = calcium, Na^{+2} = sodium

1.4 Phases of liver ischaemia reperfusion injury

I/R induced liver injury occurs in a biphasic manner (184). Which are as follows:

- 1) **Early Phase:** which occurs within 1-4 hours following reperfusion and is characterised by Kupffer cell(KC) and Polymorphonuclear leucocyte (PMN) activation(82), increased production of Nitric oxide(NO) by liver mitochondria and KCs (230;364), activation of complement cascade and production of C5a by proteolytic cleavage(185) and generation of ROS such as Superoxide (O_2^-),

hydrogen peroxide (H_2O_2) and/or hydroxyl radical (251;273). This early phase injury occurs in a PMN independent manner and is mediated by ROS generated upon reperfusion (114;184;273). Thus the early phase initiated the signal transduction processes which promotes neutrophil influx in the liver(71;265)

2) **Late Phase:** which occurs between 4-24 hours following reperfusion and is characterised by PMN influx into the post ischemic liver and organ injury (187;194;351). These neutrophils are primed and activated during the early phase (71;314). The activated neutrophils a) adhere to endothelial cells and hepatocytes and generate ROS and release proteases which damages endothelial and parenchymal cells; b) obstructs hepatic sinusoidal blood flow leading to hepatic hypoperfusion (no reflow) (187). The activated neutrophils induced cellular injury is mediated by :

- a) activation of NADPH oxidase(13;85)
- b) induction of NOS (54;55)
- c) release of products of arachidonic acid metabolism (i.e. leukotrienes, eicosanoids) and proteolytic enzymes (i.e. elastase, metalloproteinases)(414).

This initiates rapid oxygen uptake known as respiratory burst (13;414) in which superoxide anion radicals (O_2^-) and NO is produced(54). These react together to produce (H_2O_2) and peroxynitrite ($ONOO^-$) both of which are highly cytotoxic(20).

1.5 Microcirculatory failure in liver ischaemia reperfusion injury

The crucial role of microcirculatory impairment as a determinant of tissue viability after hepatic I/R has been investigated (208;340;394). Study of temporal and spatial microvascular changes with I/R using intravital microscopy showed two distinct

pathophysiologic mechanisms as key components of reperfusion injury. These mechanisms are primary sinusoidal perfusion failure “no reflow”(147) and “reflow paradox” (292;320).

In no reflow phenomenon, initially there is sinusoidal perfusion followed by progressive stasis and reduction of the sinusoidal flow (147). In reflow paradox phenomenon, reflow induces impairment of the microcirculation and tissue damage rather than improvement of the microcirculation and tissue function (402).

The severity of the sinusoidal perfusion failure is dependent on the ischaemia time(187). This sinusoidal impairment is paralleled with the changes in hepatic tissue oxygenation, hepatocellular integrity, and parenchymal function (340). This supports the major contribution of sinusoidal impairment to the reperfusion injury by increasing the hypoxic conditions during reperfusion(402).

Several mechanisms contribute to the no reflow phenomena, including narrowing of the sinusoid lumens by endothelial cell swelling(60) secondary to ischaemia-induced ATP deficiency and the consequent failure of ion transport through the cell membrane(187;291). A significant reduction of leukocyte velocity with subsequent stasis and intrasinusoidal plugging has been suggested as a hindrance for blood perfusion (404).

Apart from sinusoidal no reflow, reperfusion of microvessels is paradoxically associated with additional injury which is termed the “reflow paradox” (187;404). Reflow paradox is closely linked with the inflammatory response characterised by a network of intercellular reactions which includes leukocyte, endothelial cells, and tissue cells (37;250). The role of leukocyte has been confirmed by the observation that the degree of leukocyte infiltration of the reperfused tissue correlates with

postoperative liver function impairment and hepatocyte injury (187;194). Also, in neutropenic animals there is a substantial decrease in the severity of I/R injury (187). Along with these chain of events Kupffer cells (KC) and neutrophils are activated, which produce inflammatory cytokines (71) and reactive oxygen species(ROS) further aggravating hepatic injury(347).

1.6 Role of Kupffer cells, Neutrophils and Platelets in liver ischaemia reperfusion injury

1.6.1: Kupffer Cells

The pathogenesis of hepatic IRI, is initiated by activation of KC during ischaemia and initial stages of reperfusion (15;192;251;348). Activation of KC produces morphologic changes, which causes them to protrude into the sinusoids contributing to the reduction of blood flow within the sinusoidal lumen(47;249;348). Activated KC release proinflammatory ($\text{TNF}\alpha$, IL-1,IL-8, IL-6) and anti-inflammatory mediators (IL-10, IL-13) , as well as ROS which appears to play a pivotal role in the inflammatory response during hepatic IRI (72;192;249;389). Thus KC modulation could potentially attenuate or aggravate IR induced organ injury

1.6.2: Neutrophils

Neutrophils are activated by the ROS and proinflammatory mediators released during reperfusion, and activated neutrophils further augment the IR injury by release of ROS and several proteases(243;388).

Neutrophil recruitment during reperfusion and their adhesion to endothelial cells (EC) is mediated by the interaction between selectins and integrins expressed in the neutrophil membrane, and intercellular adhesion molecules (ICAM) expressed on EC.(388). I/R increases ICAM-1 expression in hepatic EC probably through $\text{TNF}\alpha$ and IL-1 synthesis which has been associated with acute liver rejection, while

neutralization of ICAM-1 reduces the severity of IRI(116;232;402). More recently T cells and NK-T cells have also been proposed to play an important role in hepatic IRI.(234). Resident lymphocytes within the liver can alter inflammation through the secretion of soluble mediators such as cytokines or through cognate interactions in an antigen dependent manner. Expression of these mediators will then result in the recruitment of more lymphocytes and neutrophils(45).

1.6.3: Platelets

Platelets adhere to the hepatic sinusoids and induce programmed EC death upon reperfusion of transplanted organs(353). Platelets also synthesize several factors (cytokines, TGF- β , serotonin, and calpain) which play an important role in liver I/R and regeneration (245). Platelet derived serotonin has been shown to promote tissue repair after normothermic hepatic ischaemia in mice(286). In human platelets , calpain activation is dependent on fibrinogen binding to integrin and subsequent platelet aggregation suggesting a role for this protease in regulation of post aggregation responses(333). Platelets also produce NO that leads to the production of peroxynitrite which is a potent inductor of programmed cell death in EC(150;340).

1.7 Role of cytokines in liver ischaemia reperfusion injury

Cytokines play an important role in liver IRI, both by initiating and sustaining the inflammatory response as well as modulating it's severity (351;388). Cytokines due to their ability to act in a autocrine, paracrine, and humoral manner not only initiates and maintains the inflammatory response (351), but also induce production of other inflammatory mediators like other cytokines , chemokines and eicosanoids(71;351) Tumour necrosis factor alpha (TNF α) and interleukin-1 (IL1), are two such cytokines which are produced by activated KC and most commonly implicated in hepatic IRI (72;188;369). Both these cytokines induce interleukin 8(IL8) synthesis

(383;384). IL8 is a CXC chemokine and a potent inducer of neutrophil chemotaxis and activation in vitro and in vivo(157;388). These cytokines and chemokines upregulate cellular adhesion molecules like

- 1) Selectins (P-selectin, E-selectin, L-selectin).
- 2) Integrins (MAC 1, LFA 1, VLA 4).
- 3) Immunoglobulins (ICAM-1, VCAM-1, PECAM-1).

Thus initiating the adhesion molecule cascade leading to PMN recruitment into reperfused tissues by margination, rolling, firm adhesion, diapedesis and migration into the extravascular tissue (311;351;388).

1.8 Role of adhesion molecules in liver ischaemia reperfusion injury

Neutrophils are involved in both phases of liver IRI. Neutrophil recruitment is a multistep process which involves initial contact and adhesion with the endothelium, transendothelial migration and subsequent parenchymal cell adherence and damage(70). After IRI cellular adhesion molecules (CAM) are activated and/or upregulated on the surface of neutrophils and SEC(351). The inflammatory response is achieved via sequential steps which include the activation of leukocytes by cytokines(72;311;388), and increased adhesion molecules expression(71;403). The increased adhesion molecules expression results in rolling of the leukocytes on the sinusoidal lining cells(59;311), leukocyte adhesion to the sinusoidal cell lining via intercellular adhesion molecule-1 (ICAM-1)(311;403) , and leukocyte extravasation and tissue infiltration via the interaction of integrin matrix receptors and extracellular matrix molecules such as collagen and laminin (25;261).

Figure 1.1 Role of ICAM-1 in PMN capture and transendothelial migration

In normal liver tissue, low constitutive expression of ICAM-1 can be found on the hepatic endothelium and on KC. Although ICAM-1 is not detectable in hepatocytes, it is upregulated by cytokines on virtually all liver cells(379). Intravital microscopic studies have demonstrated leukocyte adhesion to endothelial cells of both sinusoids and postsinusoidal venules after warm (292) and cold ischaemia and reperfusion(327). It has also been shown that leukocyte adhesion in postsinusoidal venules, but not in sinusoids, is mediated by ICAM-1 and that ICAM-1- mediated leukocyte adherence is responsible for the manifestation of excretory dysfunction and hepatocyte damage(403).

Pre-treatment with monoclonal antibodies to ICAM-1, lymphocyte function associated antigen-1 and CD 18 before induction of rat lobar I/R resulted in a reduced leukocyte infiltration and lipid peroxide level and enhanced the recovery of hepatic ATP with increased survival rate(261).

1.9 Role of reactive oxygen species in liver ischaemia reperfusion injury

Aerobic metabolism releases ROS which under normal circumstances are neutralized by diverse antioxidant mechanisms(389). Under stress the balance between ROS and antioxidants shifts towards ROS leading to oxidative stress and cytotoxicity,(144;400). There is large body of evidence for the role of oxygen free radicals as a mediator of the microcirculatory and parenchymal cell injury associated with I/R injury(144). Activated KC have been identified as the major source of ROS formation in the early phase of IRI (33;184). Within the liver, the cytotoxic effects of ROS translate into nitrosylation of iron-sulfur groups and tyrosine residues, inactivation of the heme group and lipid peroxidation, (144;340). ROS can be potentially inhibited by antioxidant agents and therefore several studies have focused in modulating the severity of IR injury with various pharmacologic agents including allopurinol(231), N-acetylcysteine (134;145), α -tocopherol(260), enzymatic superoxide dismutase (SOD)(269) and catalase(144;269).

Endogenous antioxidant levels decrease significantly during reperfusion, therefore the administration of exogenous antioxidants particularly in the early stages of reperfusion could significantly decrease the severity of IR injury(206;260) .

Table 1.2: The role of reactive oxygen species in ischaemia reperfusion injury

Lipid Peroxidation
Activation of proteases
↑ TNF- α , PAF & IL-1
Activation of NF κ β & AP-1
↑Expression of adhesion molecules
Microcirculatory failure
Apoptosis

Key: ↑= increased, TNF- α = tumour necrosis factor alpha, IL-1= interleukin 1
PAF= platelet activating factor, NF κ β = nuclear factor Kappa beta, AP-1 = activator protein 1

1.10 Role of nitric oxide and nitric oxide synthase in liver ischaemia reperfusion injury

Nitric oxide (NO) a potent biologic mediator is the end product of the enzyme NOS (nitric oxide synthase)(377). Since the identification of NO as the endothelium derived relaxation factor in 1987 (297) a number of physiological actions have been attributed to it which include the following ;

- 1) Decreased inflammatory cell and platelet activity(63;321)
- 2) Decreased expression of cytokines and adhesion molecules(86;252) and
- 3) NO also acts as a free radical via its conversion to peroxynitrite (ONOO⁻)(307).

NO is synthesized via oxidation of arginine by NOS and can be formed in liver by hepatocytes, KCs, and endothelial cells (377). There are two major isoforms of NOS in the liver ; a)endothelial NOS (eNOS) or constitutive NOS and b) inducible NOS (iNOS)(377). eNOS is expressed constitutively and it's activity is dependent on Ca⁺² and calmodulin(398), while iNOS is expressed by macrophages (including KCs) and

neutrophils in the liver. iNOS is upregulated by extracellular stimuli like cytokines and lipopolysaccharides (LPS), leading to production of much higher levels of NO as compared to eNOS alone(377). These inflammatory mediators (i.e. cytokines and LPS), activate NF κ β, which plays a pivotal role in expressional regulation of iNOS gene (173). iNOS activity is independent of intracellular calcium levels and produces NO at magnitudes several orders greater than eNOS (138;217).

During Ischaemia intracellular levels of NADPH and oxygen fall leading to decreased levels of NO, since NADPH and oxygen are essential for NOS activity(365). Upon reperfusion excessive Ca⁺² entry into endothelial cells activates eNOS leading to a burst in NO production which is transient due to functional impairment of endothelial cells secondary to the hypoxic insult(240;307). Also in the immediate reperfusion period a large amount of arginase is released which results in depletion of L-arginine(346). iNOS induction requires several hours and iNOS activity is required for dose dependant production of NO which has been shown previously in animal models of hepatic IRI using different doses of L-arginine (290). This dose dependant production of NO was observed only after 6 hours (290).

However, the role of NO produced during reperfusion is complex and controversial. Administration of both selective and nonselective inhibitors of NOS results in marked aggravation of post ischemic liver injury(77;222). While increasing NO availability by using NO precursor (e.g. L-arginine) or NO donor (e.g. FK409) markedly decreases reperfusion injury (222;290;345). On the contrary use of S-nitroso N acetyl penicillamine to generate high concentration of NO in isolated perfused rat liver results in functional and morphological impairment of liver sinusoids (87). In hepatocyte cell cultures NO has been shown to stimulate

apoptosis(407), while in a IRI model using isolated perfused hepatocytes NO was shown to attenuate injury at low doses but aggravate it at high doses (115).

Thus the effects of NO can be likened to a double-edged sword, wherein low concentrations of NO (generated by eNOS activation) are involved in the regulation of vascular tone and are beneficial to the outcome of IRI, while high levels of NO (generated by iNOS activation) are involved in inflammatory processes and are detrimental in IRI.

1.11 Role of complement in liver ischaemia reperfusion injury

The complement system is an integral part of the body's humoral defence mechanism and also a primary mediator of inflammatory processes(10;128). The complement system consists of a number of proteins, which circulate in the plasma in an inactive form and are activated by proteolytic cleavage (10;128). The classical or the alternative pathway can activate this system. In hepatic IRI complement cascade is activated by both the classical and the alternative pathways by the pathophysiologic events of IRI (16).

Complement activation results in three major effects:

- 1) Generation of opsonins (C3b,iC3b) which promotes binding and phagocytosis of microorganisms and particles by phagocytic cells via their complement receptors (10;128).
- 2) Generation of anaphylatoxins (C3a and C5a) which are proinflammatory mediators and activate endothelial cells, neutrophils, macrophages (KC), and mast cells(61;128;185;390). C3a and C5a also contribute to neutrophil chemotaxis, and increased vascular permeability within the liver (193;303). Activated endothelial cells lead to fibrin deposition, platelet aggregation and adhesion of neutrophils to the endothelium(61;193). These complement related

pathophysiologic events lead to failure of hepatic microcirculation which is an important determinant of postischemic liver function(270;385;402).

- 3) Formation of membrane attack complex (MAC) on cell surfaces (factors 5b-9) which
 - a) can directly induce target cell lysis (185;195).
 - b) activates endothelial nuclear factor kappa beta ($\text{NF}\kappa\beta$) to increase leucocyte adhesion molecule transcription and expression (i.e. ICAM 1, VCAM 1, P selectin) (69).
 - c) promote leucocyte activation and chemotaxis by induction of IL8 and monocyte chemoattractant protein 1 (MCP1) (69).
 - d) MAC also alters vascular tone by inhibition of endothelium dependant relaxation and decreased endothelial cyclic GMP (69).

Thus although complement activation occurs during the early phase of IRI, it also contributes to the later neutrophil mediated injury during the late phase of IRI.

1.12 Role of molecular mechanisms in liver ischaemia reperfusion injury

When the liver suffers an ischaemic insult, the alterations induced by the oxidative stress can exceed the compensatory capacity of the liver, resulting in cell death. The ischaemic event can reprogram gene expression of the surviving cells, initiating cellular mechanisms that allows them to regenerate and remodel(65;114). One of the key transcription factors involved in mediating liver regeneration following IRI is nuclear factor kappa B($\text{NF-}\kappa\text{B}$)(393;426).

$\text{NF-}\kappa\text{B}$ is normally found in the cytoplasm attached to the inhibitory protein I κB , which is degraded during oxidative stress allowing the translocation of $\text{NF-}\kappa\text{B}$ to the nucleus(426). When activated $\text{NF-}\kappa\text{B}$ induces the synthesis of iNOS, cytokines (TNF- α), chemokines and adhesion molecules (ICAM-1) (140;426). The key

mechanism for NF- κ B activation is by ROS production particularly H₂O₂(246). ROS production is a direct consequence of the IRI and via increased TNF- α production immediately following liver IRI(33;184;251). In normal hepatocyte, glutathione and other thiols dissipate excess ROS(117). ROS also activate of NF- κ B, which acts as initiator of liver regeneration. Exhaustion of these intracellular defence mechanism results in excess ROS which then initiates mitochondrial dysfunction and activation of caspases leading to cell death(117).

Antioxidant administration reduces NF- κ B activation by:

- 1) Mopping up excess ROS
- 2) By acting as an anti-inflammatory agent during the late phase of IRI (370).

Other genes that may participate in IRI include those of ET-1, NOS-3, heme-oxygenase and those of the heat stress factor proteins and ROS have been documented to either activate or modulate all these cell pathways(3).

1.13 Apoptosis and necrosis in liver ischaemia reperfusion injury

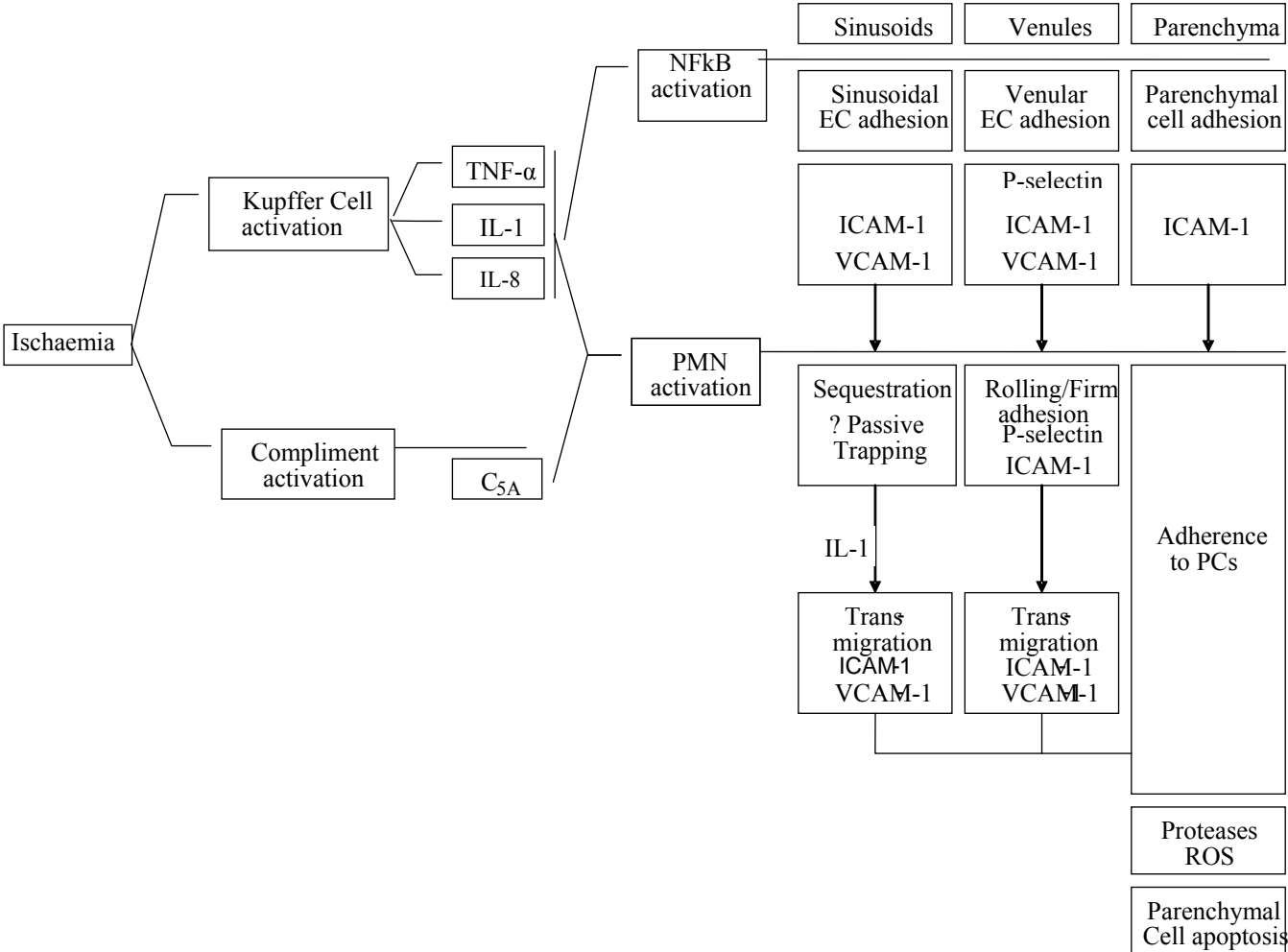
During reperfusion, TNF- α , ROS, and other mediators activate many of the proteins involved in apoptosis such as caspase-3 and caspase-8 along with release of mitochondrial cytochrome C to the cytoplasm which initiates the cascade of events culminating in DNA destruction and cell death(340). However, it is still not clear whether apoptosis or massive necrosis is the final event, which predominates cell death following IRI. Lemasters *et al*(241) have proposed the theory of necroapoptosis suggesting that both cell death mechanisms occur simultaneously during ischaemia and overlap during reperfusion. The ischaemic stimulus can culminate in necrosis or apoptosis, depending on the interaction of other factors like significant reductions in cellular ATP levels or the fat content of the liver(264;339)

Hence, variety of mediators and signals are involved in the pathogenesis of hepatic IRI, which can compensate for the absence of one mediator. Therapeutic strategies to

ameliorate IRI in liver and improve outcomes have recently been summarized by Clavien and colleagues(66).

The pathophysiologic processes involved in liver IRI is illustrated in figure 1.2. It would appear that ROS and KC activation are the two primary events in liver IRI and modulating these early events would ameliorate this injury.

Figure 1.2: Pathophysiology of hepatic ischaemia reperfusion injury



Chapter 2

Therapeutic modulation of liver ischaemia reperfusion injury with emphasis on antioxidants

IRI is a complex pathophysiological process involving multiple mediators which act in an autocrine and paracrine manner to augment the original injury. This cascade of proinflammatory events induces both acute functional and structural changes in the organ leading to organ dysfunction and failure(32;66). Thus attenuation of IRI by strategies targeting various mediators and cell populations at different levels of the cascade may be helpful in improving both short and long term organ function.

2.1: Cytokine modulation to reduce liver ischaemia reperfusion injury

Hepatic IRI is inevitable in surgical procedures for liver trauma and hepatectomy as well as liver transplantation. Cytokines are pivotal factors in neutrophil-mediated liver injury following liver IRI, while various other mediators are involved in this insult. Inflammatory cytokines such as TNF- α are associated with the induction of cellular adhesion molecules and hepatic microcirculatory impairment based on neutrophil-vascular endothelial cell interaction(71). Members of the chemokine family such as IL-8, CINC, MIP-2, and MCP-1 are involved in neutrophil infiltration in the liver and remote organs(314).The roles of various cytokines involved in liver IRI is summarized in table 2.1. Lentsch et al have recently reviewed the role of cytokines in hepatic IRI(178).

2.1.1: TNF- α

TNF- α is a powerful proinflammatory mediator which plays a central role in initiation and perpetuation of the inflammatory cascade following liver IRI(247). It also stimulates signal transduction pathways leading to increased gene expression for secondary inflammatory mediators(247). TNF- α is mainly produced by activated KCs during early reperfusion and blockade of this mediator has been shown to ameliorate liver IRI, not only by reducing the production of secondary mediators but

also, by preventing the expression of VCAM and expression of CXC chemokines which are chemotactic for neutrophils(70;72;351).

2.1.2 Chemokines (IL-8 & MCP-1):

Chemokines (IL-8 & MCP-1) are small peptide mediators whose names are derived from their function of promoting directed chemotaxis in nearby responsive cells. There are currently 4 branches of the chemokine family which include CXC,CC,CX₃C and C(351). Due to the extensive functional overlap between the various chemokines blockade of any one of these mediators does not completely ameliorate IRI. However, studies have shown that chemokine blockade leads to reduced neutrophil accumulation and subsequent cellular injury(243). The receptors for these mediators are expressed on neutrophils and they mediate the chemotactic response of these cells towards their ligands. These present a more attractive target since only two of these receptors are expressed on neutrophils(71).

2.1.3: Adhesion molecules

Adhesion to vascular endothelium occurs early during reperfusion and is responsible for activation and sequestration of neutrophils. This is mediated by adhesion molecules which are expressed on both neutrophils and endothelial cells(389). The three main classes of adhesion molecules are selectins, integrins, and immunoglobulin like adhesion molecules. Selectins are expressed on both neutrophils and EC and they capture circulating leucocytes to bring them into close proximity to the endothelial cells. There are 3 members of this family i) E-selectin which are expressed on EC, ii) L-selectins which are expressed on leucocytes and iii) P-selectin which are expressed on platelets and EC. Multiple studies have utilised small molecule inhibitors of selectin function to target these interactions and have attenuated neutrophils accumulation and liver injury(59). Integrins are expressed on leucocytes and they bind to the immunoglobulin like adhesion molecules which are

expressed on EC. The binding of these two adhesion molecules promotes transendothelial migration of leucocytes into the tissue(68). In the liver two immunoglobulin family members i.e. intercellular adhesion molecule (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) control the majority of neutrophil adhesion and transmigration blockade of which dramatically reduces neutrophil accumulation and injury(116;232;389).

Table 2.1: Summary of the role of cytokines in ischaemia-reperfusion injury

Cytokine	Function related to ischaemia reperfusion injury
TNF- α	Acts on endothelial cells to increase surface adhesion molecules, activates the coagulation cascade and Kupffer cells, activates leucocytes infiltrating the ischaemic area
IL-1	Mediates local inflammatory response by activation of leucocytes, induces synthesis of acute phase reactants, acts on endothelial cells to increase surface adhesion molecules
IL-8	Promotes invasion of leucocytes into ischaemic areas, acts as activator of neutrophils
IL-6	Stimulates production of IL-1 and TNF- α antagonists, induces liver synthesis of acute phase proteins like fibrinogen
PAF	Mediates vasoconstriction, induces platelet aggregation, promotes neutrophil adherence to endothelium, stimulates production of ROS, leads to aggregation and degranulation of neutrophils
TGF- β	Proapoptotic properties and involvement in SEC apoptosis

Key: TNF- α = Tumour necrosis factor alpha, IL-1 = interleukin 1, IL-6 interleukin 6, IL-8= interleukin 8, PAF = platelet activating factor, TGF- β = tumour growth factor beta

2.2: Prostaglandin therapy in modulating liver ischaemia reperfusion injury

Prostaglandins (PGs) are biologically active polyunsaturated fatty acids derived primarily from arachidonic acid, via the cyclooxygenase (COX) or lipoxygenase pathways to form either prostaglandins and thromboxane or leukotrienes(310). The cyclooxygenase pathway produces PGs (D, E and F), thromboxane and prostacyclin.

The lipooxygenase pathway is active in leucocytes and macrophages synthesizing leukotrienes. They are short lived hormone like chemicals which regulate cellular activities on a moment to moment basis and liver is the major organ involved in synthesis, degradation and elimination of these by-products of the arachidonic acid pathway (300).

During the last decade, substantial evidence has accumulated on the cytoprotective effects of PGs on liver IRI , which has been extensively documented in various reviews (174;300;310).

PGs have been shown to protect against liver IRI by

- 1) Increasing liver perfusion and inhibiting leucocyte & platelet aggregation by downregulating intercellular adhesion molecules (300)
- 2) Protecting SECs from apoptosis (430)
- 3) Suppressing thromboxane A₂ and increasing PGI₂ production (156)
- 4) Inhibiting Ca⁺² –calpain –mu- mediated IR injury (216)
- 5) Ameliorating protease release and ODFR generation from activated leucocytes with PGI₂ (310)
- 6) KC activation leads to synthesis of TNF- α which induces PGE₂ production, which subsequently inhibits TNF- α synthesis (229)
- 7) Intra-arterial infusion of PGE₁ has been shown to protect against liver IRI both experimentally and clinically to prevent hepatic failure following orthotopic liver transplantation (OLT) (9;175)
- 8) PGI₂ has been shown to reduce IRI following OLT by improving hepatic splanchnic oxygenation (281)
- 9) Treatment of donor liver with PGI₂ increased graft viability in living related liver transplantation both experimentally and clinically (227;342)

10) PGs also augment liver regeneration directly by protecting hepatocytes and indirectly by expressing critical factors needed for cell proliferation(26;41;181)

Although PGs have been shown to have multiple beneficial effects in both experimental and clinical scenarios, there are still questions remaining regarding its optimal dose, administration routes and, timing and because of its very short half life and metabolism by the lungs. The other important factor being the action of PGs is downstream following the initial injury hence on its own they would not completely ameliorate IRI.

2.3: Gene therapy in modulating liver ischaemia reperfusion injury

The cellular mechanisms of liver IRI are complex and multimodal and exploring genes to target specific events in this complex pathway may be a viable therapeutic strategy. Recent experimental studies using gene transfer methods have provided encouraging results in ameliorating liver IRI. Studies using gene therapy targeting specific genes in liver IRI are summarised in table 2.2.

2.3.1: Antiapoptotic Genes (BCL-2/BCL-XL and BAG-1)

The proto-oncogene Bcl-2 and Bcl-2 related gene Bcl-xL which act by blocking apoptosis and necrosis are potent regulators of programmed cell death and a key target for therapeutic manipulation (228). Overexpression of Ad-mediated Bcl-2 gene significantly reduces hepatocytes apoptosis, improves hepatic function and prolongs survival(29). Similar results have been reported by Swaitzki et al who demonstrated the cytoprotective effect of Ad-mediated Bag-1 gene transfer in rat liver IRI(322).

2.3.2: Antioxidant Genes: SOD and HO-1, Ferritin

Early activation of NF κ B and AP1, following liver IR leads to early transcriptional activation of genes which encode inflammatory cytokines and acute cellular responses further augmenting the extent of hepatic IRI(443). Gene transfer of Ad-

Cu/Zn-SOD (cytosolic superoxide dismutase) and Ad-MnSOD (mitochondrial superoxide dismutase) reduced serum transaminases and preserved histological features suggesting that reduced cytokine and chemokine production is dependent on redox-sensitive transcription factor NFκB(419;443). Heme oxygenase 1 (HO-1) is a stress responsive protein which has been shown to exhibit cytoprotective effects after liver IRI. Upregulation of HO-1 gene overexpression correlated with preserved hepatic architecture, improved LFT's, reduced T cell and macrophage infiltration and increased graft survival(6;208;394). Similarly ferritin induction due to action of HO-1 on the heme porphyrin ring causes release of Fe⁺² which plays a role in formation of OH⁻ and OH through the Fenton reaction. Ad vector carrying the ferritin heavy chain (H-Ferritin) gene has been shown to protect rat livers from IRI by inhibition of endothelial cell and hepatocyte apoptosis(27).

2.3.3: Immunoregulatory cytokines: IL-10 and IL-13

Studies have shown correlation of overexpression of IL-13 which regulates the liver IRI inflammatory response via the signal transducer and activator of transcription 6 (STAT 6) pathway while IL-10 induces HO-1 gene expression(113;207). Ad-IL-13 gene transfer has shown cytoprotective effects in both warm and cold Ischaemia models, with improved hepatic function, reduced neutrophil aggregation, and prevention of hepatic apoptosis

2.3.4: IL-1 Receptor antagonist:

IL-1 is a proinflammatory cytokine which plays a central role in liver IRI (351). Gene transfer of Ad-mediated IL-1 receptor antagonist improved hepatic function and animal survival following partial hepatic IRI with amelioration of TNF-α, IL-1 and IL-6 (158).

2.3.5: Transcription factor: NF- κ B

Activation of NF- κ B by ROS following IRI leads to overexpression of proinflammatory cytokines further augmenting the IR injury. Thus inhibition of NF- κ B activation by redox gene therapy have shown beneficial effects against apoptosis and protection against proinflammatory cytokines (443).

2.3.6: T-cell costimulation blockade: CD40-CD154

CD4⁺ T lymphocytes mediate key cellular events in liver IRI and the CD40-CD154 costimulation provides the essential second signal in the initiation and maintenance of T cell dependent immune responses (209). Blockade of the CD40-CD154 interaction by pre-treatment of liver isografts with Ad-CD401 gene significantly attenuated the inflammatory response following liver IRI with prevention of apoptosis and preservation of liver architecture (209).

2.3.7: RNA interference

RNA interference involves injection of small interfering RNA duplexes to downregulate gene expression like the genes involved in hepatic IRI providing a promising therapeutic modality (359).

Thus targeting specific genes to protect against IRI is promising; however current laboratory protocols need to be modified prior to application in the clinical setting which might be possible with further advances in gene therapy technology.

2.4: Role of antiapoptotic molecules in liver ischaemia reperfusion injury

Liver IRI triggers a cascade of cellular events leading to programmed cell death, thus inhibition of these proapoptotic pathways may provide a viable therapeutic alternative. Apoptosis can occur via a) extrinsic pathway or death receptor signalling and b) the mitochondrial pathway or the intrinsic pathway(151;433). Death receptor mediated apoptosis is in turn mediated by Type 1 and type 2 cells. Apoptosis

induction in Type 1 cells leads to activation of large amounts of caspase 8 (initiator caspase) which directly activates caspase 3 (main executioner caspase), while type 2 cells initially activate only very small amounts of caspase-8(433).

At the cellular level death receptors (Fas, TNF-receptor 1, and TNF related apoptosis inducing ligand; TRAIL receptors 1&2), which belong to the TNF superfamily and induce apoptosis directly are the first potential targets for antiapoptotic strategies against liver IRI.(433). There are few studies which have studied impact of Fas signalling in hepatic IRI and inhibition of Fas signalling has not been of conclusive benefit in warm hepatic IRI(319). Contribution of TNF-R1 signalling in the cold hepatic IRI is not established, while in warm liver IRI lack of TNF-R1 has been shown to improve survival, reduce apoptosis and decrease liver injury(319). The impact of TRAIL induced apoptosis in liver IRI has not been investigated.

The major form of apoptosis seen in vertebrate cells proceeds via the intrinsic or the mitochondrial pathway which is initiated by various second messengers like ROS, Ca^{+2} , stress kinases, ceramides and proapoptotic Bcl-2 members leading to permeabilization of mitochondrial membranes and release of nuclease and protease activators(435). Thus strategies to prevent cell death in liver IRI might include inhibition of mitochondrial membrane permeabilization or events downstream of mitochondria. Modulation of expression of Bcl-2 proteins as a strategy to protect against hepatic IRI has been shown to be effective in several studies involving warm liver IRI (29;302;338) , while similar studies in context of cold preservation injury, investigating the direct impact of Bcl-2 family are still missing. However overexpression of Bag-1 which can stabilise Bcl-2 has been shown to reduce liver injury and improve survival (322).

Inhibition of mitochondrial membrane permeabilization by a number of agents have been shown to reduce caspase 3 activation and ameliorate liver injury (171;213;271).

Similarly inhibition of caspases by pharmacological means has been shown to be effective in preventing both cold and warm liver IRI(139). However, caspase inhibition alone does not completely protect against liver IRI since direct cell lysis is independent of intracellular mediators and also due to presence of mediators which can lead to apoptosis independent of caspases (139). Calpains are cytosolic cysteine proteases which require Ca^{+2} for their activity have been investigated in cold and warm liver IR. Inhibition of calpains significantly protected livers from cold and rewarming IRI (223;352). Cathepsins B and D are lysosomal proteases which are released by stimulation of TNF-R1 and activation of caspase -8 and which in turn activate a lysosomal pathway of apoptosis (153). Increased release of Cathepsin B and D following reperfusion after 24 hours of cold storage was associated with reduction in lysosomal integrity in fatty but not lean livers and increased markers of apoptosis (18). Genetic inactivation of cathepsin B or application of specific inhibitor ameliorated signs of hepatocellular apoptosis and necrosis (18).

Recent experimental evidence has shown reversible blockade of p53 dependent transcriptional activation and apoptosis, by Pifithrin- α , a specific p53 inhibitor in a isolated perfused rat liver after 24-48 hours of cold storage with significant reduction of apoptosis and SEC detachment (103).Mitogen activated protein kinases (MAPKs) are a family of proteins involved in regulating cellular activities from gene expression to programmed cell death. Application of stress kinase inhibitor SB202190 abolished JNK (a MAPK) activation and completely inhibited caspase 3 activation (78). Similar results have been reported with use of diltiazem and FK-506 (79;80) however, due to the additional properties of these drugs these experiments do not confirm the role of JNK activation in hepatic IRI.

Thus even though a number of substances inhibiting pathways of programmed cell death have been investigated and found to be beneficial in both warm and cold liver

IRI. However, none of these substances have been used clinically. This might be because these substances act downstream from the initial stimulus which initiates IRI and also other strategies like IPC and RIPC are more easily applicable particularly in the setting of liver resectional surgery.

Table 2.2: Summary of gene therapy using specific target genes in hepatic ischaemia reperfusion injury

Target Gene	Vector	Treatment	Animal	Authors
Anti-oxidant SOD	Adenovirus	Donor	Mice	Zwacka et al;1998
Anti-oxidant SOD	Adenovirus	Donor	Rats	Wheeler et al; 2001
Anti-oxidant HO-1	Adenovirus	Donor	Rats	Amersi et al 1999
Anti-oxidant HO-1	Adenovirus	Donor	Rats	Coito et al;2002
Anti-apoptotic Bcl-2	Adenovirus	Donor	Rats	Bilbao et al, 1999;
Anti-apoptotic Bag-1	Adenovirus	Donor	Rats	Sawitzki et al 2002
Cytokines, IL-13	Adenovirus	Donor	Rats/Mice	Ke B et al 2003,2004
Cytokine antagonists, IL-1R antagonist	Adenovirus/Cationic liposomes	Donor	Rats	Harada et al 2002
Protective, ferritin	Adenovirus	Donor	Rats	Berberat et al,2003
Costimulatory blockade, CD40Ig	Adenovirus	Donor	Rats	Ke B <i>et al</i> ;2004
Transcription factor, NFκB	Adenovirus	Donor	Rats	Takahashi <i>et al</i> ;2001
TLR-4 & HO-1 signalling	Adenovirus	Donor	Wild type knockout mice	Ke B <i>et al</i> ; 2007

Key: SOD = Superoxide dismutase, HO-1 = hemeoxygenase 1, Bcl-2 = B cell lymphoma 2, Bag-1= Bcl-2 associated anthogene, IL-13 = interleukin 13, IL-1R = interleukin 1 receptor, NFκB = nuclear factor kappa beta, TLR-4 = toll like receptor 4, CD40 Ig = CD40 immunoglobulin,

2.5: Surgical strategies to modulate liver ischaemia reperfusion injury

Surgical resection of liver tumours with disease restricted to the liver or in selected cases with limited extrahepatic disease offers a curative option with improved survival in conjunction with chemotherapy(66).Pringle's maneuver and total vascular exclusion are used to minimize blood loss and need for transfusion intraoperatively(226;305). Both techniques, however result in inevitable ischaemic injury to the liver which may subsequently impair regeneration after major hepatectomy(22;23). I/R injury also occurs due to intraoperative mobilisation and compression of the liver due to retraction(327;331). Liver transplantation either from a deceased/living donor is the standard of care for selected patients with end stage liver dysfunction. However, the inherent IRI during organ harvest, storage and reimplantation is a significant cause of morbidity and mortality in these patients (65;66;331).

3 surgical strategies have been proposed to attempt to reduce liver IRI.

2.5.1: Ischaemic Preconditioning

This consists of one or more brief episodes of Ischaemia followed by a short interval of reperfusion, before a prolonged period of ischaemia. Number of extracellular mediators like adenosine, nitric oxide (NO), bradykinin and intracellular messengers such as p38 MAPK, Ik Kinase; signal transducer and activator of transcription-3(STAT-3) and transcription factors including NFκB and heat shock transcription factor 1 have all been implied in the protective mechanisms for this strategy(52;340).The benefits of ischaemic preconditioning (IP) on energy metabolism, inflammatory mediators including ROS and TNF-α, mitochondrial dysfunction , KC activation and microcirculatory dysfunction associated with liver IRI have been described(17;142;212;344). IP has been successfully applied in human

liver resection and transplantation(12;17;58;67;199;224;288). These studies demonstrate that IP decreases the extent of IRI in both major liver resection and transplantation, however clinically significant benefit has yet to be demonstrated from the use of donor IP in clinical liver transplantation. This may be due to multiple uncontrolled variables during donor organ procurement, preservation, recipient factors, as well as implantation process and postoperative management. Also these studies lack sufficient power to demonstrate survival advantages due to intergroup variations and technical factors like the time used. IP which is derived from animal studies may not be long enough to confer significant protection and improve survival. The use of IP in solid organ transplantation and indeed in major liver surgery has other limitations including the potential for vascular injury and worsening post transplant function. The other major limitation is inability to apply this technique to non heart beating organ donors, where the patient sustains cardiopulmonary arrest before organ procurement.

2.5.2: Postconditioning

Post conditioning (PC) is defined as repetitive brief cycles of reperfusion performed at the onset of reperfusion(439). The timing of postconditioning is critical (at the initiation of reperfusion) to attain optimal protection from IRI(215). The protective effect has been proposed to be via recruitment of survival kinases which are also known as reperfusion injury salvage kinases or RISK pathway(164;442). The use of PC has not yet made its way into the transplant setting but it outlines an interesting strategy which may be more beneficial to the transplanted organ than IP because of the oxidative burst at onset of reperfusion. Also this strategy may be useful to all transplants, irrespective of the organ procurement technique since it is applied just prior to reperfusion during implantation.

2.5.3: Remote ischaemic preconditioning

RIPC is a novel method of protecting the target tissue and may be defined as the induction of nonlethal stress by brief ischaemia to an organ distant from the organ undergoing IRI resulting in systemic and local tolerance to subsequent IRI(205;374). RIPC stimulus induces release of biochemical messengers which act either by bloodstream or via neurogenic pathways resulting in reduced oxidative stress and preservation of mitochondrial function. Numerous mechanisms including endothelial NO, ROS, Kinases, catecholamines and ATP channels have been proposed(205). Originally described in the canine heart, it's protective effect was subsequently confirmed in various organ systems(204-206;373). The main advantage of this strategy is that it can be readily applied during organ procurement and implantation with minimal or no adverse effects on the target organ. Further clinical trials are awaited to assess the effectiveness of this strategy.

2.6: Role of reactive oxygen species and antioxidant therapy in liver ischaemia reperfusion injury

A radical is any atom or bio molecule which contains unpaired electrons making it more reactive than the corresponding non radical(144). Oxygen is the main source of free radical formation due to its high bioavailability and the most biologically relevant radicals are the Superoxide anion (O_2^-), the hydroxyl radical (OH^-) and nitric oxide (NO). Some other species are intermediate in the metabolism of O_2 and NO but are not radicals since they do not contain unpaired electrons(146). These intermediate species along with the radical species are called ROS (H_2O_2 and hypochlorous acid) and RNS (peroxynitrite $ONOO^-$)(144). Liver IRI is at least partially induced by ROS and RNS with concomitant consumption of endogenous antioxidants, formation of mitochondrial permeability transition pores and apoptotic or necrotic cell death (143;146).

The role of ROS in liver IRI is summarised in table 2.3

Table 2.3: Role of reactive oxygen species (ROS) in liver ischaemia reperfusion injury

Function	References
Enhance pro-inflammatory gene expression (TNF- α , IL-1, IL-8, cellular adhesion molecules)	(243;253)
Induce expression of the transcription factors NF-kB and activator protein-1	(443;159)
Direct cellular damage through protein oxidation and degradation, lipid peroxidation and DNA damage	(189)
Direct induction and regulation of apoptotic and necrotic cell death	(312;319)
Inactivation of antiproteases	(189;414)
Induction of protective stress genes in hepatocytes	(19)
Formation of mediators involved in regulating sinusoidal blood flow & liver regeneration	(279;299)

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

The human body has endogenous antioxidant defence mechanism to protect it from damage by these free radicals whose primary function is to prevent initiation of oxidative damage or limit its propagation. However, oxidative stress results when these endogenous antioxidant systems are overwhelmed by excessive production of ROS and RNS(144). Hepatocytes are resistant to injury by ROS and RNS since they contain high intracellular concentration of glutathione (GSH), Superoxide dismutase(SOD), catalase and lipid soluble antioxidants(144).

Antioxidants are a heterogeneous family of molecules which can be classified on the basis of their site of action viz.

- 1) Intracellular antioxidants: SOD, catalase, glutathione peroxidase and reductase enzymes, the tri-peptide glutathione, the polypeptide thioredoxine, the enzyme hemoxygenase (HO) and peroxidases of the peroxiredoxin family.

- 2) Extracellular antioxidants: which includes the metal binding proteins (Transferrin, lactoferrin, ceruloplasmin, albumin, haemoglobin and myoglobin), various low molecular weight substances (bilirubin, melatonin, Lipoic acid, coenzyme Q, uric acid and melamins),
- 3) Exogenous antioxidants: dietary constituents (ascorbic acid, α -tocopherol and carotenoids) and plant phenols (tocopherols, tocotrienols, flavonoids, anthocyanidins and phenylpropanoids)

A large number of antioxidants have been shown to be beneficial in treatment of liver IRI and have been summarised in Table 2.4. As far as extracellular antioxidants are concerned there is a lot of experimental evidence to suggest their protective effects in hepatic IRI however, the majority have not yet been used in large or small clinical trials. Further information can be obtained from exhaustive existing reviews in the literature(56;124;144;259;264;437).

Table 2.4 List of antioxidant agents with beneficial effects in liver IRI

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 / Pentoxifylline	In vivo LMM agent	Rat	WI	IM/IP	10 mg/kg / 50 mg/kg	↓ lipid peroxidation ↑ GSH levels	Portakal, 1999
Idebenone	Coenzyme Q derivative	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

Key: Admin, administration; WI, warm ischaemia; im, intramuscular; CI, cold ischaemia; iv, intravenous; PT, prothrombin time; LMM, low molecular molecule; deriv, derivatives; In, Intra gastric; 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

Intracellular antioxidant in particular sulphhydryl group containing compounds like thiols are central to the cellular antioxidant defence in mammals. These compounds exert their protective effect by reacting with ROS and RNS and thus scavenging the free radicals generated under conditions of oxidative stress(144). The main representative of this group is glutathione (GSH) which serves as a substrate for glutathione peroxidase and directly scavenges ROS(144). Experimental evidence suggests that exogenous GSH administration ameliorates liver IRI, however in the clinical setting due to its large molecular size it is not efficiently transported into cells and thus may not be very effective particularly in situations associated with intracellular oxidative stress(146). The other limiting factor in GSH synthesis is requirement of intracellular cysteine which is extremely unstable in its reduced form. Other intracellular antioxidants like Superoxide Dismutase (SOD) and catalase have shown ambivalent results in the literature largely due to short half life and lack of uptake of the intact protein into cells, to bypass these difficulties newer derivatives of SOD and catalase have been developed by conjugation with carbohydrates, which facilitates their uptake into liver non parenchymal cells with promising results(144). Allopurinol a xanthine oxidase inhibitor, in low doses has not shown any protective effects against liver IRI but high doses when administered intraperitoneally have shown to be beneficial in liver IRI(200).

Hence, considerable research has focused on N-acetylcysteine (NAC), a) which is the redox state of cysteine, b) has a small molecular size and c) is rapidly hydrolyzed to cysteine intracellularly to provide the substrate for GSH synthesis and hence replenishing the intracellular GSH stores under conditions of extreme oxidative stress(144;146).

NAC is a well documented substance in medicine. Its defined volume of distribution is 0.33 L/kg, its renal clearance is 0.21 L/hr/kg, and its elimination half-life is 2.27

hours(30). Originally, NAC has been used to liquefy mucus in bronchi; NAC is also the antidote for paracetamol poisoning(166). It should also be mentioned that cysteine derivatives have been intensively investigated in military medicine because of their protective effects against radiation and that NAC has been investigated as a modulator of radiographic contrast agent-induced nephrotoxicity(40). The role of NAC in Ischaemia-reperfusion injury is shown in Table 2.5 (357).

Table 2.5: Postulated roles for NAC in Ischaemia reperfusion injury

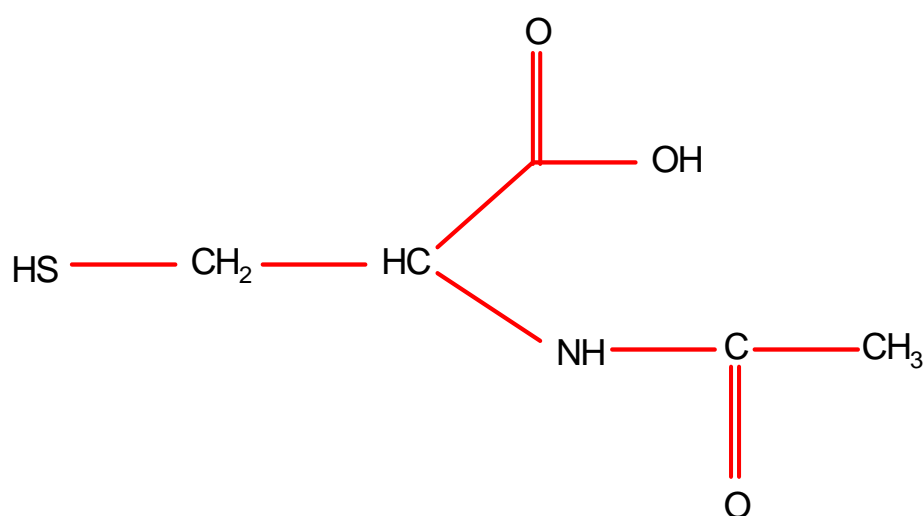
<p>Against ROS:</p> <ul style="list-style-type: none"> Redox mechanism cysteine-cystine Maintained tissue glutathione levels ↑ glutathione reductase activity <p>At cellular level</p> <ul style="list-style-type: none"> Indirect protection against ROS by formation of mixed disulfides with SH groups of membrane peptide/enzymes Effect on platelet function Action under low pH (ischaemic) conditions Inhibit expression of iNOS mRNA Block upregulated NF-κβ activity <p>At microcirculatory level</p> <ul style="list-style-type: none"> Interaction with neutrophils NAC induced ACE inhibition Modulation of coagulation Effect on adhesion processes (via adhesion molecules)
--

Key: ROS = reactive oxygen species, SH= sulphhydryl, NAC = N-acetylcysteine, ACE = acetyl coenzyme,

The diversity in the pharmacological uses of NAC is due to the multiple chemical properties of the cysteine 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(422). 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(429), antiproliferative effects(214) and direct reduction of functional protein thiols at the cell surface(235).

Figure 2.1: Chemical structure of N-acetylcysteine



2.7: Role of N-acetylcysteine in liver ischaemia reperfusion injury

NAC has been recognized to protect the liver from warm and cold I/R injury (98;401) where it was used as a glutathione donor as it increased reduced glutathione levels in the liver after reperfusion (132;276). It was also seen to enhance sinusoidal perfusion by intravital microscopy on reperfusion and transplantation(220;221), and reducing neutrophil sequestration(381). Its use during preservation revealed reduced sinusoidal oxidative stress, and, under a glutathione-depleted conditions, reduced hepatocellular and sinusoidal oxidative stress(277). The use of NAC-pretreatment in

a large animal transplantation model showed that NAC pre-treatment was associated with less cytolysis, transaminase elevation, better coagulation, reduced the incidence of graft dysfunction and primary non-function(313) although this is still controversial (257). NAC was also observed to reduce remote lung injury following liver I/R (412;413). Further investigation of the mechanisms by which NAC was able to produce these effects revealed that it inhibited TNF- α and IL-10 (239), maintained tissue glutathione levels, reduced the rise in malondialdehyde, glutathione peroxidase and superoxide catalase, following I/R, and increased glutathione reductase activities (341). The effect of oxidative stress on NF- κ B and iNOS was elucidated when NAC was seen to inhibit the expression of iNOS mRNA and block upregulated NF- κ B binding activity on reperfusion (177). NAC was seen to inhibit the kinase JNK1/SAPK1, a member of MAPK family, which regulates cell adaptation to stressful conditions(78). The clinical benefit of NAC in liver I/R is ambivalent(60;221). This probably relates to differences in the method of NAC administration and the duration of follow up.

2.8: Role of N-acetylcysteine in clinical liver transplantation

The various clinical trials using NAC in liver transplantation are summarised in Table 2.6. In the first reported trial in clinical liver transplantation, NAC failed to show any beneficial effect of the intraoperative administration of NAC on hemodynamics and graft function in liver transplantation in cirrhotic patients (363). However it was later reported to produce a distinct reduction in I/R injury and improved liver function with less elevated peak transaminases, better microcirculation, improved liver synthetic function and a lower incidence of PNF (382). NAC was also observed to increase selectin shedding following OLT (376). Furthermore, it was observed to inhibited the increase in circulating ICAM-1 and

VCAM-1 24 hr after reperfusion in OLT and reduced the rise in alpha-glutathione S-transferase after reperfusion of the donor liver (408). Although not all the effects of NAC observed in OLT were beneficial (375). Taut et al observed increased hepatic glutamine and urea production in patients treated with NAC. This would increase the oxygen and energy demand from a recently implanted graft resulting in metabolic disturbances (375).

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(38). The second failed to show any clear protection on postoperative graft function(363). The third clinical study showed that NAC administration was associated with better liver function, less hepatocellular injury and lower incidence of primary graft dysfunction(382). 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(408). In the fifth study NAC was used in combination with prostaglandin E₁ in paediatric 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(39). The most recent published study(211) 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.

Table 2.6: NAC Experience with human liver transplantation

First Author	n=number of Patients	Type of study	Use of N-acetylcysteine	Parameters
Bromley et al 1995	50 (25 in each group)	Prospective randomised double blind	150mg/kg NAC over 15 min followed by 50mg/kg over 4 hrs and 100mg/kg over 16 hours versus 5% dextrose in control group upon induction	No significant differences in mortality, morbidity or postoperative graft function.
Thies et al 1998	60 (30 in each group)	Prospective randomised	Liver flushed with 1L ringer lactate + NAC 1gm/l versus 1l ringer lactate alone for placebo & 150mg/kg NAC over 15 min followed by 50mg/kg over 4 hrs and 100mg/kg over 16 hours versus 5% dextrose in control group	↑Portal blood flow, ↓AST & ALT in NAC group, ↓ need for coagulation products, ↓ incidence of PNF and ↓ histological grade of severe IRI
Steib et al 1998	60 (30 in each group)	Prospective randomised	150mg/kg NAC over 15 min followed by 50mg/kg over 4 hrs and 100mg/kg over 16 hours versus 5% dextrose in control group at time of graft implantation	No significant differences were seen between the 2 groups on postoperative graft function or haemodynamics
Weigand et al 2001	20 (10 in each group)	Prospective randomised	Liver flushed with 1L ringer lactate + NAC 1gm/l versus 1l ringer lactate alone for placebo & 150mg/kg NAC over 15 min followed by 50mg/kg over 4 hrs and 100mg/kg over 16 hours versus 5% dextrose in control group	↓ circulating ICAM-1 and VCAM-1 at 24 hours after reperfusion and ↓α-GST in the NAC group. No change in cytokines or liver enzymes
Bucuvalas et al 2001	25 (12 in NAC + PGE ₁ group and 13 in control group)	Open label pilot study	IV NAC 70mg/kg upon reperfusion and thereafter 12 hourly for 6 days + PGE ₁ 0.4 µg/kg/h, for 6 days	No significant differences in mortality, morbidity or postoperative graft function
Khan et al 2005	22 (9 in each group, 4 were excluded post randomisation)	Prospective randomised	NAC administered to donor prior to crossclamp.150mg/kg NAC over 15 min, followed by NAC 1gm/l in citrate for portal vein flush x 3 litres and NAC 1gm/L in UW during bench perfusion prior to organ storage	No significant differences in mortality, morbidity, postoperative graft function or rejection episodes

NAC – N-acetylcysteine, α-GST- α-Glutathione S-transferase, PNF- Primary non function, IRI- ischaemia reperfusion injury, PGE₁- Prostaglandin E1

Another interesting effect of NAC administration is the reversal of the effects of ischaemic preconditioning. ROS are thought to be essential for the preconditioning response against I/R injury(190;354) suggesting a role for ROS in redox signaling. Two studies found that the inhibition of ROS by NAC was detrimental to liver protection from ischaemic preconditioning(354;378). These findings suggest that small amounts of ROS are acting as signalling molecules for the body's response against IRI.

NAC has been investigated extensively in the treatment of I/R in other organs where it was found to ameliorate the IRI (44;83;121;211;357). Renal IRI 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 (334). This protective effect is associated with less oxidative stress as indicated by less lipid peroxidation and maintenance of GSH levels. Donor pretreatment with NAC preserved renal metabolism and improved outcomes of I/R injured kidney transplants (133;248).

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 an animal model of multiple sclerosis and characterized by significant oxidative stress (289). Also AD4 protected human red cells from oxidative stress more efficiently than NAC (152).

In summary, NAC can scavenge ROS, increase glutathione levels, undergo auto-oxidation (and produce H₂O₂) and serve as reducing agent. Activation of NF-κβ in response to a variety of signals (IL-1, TNF, and H₂O₂) can be inhibited by NAC, suggesting ROS as common signalling modulators. In liver, NAC diminishes oxidative stress by various agents, and gives some protection against fibrosis, viral infections and toxicity (434).

NAC has been an extensively utilized tool for investigating redox sensitivity of biological or pathological processes. However, due to multiple activities of NAC and the possibility of direct modification of certain signals and signalling proteins, caution is warranted in interpretations. Such putative redox-sensitive mechanisms should be confirmed by additional approaches such as overexpression of antioxidant enzymes and proteins.

Chapter 3
Glycine review

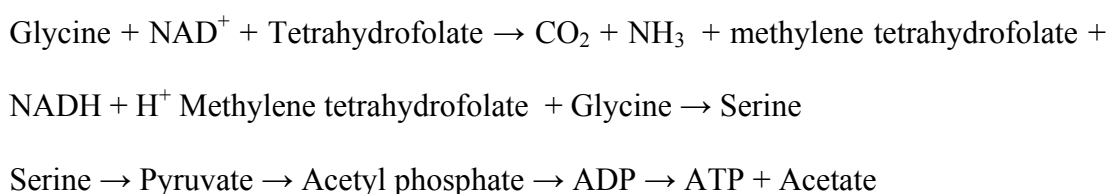
3.1 Introduction

Liver IRI is characterised by series of morphologic changes, which are reviewed in chapter 1. Briefly, the morphologic changes include disturbances of microvascular perfusion secondary to injury of the sinusoidal lining signified by endothelial cells and Kupffer cell (KC) disturbances (47) (186), intrahepatic accumulation of white blood cells (WBCs) (186) and impaired hepatocellular function (47;186). Activated KCs have been identified as a key event in the initiation and perpetuation of the IRI. Activation of KC's occurs early during organ harvest for transplantation due to insitu inevitable organ manipulation with standard harvesting techniques (328). These activated KCs are a major source of potent mediators like reactive oxygen species (ROS) , tumour necrosis factor- α (TNF- α), cytokines (interleukin-6 IL-6, interleukin IL-8), eicosanoids and chemokines (192;369). These mediators potentially impair liver function by via mechanisms including disturbance of the intrahepatic microcirculation, hypoxia, increased oxygen consumption and depletion of intrahepatic glycogen reserves (331) . Activated KCs increase oxygen uptake of the liver after cold storage. This may be due to KC derived prostaglandin E₂ (PGE₂) which stimulates oxygen uptake in hepatocytes(174). Thus KC modulation has been targeted to ameliorate liver IRI either by destruction using gadolinium chloride (GDCL₃) a rare earth metal (162) or inactivation by methylpalmitate a non hydrolysable fatty acid ester (263)

3.2: Glycine

Glycine, a non-toxic, nonessential amino acid in pharmacological doses blocks KC activation, along with neutrophils and possible lymphocyte activation, thereby providing a broad cytoprotective effect (326;441). Physiological levels of glycine ranges from 200-400 μ mol/L in humans. Glycine is a part of the normal human diet. The human body is able to synthesize glycine in the absence of dietary intake from

threonine in the central nervous system and serine in the periphery. The average daily diet provides up to 2 grams of glycine as it is the second most common amino acid found in proteins, with fish, meat and dairy products being rich sources(418;441). Excess glycine is catabolised by the glycine cleavage system, a four enzyme complex (designated as p1-4) which is present in the inner mitochondrial membrane and is most abundant in hepatocytes(272). The catabolic pathway allows consumption of glycine in energy generation by the following reactions(316):



3.3: Mechanism of action of glycine

The cytoprotective and anti-inflammatory effect of glycine depends on the structural characteristics, which glycine shares with other amino acids like taurine, alanine and strychnine (411).

Glycine is an inhibitory neurotransmitter in the central nervous system where it hyperpolarizes the post synaptic spinal motor neurons and has been used therapeutically in central nervous system disorders(35;237). Weinberg *et al* first reported the cytoprotective effect of glycine during the investigation of the mechanism of antioxidant glutathione and its constituent amino acids(409). This cytoprotective effect was independent of cellular ATP levels

Glycine inhibits nonlysosomal calcium dependant proteases and protects hepatocytes against anoxic damage(441). Ozaki *et al* demonstrated that glycine could protect livers *in situ* from reperfusion damage by minimising lipid peroxidation (295).

Glycine could stabilise the cell membrane by inhibiting phospholipase A₂, leading to a reduction of arachidonic acid and eicosanoids which influence hepatic microcirculation(332). Carolina rinse solution which contains glycine prevents reperfusion injury to livers in both experimental and human liver transplantation(14). The protective effect of glycine is mediated via mechanisms involving proinflammatory mediators, hypoxia reduction, reperfusion enhancement and toxin attenuation in various animal species (154). Glycine has been shown to inhibit KC activity when administered either orally or intravenously (326;327).

3.4: Glycine receptors

The anti-inflammatory and cytoprotective effects of glycine are mediated via glycine receptors (GlyR). GlyR stimulation produces hyperpolarization of cell membrane by Cl⁻ influx and consequent inhibition of excitation under inflammatory stimuli(254). GlyR were originally discovered in the CNS, where glycine acts as an inhibitory neurotransmitter(254), later GlyR were found to be present on peripheral cells(91).

3.4.1: CNS glycine receptor

GlyR are primarily localised to the post synaptic membrane in the spinal cord, brain stem, caudal brain and in the retina where they mediate reciprocal inhibition necessary for spinal cord reflexes and muscle tone(254). In the brain, glycine acts as the principal mediator of fast inhibitory neurotransmission. Activation of the anionic (Cl⁻ and bicarbonate) channels by glycine and gamma amino butyric acid respectively causes post synaptic membrane hyperpolarization and suppression of neuronal firing(36).

3.4.2: Peripheral glycine receptor

Peripheral GlyR are present in almost all cell types including white cells i.e. Kupffer cells (KC) (130;180), alveolar macrophages(130), splenic macrophages(130),

Monocytes(360), Neutrophils(130;417), lymphocytes(361), epithelial cells such as hepatocytes(309) and endothelial cells(428;438). The structure of peripheral GlyR is similar to the central GlyR. Molecular evidence for the presence of GlyR on peripheral cells is based on demonstration of mRNA and protein expression of GlyR subunits in KCs, alveolar and splenic macrophages and neutrophils(130). Yamashina *et al* demonstrated evidence for GlyR β subunit on endothelial cells using western blot analysis(428).

Functional evidence for the effects of glycine in peripheral inflammatory cells has been demonstrated by amelioration of TNF- α (180;360;420), IL1- β (360) ,IL-10(360), decreased production of oxygen derived free radicals(417;420). In addition glycine prevents the apoptosis of cultured sinusoidal endothelial cells (SEC) caused by deprivation of the vascular endothelial growth factor (VEGF) possibly by a Bcl2 dependant mechanism, since the fall in Bcl2 induced by VEGF deprivation is totally prevented by glycine. This effect is also mediated by GlyR as it is prevented by low dose 1mM strychnine, an antagonist of glycine at low doses(438).

3.5: Mechanism of anti-inflammatory action of glycine

Intracellular calcium is mostly sequestered in the endoplasmic reticulum, keeping the concentration of cytoplasmic calcium low while the extracellular fluid has a relatively higher concentration of calcium(28;120). Mammalian cells express two types of Ca⁺² channels viz. voltage gated and receptor gated. Cell injury leads to an increase in intracellular calcium flux by opening of voltage gated Ca⁺² channels which in turn are activated by membrane depolarization (267;391;395). Activation of these channels allows cytoplasmic influx of Ca⁺² along the concentration gradient and glycine prevents this rise in intracellular Ca⁺² (417;420). This is achieved by stimulating chloride ion (Cl⁻) conductance by activating GlyR which increases

intracellular Cl^{-1} and thus hyperpolarizes the cell membrane preventing depolarization in response to inflammatory stimuli and preventing activation of voltage gated Ca^{+2} channel activation.(180;417).

Besides membrane hyperpolarization, several other studies have proposed other modes of action for glycine. Inhibition of Ca^{+2} dependent degradative, nonlysosomal proteases, including calpains following recovery of intracellular pH after reperfusion(282), KC membrane stabilization by suppressing changes in plasma membrane proteins(210) and inhibition of an organic anion channel(154).

3.6: Cytoprotective effect of glycine

The cytoprotective effect of glycine has been shown to be independent of Ca^{+2} homeostasis and Cl^{-1} conductance in cultured cells(111;136;410) while others have suggested a role for Cl^{-1} Channel blockade in cytoprotection(274) suggesting that the basis of this effect needs further clarification. Calcium has been shown to be the final common pathway leading to cell death induced by toxic influences(324)and increased intracellular Ca^{+2} is a common feature between apoptotic and necrotic cell death(392). Glycine can provide cytoprotection despite allowing a rise in intracellular Ca^{+2} ; a effect which is probably mediated by blocking pre lytic opening of large cell membrane pores (death channels) and hence preventing cell lysis. In addition glycine also prevents the cell membrane bleb dilatation in response to increasing membrane permeability(284).

Hepatic IRI leads to cellular ultra structural changes like development of vesicular profile, alteration of junctional complexes and development of electron densities(129). Cell swelling and bleb formation has also been described in hepatocytes after cold and warm ischaemia(46;242). Similar cell membrane blebbing, followed by bleb dilatation and cell lysis has been shown to occur in a

variety of invitro models of cellular injury and these models have been used to study the cytoprotective effect of glycine(48;95;96;111;284). The cytoprotective effect was found to be independent of the calcium flux although glycine did delay the rise in intracellular calcium but did not prevent it completely. This cytoprotective effect of glycine has been shown to depend on it's effect at the cell membrane rather than it being a intracytoplasmic action.(97)

Table 3.1: Mechanisms of action of glycine

	Cytoprotective effect	Modulatory Effect
Cells most studied	Renal cells Hepatocytes Endothelial cells	Immune cells Macroglial Cells Endothelial Cells
Main effect	Protection against ischaemic necrosis	Modulation of proliferation, migration, apoptosis
Active concentrations	Upto 10 mM	0.1-1 mM
Ca⁺² Flux modulation	No	Yes
Uptake of Chloride	No	Yes
Chloride dependency	No	Yes
Molecular Findings	Only β subunits	Both α & β subunits
Suggested Mechanism	Glycine sensitive death pathway. Unknown role of GlyR	GlyR dependent modulation of calcium signalling
Pharmacological Findings	Effects mimicked by related amino acids but not by taurine	Effects mimicked by taurine and β -alanine

Key: Ca⁺² = calcium, α = alpha, β = beta, GlyR= glycine receptor

3.7: Glycine and hepatic ischaemia reperfusion injury

In the liver IRI is a serious problem after liver transplantation and complex resectional surgery which may lead to liver dysfunction and poor outcome. Thus, a nonessential amino acid like glycine with its cytoprotective and anti-inflammatory properties might provide just the solution for this problem. Glycine specifically acts on KCs and Neutrophils, which play a central role in initiating and sustaining the IRI. The protective effect of glycine on IRI has been demonstrated in a wide variety of models of ischaemic injury either in cell culture experiments or in vivo in various different animal models.

3.7.1: Effect of glycine on cell culture models

Glycine has been shown to prevent injury to both cultured hepatocytes and SECs from the effects of hypoxia, toxins and potassium cyanide (KCN) (53;81;285). ATP depletion by KCN induces proteolytic activity, in cultured hepatocytes which is a possible mechanism of cell death(94).Glycine has been shown to reduce this proteolytic effect in a dose dependent manner which corresponds to its cytoprotective effects(94;119;282). Mitochondrial permeability transition (MPT) has been proposed as a mechanism for loss of cell viability after reperfusion(308). In this process large pores open in the mitochondrial inner membrane which conduct both positively charged and negatively charged particles leading to mitochondrial depolarization and decoupling of oxidative phosphorylation(285;308).Glycine has been shown to improve cell viability without any effect on return of intracellular pH towards normal after reperfusion and MPT to occur(284). This would suggest that the effect of glycine is downstream of MPT and ATP generation(308).There are conflicting reports on the intracellular changes in cultured hepatocytes. Glycine prevented the intracellular accumulation of Na^{+1} associated with KCN or hypoxia induced ATP depletion in cultured hepatocytes preventing cell lysis(53). The

proposed mechanism being inhibition of Cl^{-1} entry into the cell which normally precedes Na^{+1} influx. Glycine has also been demonstrated to inhibit stress induced accumulation of intracellular Ca^{+2} thus preventing cell death(309).In cultured SECs glycine acts by blocking glycine sensitive large pores thereby preventing anoxic cell death in as yet incompletely understood manner(284).

3.7.2: Glycine and isolated liver perfusion

Several liver perfusion models have shown beneficial effects of glycine(81;327;440). den Butter et al have shown better cold preservation with UW solution than when glutathione was replaced with glycine in a isolated perfused rabbit liver after 48 hours of cold preservation. This effect was only found with cold preservation, however when glycine was added to the reperfusion medium it significantly reduced hepatocellular injury (90). Currin et al reperfused UW stored liver with Krebs-Henseliet buffer alone (KHB) Buffer + glycine or Carolina rinse solution.

KHB alone liver demonstrated extensive endothelial cell death, while KHB + glycine or Carolina rinse solution showed equivalent benefit.(81). Zhong et al have shown glycine to be beneficial in low flow reflow model of liver circulation where low flow caused pericentral hypoxia and normoxic periportal areas. Reflow produced injury in the periportal areas which was ameliorated by glycine (440). However, it is impossible to extrapolate this data to the clinical scenario because of the limitations of extracorporeal perfusion models like non physiological perfusates, temperature and haemodynamics.

3.8: Experience with animal liver transplantation

Glycine has shown significantly reduced hepatocellular injury with improved survival in various animal models(14;24;89;90;315;326;436). Glycine has been administered both orally as well as intravenously to the donor or recipient, as a part

of preservative solution, during cold preservation or as a part of pre-reperfusion flush solution and in perfusate during insitu normothermic reperfusion and before cold preservation in non heart beating donor model. den Butter et al have reported 100% 7 day survival with use of glycine in preservative solution instead of glutathione(90). Similar results have been reported with the use of glycine in Carolina Rinse Solution(CRS) but not when either were used alone(14). Schemmer et al have shown that operative manipulation during liver retrieval initiates a KC based inflammatory cascade which is ameliorated by oral glycine administration to the donor. An equivalent benefit was seen when glycine was administered intravenously (326). Zhang et al have shown improved viability of the liver of the brain-death donor rat after pretreatment with glycine (436), while Benko et al have more recently shown decrease of liver injury and improvement of liver function after pretreatment with oral glycine for 5 days in a rat model of partial hepatectomy (24). Rentsch et al showed better perfused sinusoids with reduced neutrophilic infiltration in livers obtained from donor rats administered glycine with significantly better survival as compared to control, gadolinium chloride or methylpalmitate groups. This study is important since it shows that glycine ameliorates both the early and late phase of liver IRI as reviewed in chapter 1(315)

3.9: Glycine and human liver transplantation

There is limited clinical experience with the use of glycine in human liver transplantation; however, glycine has shown promise in reducing the hepatic IRI. The various clinical trials using glycine in liver transplantation are summarised in Table 3.2.

Table 3.2: Experience with Glycine in human liver transplantation:

First Author	No of Patients	Type of study	Use of glycine	Parameters	Complications	
					<i>Glycine group</i>	<i>Control group</i>
Arora (7)	50	Double Blind Randomized Controlled trial	Hepatic arterial (150 ml) and portal vein (350 ml) flush of 2mM glycine solution before reperfusion	↓ALT day 1-3 post OLT	ITBS 1 patient	ITBS 4 patients Re-transplant 3 in 6-8 months
Schemmer (329)	4	Controlled observation study	300 mM, 250 ml glycine infusion over 1 hour before reperfusion and than daily for 1 week post OLT	↓ALT (24 hour) and AST(48 hour) peaks [‡]	No PDF	PDF 11%
Schemmer (330)	7(includes from above study)	Controlled observation study	300 mM, 250 ml glycine infusion over 1 hour before reperfusion and than daily for 1 week post OLT	↓ALT (24 hour) and AST(48 hour) peaks [‡]	--	--

[‡], significant (P<0.05) vs historical controls

PDF, primary dysfunction

ITBS, ischemic type biliary stricture

↓, reduced

Values not published

In a prospective double blind randomised controlled trial involving 50 patients, dual vessel rinse with glycine solution through the hepatic artery and portal vein prior to reperfusion significantly reduced ALT rise for 3 days post OLT(Orthotopic liver transplantation) and reduced incidence of ischaemic type strictures and overall morbidity(7). Schemmer et al also reported similar results in a study of glycine in human liver transplantation. They administered 250 mls of 300mM glycine infusion over 1 hour before OLT and then daily for 1 week post OLT. Their patient group were matched with historical control, however the historically matched controls had 11% incidence of primary graft failure, while there were none with glycine pre-treatment(329;330). Although the study designs of these two latter studies were not robust, their results admittedly suggest some benefit without significant side effects.

3.10: Dosage and clinical safety of glycine

The normal daily diet contains approximately 2 grams of glycine, but up to 40-90gm/day has been used for up to 6 weeks, in clinical trials without any serious adverse effects(169). This high dose oral intake resulted in increased blood levels to more than 3 times normal values of 300 μ M. Schemmer et al have used 250ml infusion of 300mM glycine administered over 1 hour in liver transplant recipients(329). This infusion increased serum glycine levels to 1952 \pm 210 μ M. The half life of glycine after intravenous infusion or body cavity irrigation depends on the dose administered and can vary between approximately half an hour up to 4 hours(155).

Oral treatment with glycine, is well tolerated with only minor side effects like nausea, vomiting and upper abdominal discomfort have been reported. These too have resolved following cessation of therapy(169). Similarly high dose intravenous

glycine administration for 4 days in a study of its availability in healthy volunteers did not report any serious adverse effects (84).

3.11: Glycine Toxicity

Although oral and intravenous glycine usage has been not associated with serious side effects, the following reports of glycine toxicity are mentioned in the literature following its use as an irrigant solution.

Glycine 1.5% solution when used as an irrigant in urological and gynaecological procedures may be absorbed through cut venous sinuses. Systemically absorbed glycine is primarily metabolised by transamination to serine, and by deamination to ammonia, which is normally removed by its conversion to urea. A portion of the absorbed glycine is excreted by the kidneys. Absorption of large amounts of irrigating solution can also result in pronounced hyponatremia and water intoxication (183). Absorption of this solution has also reportedly been implicated with neurotoxicity, cardiotoxicity and nephrotoxicity(137;183;255).

Increased serum concentration of ammonia following glycine administration would predispose patients with pre-existing impaired hepatic function to encephalopathy. The two neurotoxic metabolites of glycine (Glyoxylic acid and glycolic acid) increase after glycine administration.(255).Transient cardiac arrhythmias and even cardiac arrest have been reported following the use of glycine as an irrigant in urological and gynaecological procedures(42;137).

Fibrinolysis and minor haemolysis has also been reported following use of glycine as an irrigant, which may be due to osmolar changes or due to direct effect of glycine(183). Similarly hyperoxaluria following glycine use as an irrigant may affect renal function particularly in event of hypovolemia and oliguria(8;123)

High concentration of glycine in preservation solution may be lead to anuria(256), while Olsson et al found that infusion of 1.5% glycine solution increased mortality in mice, independent of it's effect on osmolality(293).

In conclusion there is good molecular evidence for the presence of glycine receptors in peripheral macrophages with considerable evidence towards it's mechanism in hepatic IRI. There is however little evidence of the effect of glycine on portal blood flow, bile flow and biliary composition and cytokines like IL-8 which are responsible for the initiation and maintenance of the latter phase of liver IRI.

Hence in this thesis we propose to use a well established animal model of partial lobar liver IRI to study the effects of a single dose of IV glycine administered prior to the ischaemic injury and its effect on liver microcirculation, hepatic mitochondrial activity, bile flow and composition, portal blood flow, cytokine expression (TNF- α and IL-8) and serum ALT and AST. We propose to use 1 hour of ischaemia and 6 hours of reperfusion in order to understand the changes in both the early phase and initial part of the late phase of liver IRI. In addition in order to assess the effect of ROS on liver IRI we propose to use NAC prophylactically within a randomised double blind controlled clinical trial for all patients undergoing elective liver resection. The next chapter describes the animal model used including the various animal groups and the materials and methods used in the various studies in the thesis

Chapter 4

Animal model, materials and methods

4.1 Animal model of lobar liver I/R injury

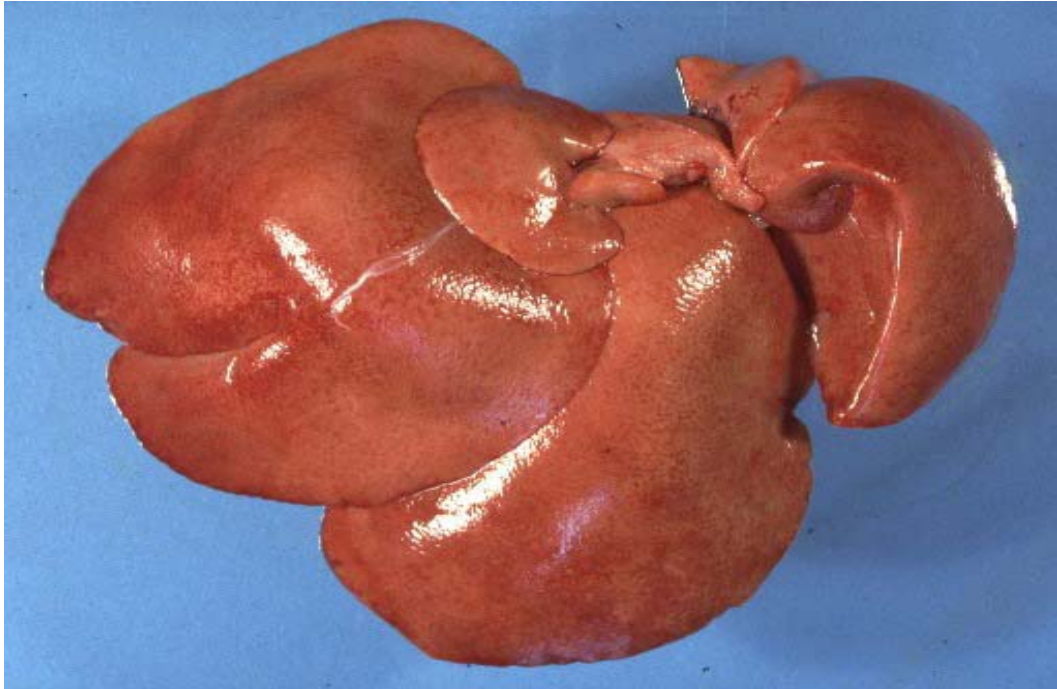
This study was conducted under a license granted by the home office in accordance with the Animals (Scientific Procedures) Act 1986. New Zealand white rabbits (3.2 ± 0.4 Kg, n=18) were used for the experiments. All animals were kept in temperature controlled environment with 12-hour light-dark cycle. Animals were kept without food overnight prior to the experiments. The hospital's ethics committee approved the experimental research protocols.

Anaesthesia was induced by an intramuscular injection of 0.5ml/kg of fentanyl citrate and fluanisone (Hypnorm; Janssen Animal Health, Buckinghamshire, UK). Following tracheostomy anaesthesia was maintained with isoflurane (1.5-3%) through an anaesthetic circuit. Body temperature was maintained at 37-38°C by a warming blanket (Homeothermic blanket control unit; Harvard Apparatus, Southmattick, Massachusetts, USA) and monitored with a rectal temperature probe. Haemoglobin oxygen saturation and heart rate were continuously monitored and recorded by a pulse oximeter (Ohmeda Biox 3740 pulse oximeter; Ohmeda Louisville, Colorado, USA). Ear marginal vein was cannulated with radioopaque catheter 20 gauge for administration of fluids, anaesthetics, and blood sampling. Ear marginal artery was cannulated with a 20 gauge radioopaque catheter and connected to a pressure transducer for monitoring the mean arterial blood pressure.

After induction of anaesthesia a midline laparotomy was performed. The ligamentous attachments of the liver were divided and the liver was exposed. The rabbit liver is divided in left and right regions by a deep cleft with the right and left lobes further divided into anterior and posterior lobules as depicted in figure 4.1. There is a quadrate lobe, which is behind the gallbladder. Lastly, there is a small circular lobe called the caudate lobe next to the right kidney. The gallbladder is deep

within the abdominal cavity. There are separate openings for the bile duct and pancreatic duct into the duodenum.

Figure 4.1: Rabbit liver displaying the lobar anatomy



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 causing mesenteric venous hypertension(225). After 60 minutes of ischaemia the vascular clip was removed and reperfusion allowed for upto 6 hours. 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

The portal vein flow was monitored continuously using a 3mm transonic Doppler flow probe (Transonic Medical Flowmeter system, HT207; Transonic Medical System, Ithaca, New York, USA).

4.2 Experimental groups and protocol

Three groups of animals were used (n=6) in each group. Sham group of animals underwent anaesthesia and laparotomy but no ischaemia or reperfusion was induced. The Control group had 1 hour of ischaemia and 6 hours of reperfusion and the Glycine group, which received an additional 300mosm of glycine given intravenously at a dose of 5ml/kg body weight prior to induction of ischaemia by slow intravenous infusion over 15 minutes. An equivalent volume of normal saline was injected in the control group of animals.

4.3 Hepatic microcirculation

Hepatic microcirculation (HM) was measured by a surface laser Doppler flowmeter (LDF) (DRT4, Moor Instruments limited, Axminster, UK) in flux units. LDF is an established method of measuring tissue microcirculation continuously in an easy way that does not interfere with blood flow to the tissues(4;415;416).

A monochromatic laser light was generated from a 2mV- helium neon laser at 632nm and delivered to the tissues via optical fibres. The light reflected back from the tissues was carried back through the optical fibres and detected by a photo detector. Only the photons which are scattered by moving red blood cells (RBC's) will have a Doppler frequency shift unlike those reflected back from static tissues. Mixing of these two components by the photo detector generates an electrical signal that contains the Doppler frequency shift information. This electrical signal is further processed to produce an output voltage that is related in a linear fashion to the total number of moving RBC's in the area in cubic millimetres multiplied by the mean

velocity of the RBC's. 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.(4;145;335;415).

The LDF measures flow in arbitrary perfusion unit (flux), a quantity proportional to the product of the average speed of the blood cells and their number concentration (often referred to as blood volume). This is expressed in arbitrary 'perfusion units' and is calculated using

$$\text{Flux} = C_{\text{mrbc}} \times V_{\text{mrbc}}$$

where, C_m = concentration, V_m = Velocity of RBC's (red blood corpuscles)

Due to the variation of the signal across the surface of tissues it is not possible to apply a conversion factor that so that the LDF signal can be expressed in absolute flow units(4;415). The application and reproducibility of LDF measurement for assessment of liver microcirculation has been validated in both experimental animals and human liver transplantation (335;336;416).

Hepatic microcirculation was assessed using a commercially available dual channel surface laser Doppler flowmeter (DRT4, Moore instruments Ltd., Devon, UK) (Figure 4.1).

At the start of each study the LDF was calibrated against a standard reference (Brownian motion of polystyrene micro-spheres in water) provided by the manufacturer. In order to curtail any disturbance to blood flow due to the pressure exerted by the LDF probe on the hepatic surface, the probe was attached to a probe holder so that the probe itself was just in contact with the hepatic surface without applying any pressure by the probe weight. The probe was applied to same point in all experiments in order to minimise any error due to anatomical variations in the hepatic microcirculation.

Data from the LDF was recorded and collected continuously. The LDF data was collected before the application of ischaemia as baseline and at end of 1 hour of ischaemia, and at 1, 2, 4 and 6 hours after onset of reperfusion as a mean of 2 minute data



Figure 4.2: Dual channel surface laser Doppler flowmeter and its probes (DRT4, Moor Instruments Ltd., Devon, UK).

4.4: Intracellular oxygenation/mitochondrial activity

Cytochrome oxidase activity as a reflection of intracellular oxygenation was continuously measured using near infrared spectroscopy (NIRS). In all tissues a number of colour bearing compounds (chromophores) viz Oxyhaemoglobin (HbO_2), Deoxyhaemoglobin (Hb) and cytochrome oxidase (CytOx) are present in variable concentrations. They have different absorption spectra in the near infrared (NIR) light and their absorption characteristics are oxygen dependent (101;102).

CytOx is the terminal complex of the mitochondrial respiratory chain(49). It takes electrons from the cytochrome C and catalyses the reduction of oxygen to water with the concomitant synthesis of ATP through the oxidative phosphorylation process(50) (101). In hepatocytes, approximately 90% of the oxygen is consumed by mitochondrial cytochrome oxidase. CytOx contains four redox active metal sites: two haem iron (Haem a and Haem a₃) centres, the copper-copper dimer (Cu_A) and the binuclear Haem-copper coupled centre (haem a₃/ Cu_β). These four metal centres accept or donate electrons during 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_β and Haem a₃. 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 (49;50;101). In the absence of oxygen, electron transfer to oxygen cannot take place. Electrons accumulate on the haem and copper atoms and CytOx becomes reduced. With oxygen availability the electrons are rapidly transferred from the metal centres to oxygen and CytOx becomes oxidised. Many factors can affect the CytOx redox state in vivo, but the most significant factors are the oxygen concentration (50;73), nitric oxide in physiological circumstances and oxidants such as ROS and RNS in oxidative stress situations(349;350).

All four centres exhibit different absorption characteristics dependin on their redox state. The copper centres are optically sensitive in the NIR region in contrast with the haem centres that absorb visible light(50;73). Absorption of the NIR light by CytOx occurs primarily at the Cu_A centre within the CytOx. The oxidised Cu_A centre has a characteristic shape spectrum with a broad peak centred around 845nm (50;73). 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(50;73).

The redox state of CytOx Cu_A is dependent on cellular oxygen availability (50;74;99;371). 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 CytOx becomes reduced. The increase in the reduction state of CytOx reflects severe cellular hypoxia.

4.4.1: Basic principles of NIRS

Biological tissues interact with light in a multitude of ways including scattering, reflection and absorption which is dependant on the type of tissue illuminated and the wavelength of light used (101). Light in the visible range of the spectrum (450-650nm) is strongly attenuated due to its absorption by haemoglobin and its scattering in tissues. This attenuation is inversely related to the wavelength of light. This leads to the failure of light to penetrate for more than 1cm into tissues (101;218). 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 upto 8 cms(101;218).

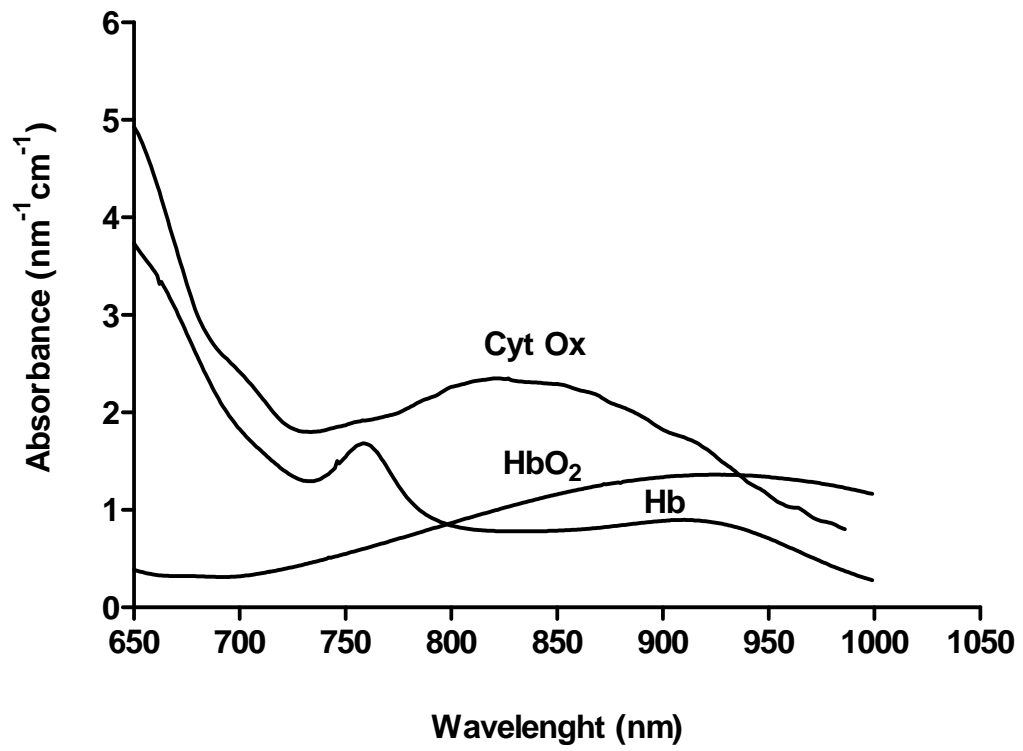


Figure 4.3: Absorption spectra of HbO₂, Hb, and Cyt Ox in the NIR light region(201)

NIRS depends on two basic principles;

- 1) The relative transparency of biological tissues to light in the near infrared region of the spectrum.
- 2) The existence of different tissue chromophores with characteristic absorption spectra in the NIR light spectrum(101;294;387).

In tissues with homogenous 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:

- 1) an additive term, G , due to scattering losses and
- 2) a multiplier B , to account for the increased optical pathlength due to scattering.

This law can be used to convert the obtained optical densities to concentration of CytOx in $\mu\text{mol/L}$ per optical pathlength (75;101;425):

$$A = \alpha \cdot c \cdot d \cdot B + G$$

Where A is the attenuation of light, α is the absorption coefficient of the chromophores ($\mu\text{mol}^{-1} \cdot \text{cm}^{-1}$), c is the concentration of the absorption compound ($\mu\text{mol/L}$) and d is the distance between the entry and exit points of light into the tissues (cm). B is the differential path length factor (DPL) which accounts for the increase in optical path length due to light scattering (which causes the optical path length to be greater than d) and G is a constant 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 particular tissue, 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:

$\Delta c = \Delta A / \alpha \cdot d$. 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 ($\mu\text{mole/L}$) from an arbitrary zero at the start of the measurement. The absorption coefficient of Cyt Ox was obtained in vivo by replacing the blood in the brains of experimental animals with a blood substitute (fluorocarbon) and exposed to 100% O_2 or N_2 to obtain the oxidised and reduced Cyt Ox spectra (75;101;425). B depends on the amount of scattering in the medium which can be measured by “the time of flight” method(88).

For simultaneous calculation of the changes in concentration of a number of chromophores from changes in attenuation at a number of wavelength, an algorithm can be used which incorporates the relevant absorption coefficient for each chromophore at each wavelength(75;99).

4.4.2: Near Infrared Spectrophotometer

The NIR spectrophotometer used in this study is the NIRO-500 (Hamamatsu Photonics K.K, Hamamatsu, Japan) (Fig2.). This spectrophotometer is the commercial version of an instrument developed by colleagues in the department of Medical Physics and Bioengineering, University College London (75;99). In the NIRO 500, the light source is monochromatic, generated from semiconductor laser diodes(LD). The light is produced at four wavelengths (774,826,849,906 nm). The choice of the wavelengths is based on 765nm, the absorption maximum for Hb; 810nm 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; 845nm, the absorption maximum for oxidised Cyt Ox; and 900nm, a reference wavelength(99;425)

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(294). Photons emerging from the liver are collected by the second bundle of optical fibres and detected by a photomultiplier tube(PMT) light detector(294) (Figure 4.3). The incident and transmitted light intensities are recorded and from these the changes in the concentration of tissue chromophores ($\mu\text{mol/l}$) are calculated using an algorithm incorporating the known chromophores absorption coefficients and an experimentally measured optical pathlength.

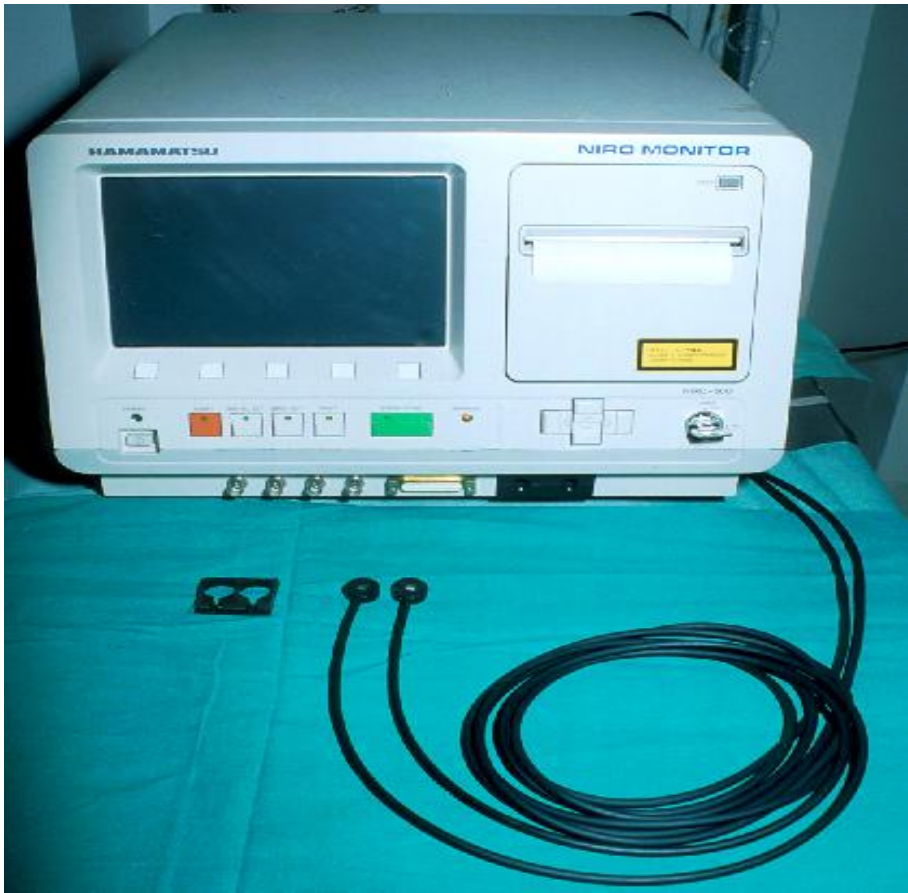


Figure 4.4: Near infrared spectrometer probes and a rubber optode holder (NIRO 500, Hamamatsu Photonics KK, Hamamatsu, Japan)

The standard NIRO 500 algorithm was developed using the wavelength dependant pathlength for the brain tissue (110). 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 it's hepatic application(99;101). The absorption coefficient of a freshly dissected and viable blood free pig liver was measured. The absorption coefficient was found to be 0.04mm^{-1} at 800 nm which is four times larger than that of normally perfused brain tissue although the transport and scattering coefficient was similar at 1.0mm^{-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 0.1mm^{-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 in the liver was assumed to be 12% by volume at an average haemoglobin saturation of 60%(238).

4.4.3: 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 tissue oxygenation and blood volume. 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 would minimise artefacts secondary to changes in the distance between the probe ends and allow as a satisfactory contact between the liver surface and probe ends

The NIRS spectrometer has the facility to set the attenuation and therefore chromophore concentration changes were set to zero with the initial NIRS setting.

This function is important so that all measurements are from a initial arbitrary zero, which ensures that artefacts such as system drift, optode movement and excessive light have a minimal effect on the data. A laptop computer connected to the NIRS spectrometer continuously collected the NIRS data. The data essentially 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 of Cyt Ox ($\mu\text{mol/l}$) using the previously defined algorithm in the NIRO 500. This data was then transferred to excel[®] data sheets (Microsoft Corporation, Seattle, USA) for analysis. The data was collected at the relevant time points as mean of 2 minute measurements and calculated in regard to the baseline value (zero set at start of experiment).

4.4.4: Interpretation of NIRO 500 measurement

NIRS allows continuous monitoring of the changes in Cyt Ox. Measurement of CytOx redox state provides a good index of intracellular oxygen. Cyt Ox becomes more reduced with decreasing the blood flow or oxygen saturation and more oxidised with increasing the blood flow, or oxygen saturation(34;48;73). Changes in the level of glucose, as a substrate for the electrons generated in the respiratory chain, also affects the redox state of Cyt Ox. With hypoglycaemia Cyt Ox becomes more oxidised and with glucose administration it becomes more reduced (34;48;73). The cell metabolic activity can affect the redox state of Cyt Ox, with increasing metabolic activity, it becomes more oxidised providing that adequate oxygen and substrate are available (73;101;145).

4.5: Ultrasonic transit time flowmetry

In this study blood, flow through the PV (PVBF) was measured using ultrasonic transit time flowmetry.

4.5.1: Principle of ultrasonic transit time flowmetry

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. In the same way that a swimmer will move quicker in the downstream direction than in the upstream direction, the speed of the ultrasound is affected by the flow of liquid passing the 'acoustic window' of the flow probe. 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(337;372). The ultrasonic transit time flowmetry has been demonstrated to provide an accurate method for hepatic blood flow measurement(11;197;346).

4.5.2 Ultrasonic transit time flowmeter

PVBF, was measured continuously using a dual ultrasonic transit time flowmeter (HT207, Transonic Systems Inc, NY, USA) (Fig 2.4.1) . The flowmeter perivascular probes 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 (372). Also, it requires accurate selection of the probe size which was determined by the outer diameter of the blood vessel.

4.5.3 Ultrasonic transit time flowmeter data collection and 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 was calculated as a mean of 1-minute data.

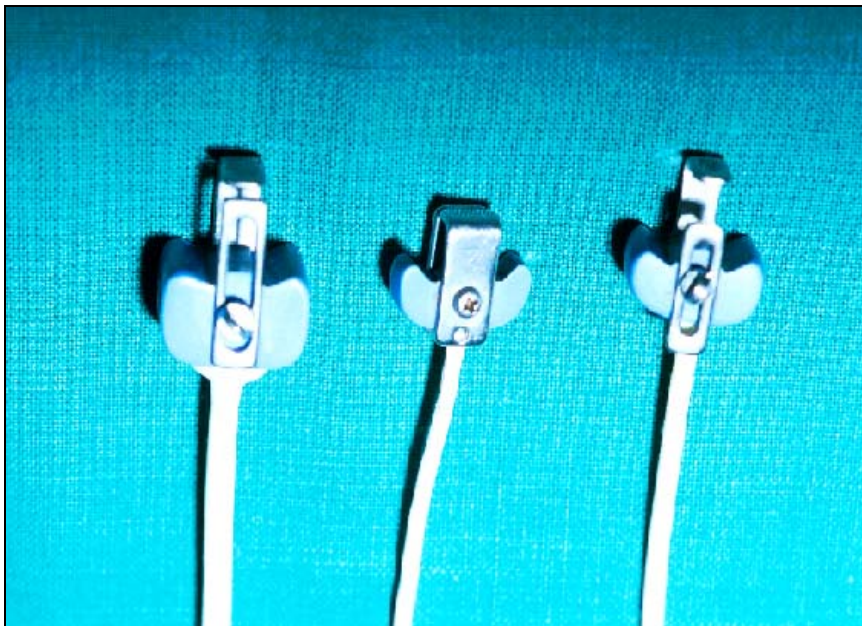


Figure 4.5: Dual ultrasonic transit time flowmeter (HT207, Transonic Systems Inc., NY, USA) and the perivascular probes.

4.6: TNF- α ELISA

For the TNF- α ELISA (BD Pharmingen, Oxford, UK) the primary antibody (capture antibody) was a purified goat anti-rabbit TNF- α and the secondary antibody (detection antibody) a biotinylated goat anti-rabbit TNF- α antibody. Both the antibodies were titrated to determine optimal concentrations for ELISA capture and detection. To obtain linear standard curves doubling dilutions of recombinant rabbit TNF- α protein ranging from 10,000 – 80 pg/ml were included in each ELISA plate.

4.7: IL-8 ELISA

For the IL-8 ELISA an OptEIA™ Rabbit IL-8 set (BD Pharmingen, Oxford, UK) was used. The set contained an anti-rabbit IL-8 antibody for capture and a biotinylated anti-rabbit IL-8 antibody as a detection antibody. To obtain linear standard curves doubling dilutions of recombinant rabbit IL-8 ranging from 200-0 pg/ml were used. All ELISA's were performed in 96 well Nunc Maxisorp™ plates (Fisher scientific, Leicester, UK). All assays were performed at room temperature and all standards and samples were run in duplicate. Any samples outside the detection range of the assay were repeated after appropriate dilution. (BD Pharmingen, Oxford, UK). The ELISA was carried out as per the manufacturer's recommended assay procedure.

The assay procedure remained the same for both the cytokines. All chemicals used in the assay were obtained from BD Pharmingen, Oxford, UK. In brief the microwells were coated with 100 μ l per well of capture antibody diluted in coating buffer (0.1 M Sodium Carbonate, pH 9.5). The plates were sealed and incubated overnight at 4°C.

The wells were aspirated and washed 3 times with \geq 300 μ l/well of wash buffer (PBS with 0.05% Tween-20). Any residual buffer was removed by inverting the plate on to a blotting paper after the last wash. The plates were then blocked with \geq 200 μ l/well Assay diluent (PBS with 10% Fetal bovine serum, pH 7.0) and incubated at room

temperature for 1 hour. The wells were then washed and aspirated 3 times with wash buffer as before. Standards and samples were then prepared in Assay Diluent and 100 µl/well of each standard, sample and control (Assay Diluent only) were pipetted into appropriate wells and incubated at room temperature for 2 hours after sealing the plate. The wells were then aspirated and washed with wash buffer as before, but the wash was repeated 5 times. 100 µl/well of Working Detector (Detection Antibody + Avidin-horseradish peroxidase conjugate) was added to each well. The plate was then sealed and incubated at room temperature for 1 hour. The wells were then washed and aspirated with wash buffer as before but the wash was repeated 7 times and each well was soaked for 30seconds for each wash. 100 µl/well of substrate solution (Tetramethylbenzidine and hydrogen peroxide) was added to each well and the plate was incubated at room temperature for 30 minutes in the dark without the plate sealer. 50 µl/well of Stop Solution (1M H₃PO₄ or 2 N H₂SO₄) was added and absorbance was read at 450nm within 30 minutes and a wavelength correction by subtracting absorbance at 570nm was applied. The mean absorbance for each set of duplicate standards, controls and samples were calculated. A standard curve was then plotted with TNF-α / Il-8 concentration on the X-axis and absorbance on the Y-axis and the individual sample cytokine concentration was then determined.

4.8: Liver transaminases

Alanine aminotransferase (ALT), Aspartate aminotransferase (AST) were measured in serum. Blood samples were centrifuged at 2000g 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 analyzer (Hitachi® 747, Roche Diagnostics, Lewes, UK).

4.9: Data collection and statistical analysis

Data from the pulse oximeter and blood pressure monitor was collected continuously on a laptop computer. The data was averaged for one minute before the induction of Ischaemia (baseline), at the end of Ischaemia and 1, 2, 4 & 6 post reperfusion. Serum samples were taken at 1, 2, 4 and 6 hours following reperfusion for TNF α , IL-8, ALT and AST assay. Values are expressed as mean \pm SD. For statistical analysis, Student's t-test for paired samples and one-way analysis of variance (ANOVA) with Bonferroni adjustment for multiple comparisons was used. $P < 0.05$ was considered statistically significant. All statistical analysis was performed on Graph pad Prism version 4.

4.10: Bile flow and Proton nuclear magnetic resonance ^1H NMR spectroscopy

Bile samples were taken at baseline and each hour thereafter and stored at -80°C . Bile volume was expressed as $\mu\text{L}/\text{min}/100\text{g}$ of liver weight. ^1H NMR 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 [$^2\text{d}_4$] trimethylsilylpropionate (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, which enabled an accurate comparison bile levels between the groups. One dimensional NMR spectra were obtained at 500 MHz with a sweep width of 6 kHz and 32 k data points in 64 scans were collected in both normal and spin echo mode. 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 Santiago de

Compostela, Spain). All spectra were integrated using a fixed range for each peak and published peak assignments(268).

Results are presented as mean \pm standard deviation (SD). The statistical analysis was performed using a one-way analysis of variance (ANOVA) with multiple comparisons adjusted for by the Bonferroni test. Statistics were calculated using commercially available software (SPSS release 11.0.0; SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

4.11 Summary of the methods used

This section of the thesis focuses on the effect of glycine on liver IRI in a well established intermediate animal model of partial lobar liver IRI. A sham operated group was specifically used in order to evaluate the effects of prolonged surgery and anaesthesia as is commonly encountered in the clinical setting of liver resections or liver transplantation surgery. The employment of a tracheostomy permitted a stable haemodynamic model which allowed assessment for this prolonged duration(134;145). A time period of 6 hours of reperfusion was chosen in order to allow assessment of changes not only in the early phase but also during the beginning of the late phase of IRI(184). Systemic haemodynamic parameters (BP and Heart rate) were monitored by arterial line and temperature with a PR probe.

The intermediate size of the model allowed for the simultaneous placement of both the LDF and NIRS probes and frequent sampling of blood to evaluate plasma levels of the cytokines studied without haemodynamic compromise(145).

Laser Doppler Flow was used to study changes in hepatic microcirculation, while changes in the portal blood flow were monitored with ultrasonic transit time flowmetry probes. The NIRS was utilised to study changes in tissue CytOx, which reflects intracellular tissue oxygenation and mitochondrial activity.

Bile flow was monitored and bile composition analysed by ^1H NMR spectroscopy. This is the first time ^1H NMR spectroscopy has been used to study the effect of glycine on bile composition in a *in vivo* model of liver IRI.

Finally the two key cytokines (TNF- α and IL-8) involved in liver IRI were analysed by ELISA while liver enzymes (ALT & AST) were analysed by a commercially available clinical chemistry autoanalyser.

The next chapter summarizes the experimental findings and effects of intravenous glycine on hepatic haemodynamics, hepatic microcirculation, and portal blood flow following liver IRI.

Chapter 5

**The role of glycine in liver ischaemia reperfusion injury and
its effect on hepatic microcirculation, tissue oxygenation and**

Portal blood flow

5.1 Introduction:

The effects of intravenous glycine on hepatic haemodynamics, hepatic microcirculation and portal blood flow following liver IRI are studied in the invivo model of partial lobar liver IRI as described in the methods chapter sections 4.1 – 4.5.

The effects of liver IRI on hepatic microcirculation are reviewed in chapter 1. In brief the pathophysiological changes of liver IRI obstruct hepatic sinusoidal blood flow leading to hepatic hypoperfusion (194). The severity of the sinusoidal perfusion failure is dependent on the ischaemia time (187) leading to primary sinusoidal perfusion failure ‘no reflow’(147) and ‘reflow paradox’ (292;320).

Sinusoidal impairment leads to reduced hepatic tissue oxygenation and hepatocellular integrity, culminating in deterioration of parenchymal function (340). Thus impairment of microcirculation and sinusoidal perfusion augments the reperfusion injury by increasing the hypoxic conditions during reperfusion (402).

Glycine is a nonessential amino acid and an inhibitory neurotransmitter in the central nervous system. It has been shown to be protective against hypoxia, Ischaemia and ATP depletion induced cell death in various experimental models (210;258;420;432;440) . There are several experimental studies which have shown beneficial effects of glycine on warm liver I/R injury (14;24;258;327;358;440) . The aim of the present study was to investigate the effect of glycine on cellular energy production by studying mitochondrial activity as expressed by redox state of cytochrome oxidase and the effect of glycine on hepatic microcirculation.

5.2 Animal model and methods

The animal model and groups including the various methods used in this study have been described in detail in chapter 4 section 4.1- 4.5.

5.2.1: Animal model

Three groups of animals were used (n=6) in each group. Sham group of animals underwent anaesthesia and laparotomy but no ischaemia or reperfusion was induced. The Control group had 1 hour of ischaemia and 6 hours of reperfusion and the Glycine group, which received an additional 300mosm of glycine given intravenously at a dose of 5ml/kg body weight prior to induction of ischaemia by slow intravenous infusion over 15 minutes. An equivalent volume of normal saline was injected in the control group of animals.

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. The portal vein flow was monitored continuously using a 3mm transonic Doppler flow probe (Transonic Medical Flowmeter system, HT207; Transonic Medical System, Ithaca, New York, USA).

5.2.2: Data collection and statistical analysis

Data from the pulse oximeter and blood pressure monitor was collected continuously on a laptop computer. The data was averaged for one minute before the induction of Ischaemia (baseline), at the end of Ischaemia and 1, 2, 4 & 6 hours post reperfusion.

A laptop computer connected to the NIRS spectrometer continuously collected the NIRS data. A software program called ONMAIN[®] (Hamamatsu Photonics K.K, Hamamatsu, Japan) was used to convert these data into changes in concentration of Cyt Ox ($\mu\text{mol/l}$) using the previously defined algorithm in the NIRO 500. This data

was then transferred to excel[®] data sheets (Microsoft Corporation, Seattle, USA) for analysis. The data was collected at the relevant time points as mean of 2 minute measurements and calculated in regard to the baseline value (zero set at start of experiment).

Data from the continuous measurement of portal blood flow 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 was calculated as a mean of 1-minute data.

Data from the LDF was recorded and collected continuously. The LDF data was collected before the application of ischaemia as baseline and at end of 1 hour of ischaemia, and at 1,2,4 and 6 hours after onset of reperfusion as a mean of 2 minute data.

5.3: Results

5.3.1: Systemic Hemodynamic parameters

Heart rate was stable in all 3 groups throughout the entire duration of the experimental protocol with no significant differences between the groups. In the sham group, the mean arterial blood pressure remained stable throughout the duration of the experiment. In the control and glycine groups the mean arterial blood pressure fell in the first hour of reperfusion and this was statistically significant between the sham & control and control & glycine ($p < 0.05$) groups, while there was no difference between the sham and glycine groups at this time point. During the second and fourth hours of reperfusion there was statistically significant drop in mean arterial blood pressure in the control group as compared to sham while there was no difference observed between the control and glycine groups. However, it was only in the sixth hour that the mean arterial blood pressure was significantly better in the sham and glycine groups as compared to control (**fig 5.1 and fig 5.2**).

Figure 5.1: Heart rate

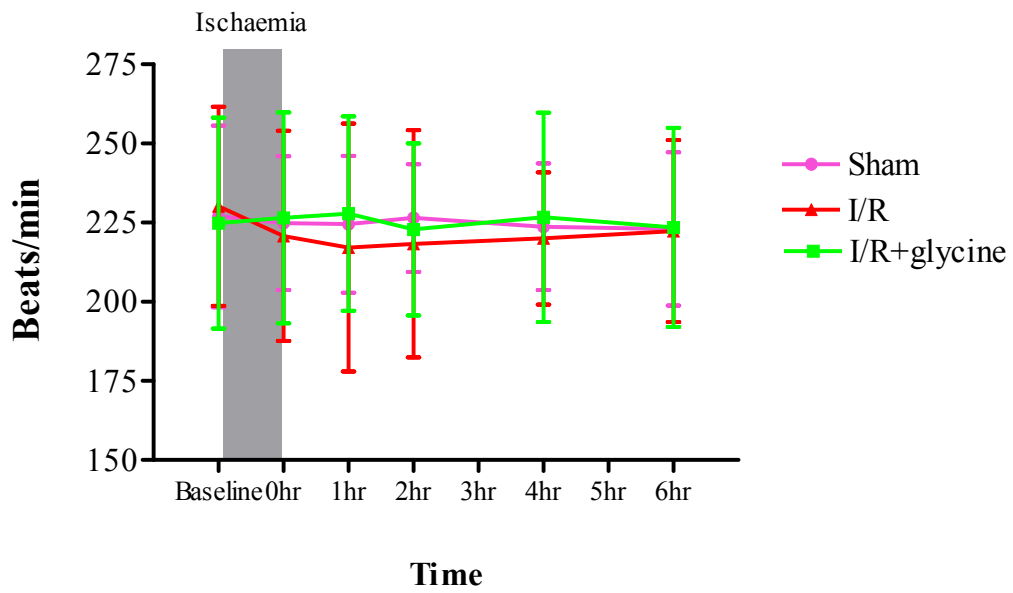
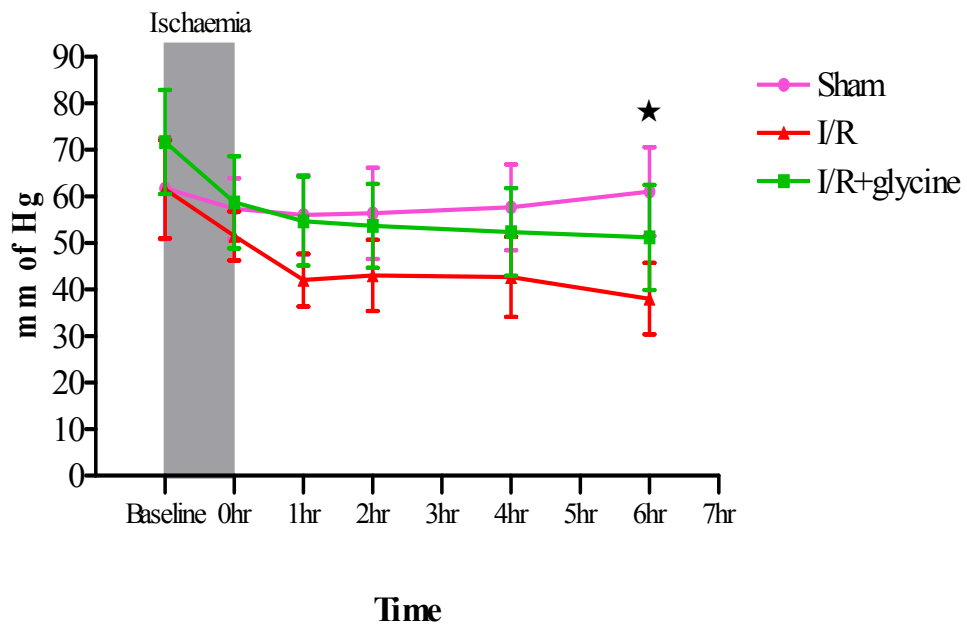


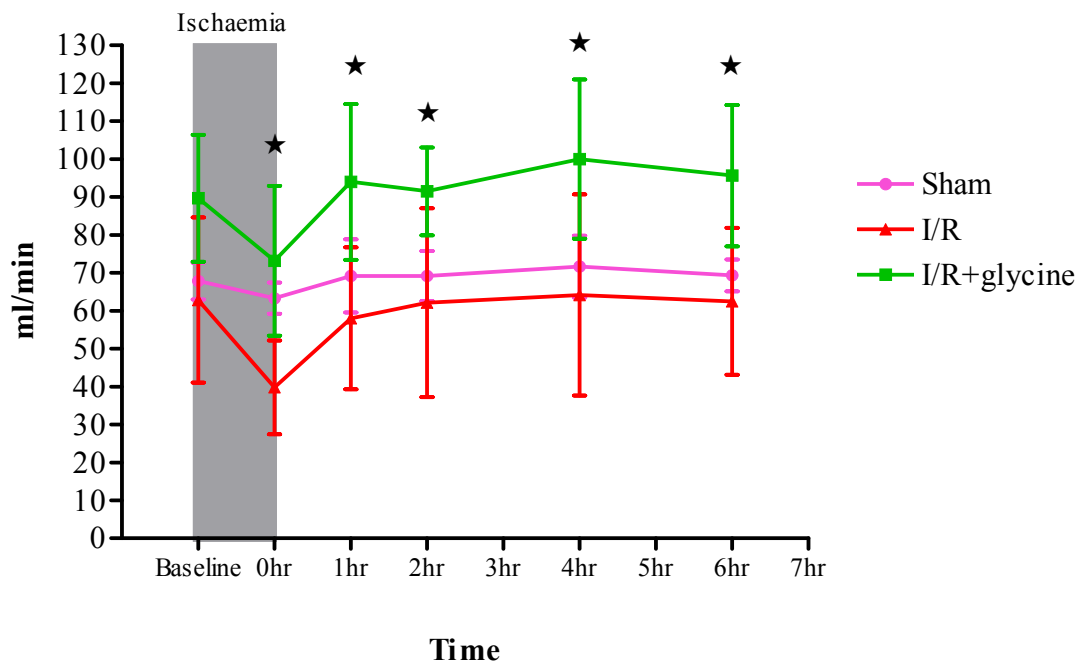
Figure 5.2: Mean arterial blood pressure



5.3.2: Hepatic Portal vein blood flow

The baseline portal blood flow was higher in the glycine group and this was statistically significant as compared to the control group but not as compared to sham. During Ischaemia portal blood flow was reduced in both the control and glycine groups ($p < 0.05$). The reduction started one min after the induction of Ischaemia and was maximal by 15 min. During the reperfusion period, the portal blood flow improved in the glycine group and was statistically significant at 1, 2, 4, and 6 hours of reperfusion as compared to the control group. There was no statistically significant difference observed in the portal blood flow between sham & control and sham & glycine groups, except in the sham v/s glycine group at 6 hours of reperfusion wherein the portal blood flow was significantly better in the glycine group as compared to the sham group (**fig 5.3**).

Figure 5.3: Portal blood flow

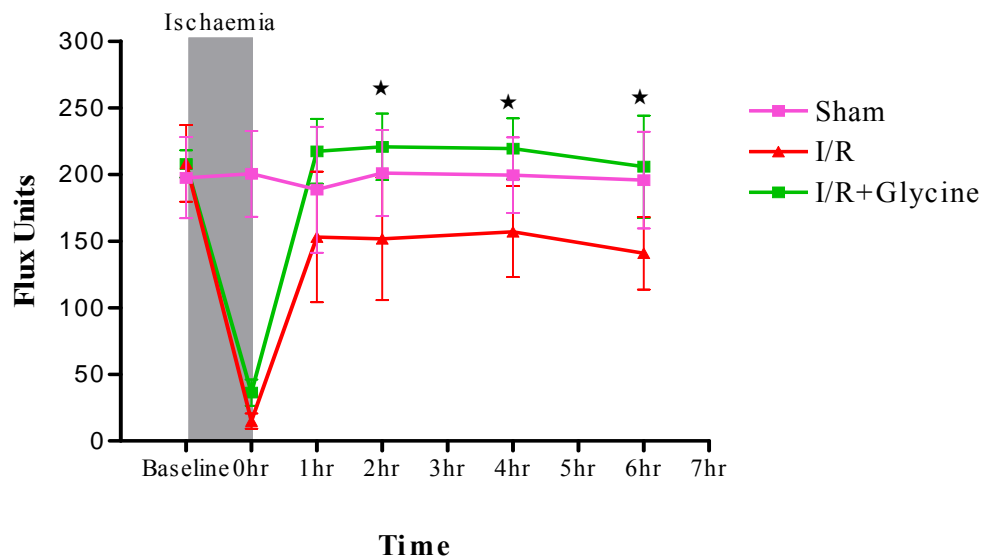


* = $p < 0.05$; Glycine+I/R v/s I/R alone

5.3.3: Hepatic microcirculation by LDF

There was no significant difference in the hepatic microcirculation over eight hours of sham operation. Following I/R the flow in the microcirculation was reduced in the Control group as compared to sham operated animals at 2, 4, and 6 hours of reperfusion and this was statistically significant (**fig 5.4**). In the glycine, treated group there was a significant improvement in the hepatic microcirculation as compared to the control group at 2, 4, and 6 hours of reperfusion.

Figure 5.4: Hepatic microcirculation (LDF)

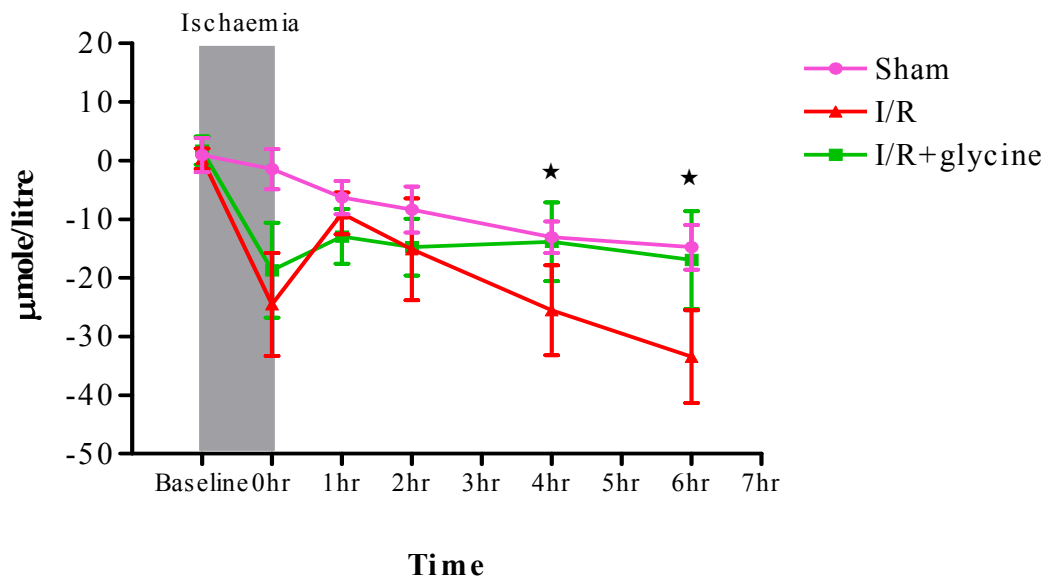


* = $p < 0.05$; Glycine+I/R v/s I/R alone

5.3.4 Hepatic tissue oxygenation by NIRS

There was no difference in the baseline CytOx levels between the three groups. During Ischaemia, there was a significant reduction in CytOx, which was maximal at 15-20 minutes after induction of Ischaemia. After reperfusion, in the control group CytOx levels returned towards baseline values during the first hour of reperfusion, but subsequently reduced. In the glycine group, a similar trend was observed immediately upon reperfusion, however the CytOx levels returned towards values observed in sham operated animals by the fourth hour of reperfusion and then decreased towards the end of the sixth hour. The improvement in CytOx levels was statistically significant at the fourth and sixth hour of reperfusion as compared to the control group ($P<0.001$) (fig 5.5).

Figure 5.5: Hepatic tissue oxygenation (CytOx)



* = $p<0.05$; Glycine+I/R v/s I/R alone

5.4 Discussion

In this study a rabbit lobar warm IRI model was used with continuous monitoring of systemic and hepatic haemodynamic parameters along with monitoring of hepatic microcirculation. A sham operated group was specifically used in order to evaluate the effects of prolonged surgery and anaesthesia as is commonly encountered in the clinical setting of liver resections or liver transplantation surgery. The employment of a tracheostomy permitted a stable haemodynamic model which allowed assessment for this prolonged duration (134;145). A time period of 6 hours of reperfusion was chosen in order to allow assessment of changes not only in the early phase but also during the beginning of the late phase of IRI (184).

In the sham operated animals the systemic and local haemodynamic parameters remained stable throughout the duration of the operation. However gradual deterioration in microcirculation and levels of Cytoc were noted although this was not statistically significant. This could be attributed to the prolonged anaesthesia and surgical procedure. The portal blood flow remained stable during the procedure.

There was a significant drop in portal blood flow during induction of ischaemia which was observed in the Control group (v/s Sham), which may be due to occlusion of the portal pedicle of the median and the left lobe. A similar drop was observed in the glycine treated group but it was significantly less as compared to control. Also the portal blood flow was observed to be significantly better than control group at all time points throughout the operation in the Glycine group. This could be due to splanchnic vasodilatation induced by the bolus dose of glycine given at the start of the operation. A similar effect was not observed either in the sham or control group animals even though they received a similar volume of normal saline at the start of the operation. This splanchnic vasodilatation has not been previously reported.

Changes in CytOx levels are indicative of the level of intracellular oxygenation and mitochondrial activity(101;145).In the present study, in the control group, CytOx levels fell significantly during the Ischaemia period and then started to improve during the first hour of reperfusion. Following this initial partial recovery, there was a gradual decrease in CytOx levels, which by the sixth hour were similar to levels during Ischaemia. This fall in CytOx levels after initial partial recovery could be attributed to reperfusion injury and its associated changes. Interestingly, in the glycine treated group, a similar trend was observed and the fall in CytOx was significantly less (v/s Control) by the end of fourth hour of reperfusion when the levels returned to those observed in the sham operated group and this was maintained up to the sixth hour of reperfusion. Thus, glycine not only protects during the early phase but also significantly decreases the extent of cellular injury in the early part of the late phase of hepatic IRI.

Similar changes were observed in hepatic microcirculation, which was reflected by the changes in LDF. In fact, significant recovery of microcirculation was observed at two hours of reperfusion and which was maintained up to six hours of reperfusion. Thus, recovery of microcirculatory changes prior to recovery of CytOx would suggest that recovery of microcirculation is paramount prior to recovery of cellular injury. The LDF data and the CytOx data suggests that inhibiting KC activation during the early phase of IRI not only ameliorates the early phase but also improves the early part of the late phase of IRI.

5.5: Conclusion

In conclusion in the experimental model used in this study, intravenous glycine ameliorated the haemodynamic alterations in both the early and beginning of the late phase of hepatic IRI. The effect on portal blood flow has not been reported before.

However the microcirculatory changes are a part of the initial pathophysiologic changes in liver IRI as reviewed in Chapter 1. The experimental work in the next chapter assesses the effect of glycine on two other key pathophysiologic phenomenon (cytokine expression and hepatic enzymes) which reflect and augment the changes in the microcirculation.

Chapter 6

The Role of glycine in liver ischaemia reperfusion injury and its effect on cytokine expression and liver function

6.1: Introduction

In this chapter the effect of a single dose of intravenous glycine on cytokine expression and liver function following warm liver IRI is studied in a *in vivo* model of partial lobar liver IRI as described in the methods chapter sections 4.1 and 4.6- 4.8. The role of cytokines in liver IRI has been reviewed in chapter 1. In brief, hepatic I/R injury is initiated by activation of KC in the liver during Ischaemia (72;192;194). However it is the cytokine release by these activated KC which plays a pivotal role in the inflammatory response during hepatic IRI(72;192;251). Cytokines play an important role in liver IRI, both by initiating and sustaining the inflammatory response as well as modulating its severity (351;388). Cytokines due to their ability to act in an autocrine, paracrine, and humoral manner not only initiates and maintains the inflammatory response (351), but also induce production of other inflammatory mediators like other cytokines, chemokines and eicosanoids(71;351).

Tumour necrosis factor alpha (TNF α) and interleukin-1 (IL1) are the key cytokines which are produced by activated KC and are most commonly implicated in hepatic IRI (71;369). These two cytokines in turn induce IL-8 synthesis, which initiates the late phase of liver IRI (71;388).

Inflammatory cytokines such as TNF- α are associated with the induction of cellular adhesion molecules and hepatic microcirculatory impairment based on neutrophil-vascular endothelial cell interaction (71). Members of the chemokine family such as IL-8, CINC, MIP-2, and MCP-1 are involved in neutrophil infiltration in the liver and remote organs further accentuating liver injury (314;351).

Thus suppression of these inflammatory mediators may not only reduce liver injury inherent to IRI but also ameliorate remote organ injury. Serum levels of liver enzymes viz AST & ALT reflects hepatocytes integrity/damage since they are

cytosolic enzymes and hence released in event of injury/damage to the hepatocytes(134;145;198).

Glycine a non essential amino acid and inhibitory neurotransmitter has been shown to inhibit KC activity when administered either orally or intravenously (179;210;326;329;432). There are several experimental studies which have shown beneficial effects of glycine on warm liver I/R injury(14;258;327;358;440).

The aim of the present study was to investigate the effect of glycine on cytokine production (TNF α and IL-8) and liver enzymes (AST & ALT).

6.2: Animal model and methods:

The animal model and groups including the various methods used in this study have been described in detail in chapter 4 sections 4.1 and 4.6 - 4.9.

6.2.1: Animal model

In brief, this study was conducted under a license granted by the home office in accordance with the Animals (Scientific Procedures) Act 1986. New Zealand white rabbits ($3.2 \pm 0.4\text{Kg}$, $n=18$) were used for the experiments.

Ear marginal vein was cannulated with radio opaque catheter 20 gauge for administration of fluids, anaesthetics, and blood sampling. Ear marginal artery was cannulated with a 20-gauge radio opaque catheter and connected to a pressure transducer for monitoring the mean arterial blood pressure. Lobar liver IRI was induced as per the experimental protocol described previously. Three groups of animals were used ($n=6$) in each group. Sham group of animals underwent anaesthesia and laparotomy but no ischaemia or reperfusion was induced. The Control group had 1 hour of ischaemia and 6 hours of reperfusion and the Glycine group, which received an additional 300mosm of glycine given intravenously at a dose of 5ml/kg body weight prior to induction of ischaemia by slow intravenous infusion over 15 minutes. An equivalent volume of normal saline was injected in the control group of animals. Blood samples were collected at 1, 2, 4 and 6 hours following reperfusion for assessment of serum cytokines (ELISA) and transaminases.

6.2.2: TNF- α ELISA

For the TNF- α ELISA (BD Pharmingen, Oxford, UK) the primary antibody (capture antibody) was a purified goat anti-rabbit TNF- α and the secondary antibody (detection antibody) a biotinylated goat anti-rabbit TNF- α antibody. Both the antibodies were titrated to determine optimal concentrations for ELISA capture and

detection. To obtain linear standard curves doubling dilutions of recombinant rabbit TNF- α protein ranging from 10,000 – 80 pg/ml were included in each ELISA plate.

6.2.3: IL-8 ELISA

An OptEIA™ Rabbit IL-8 set (BD Pharmingen, Oxford, UK) was used. The set contained an anti-rabbit IL-8 antibody for capture and a biotinylated anti-rabbit IL-8 antibody as a detection antibody. To obtain linear standard curves doubling dilutions of recombinant rabbit IL-8 ranging from 200-0 pg/ml were used. All ELISA's were performed in 96 well Nunc Maxisorp™ plates (Fisher scientific, Leicester, UK). All assays were performed at room temperature and all standards and samples were run in duplicate. Any samples outside the detection range of the assay were repeated after appropriate dilution. (BD Pharmingen, Oxford, UK). The ELISA was carried out as per the manufacturer's recommended assay procedure. The assay procedure remained the same for both the cytokines. All chemicals used in the assay were obtained from BD Pharmingen, Oxford, UK.

Alanine aminotransferase (ALT), Aspartate aminotransferase (AST) were measured in serum. Blood samples were centrifuged at 2000g 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 analyzer (Hitachi® 747, Roche Diagnostics, Lewes, UK).

6.2.4 Data collection and statistical analysis

Data from the pulse oximeter and blood pressure monitor was collected continuously on a laptop computer. The data was averaged for one minute before the induction of Ischaemia (baseline), at the end of Ischaemia and 1, 2, 4 & 6 hours post reperfusion.

Serum samples were taken at 1, 2, 4 and 6 hours following reperfusion for TNF α , IL-8, ALT and AST assay. Values are expressed as mean \pm SD. For statistical analysis, Student's t-test for paired samples and one-way analysis of variance (ANOVA) with Bonferroni adjustment for multiple comparisons was used. $P < 0.05$ was considered statistically significant. All statistical analyses were performed on Graph pad Prism version 4.

6.3 Results

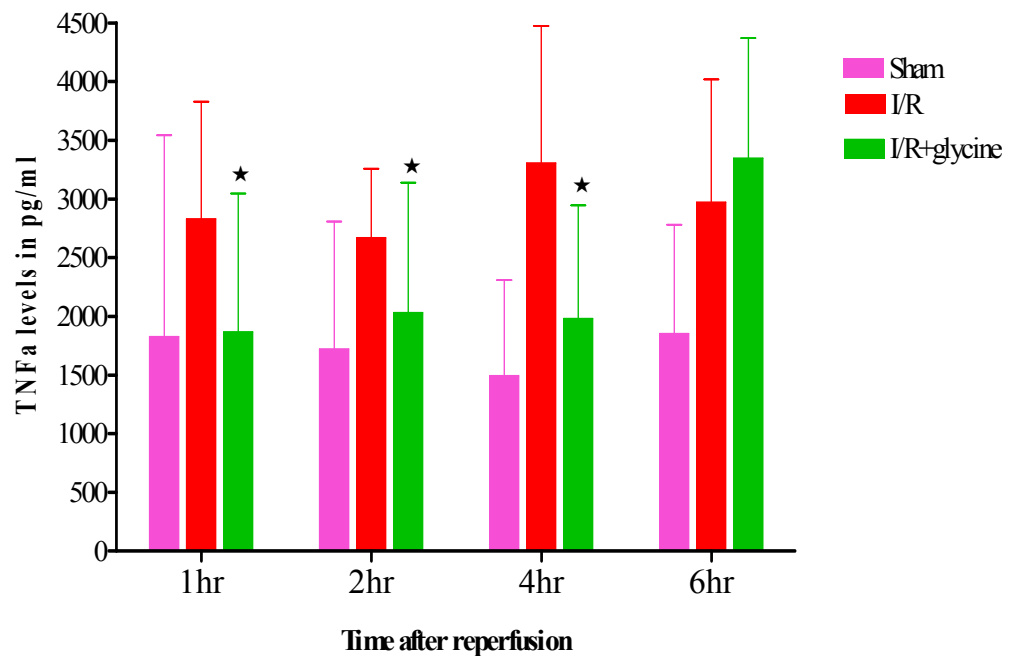
6.3.1 Systemic haemodynamic parameters

The results for systemic haemodynamics parameters are as described previously in chapter 5 section 5.3.1 and figures 5.1 and 5.2.

6.3.2 Serum TNF- α levels

There was no significant change in serum TNF- α levels over the duration of the experiment in the sham group. IR alone was associated with significantly elevated TNF- α levels. glycine administration produced a significant reduction in TNF- α levels following I/R at 1, 2 and 4 hours post reperfusion.(fig 6.1)

Figure 6.1: Serum TNF- α

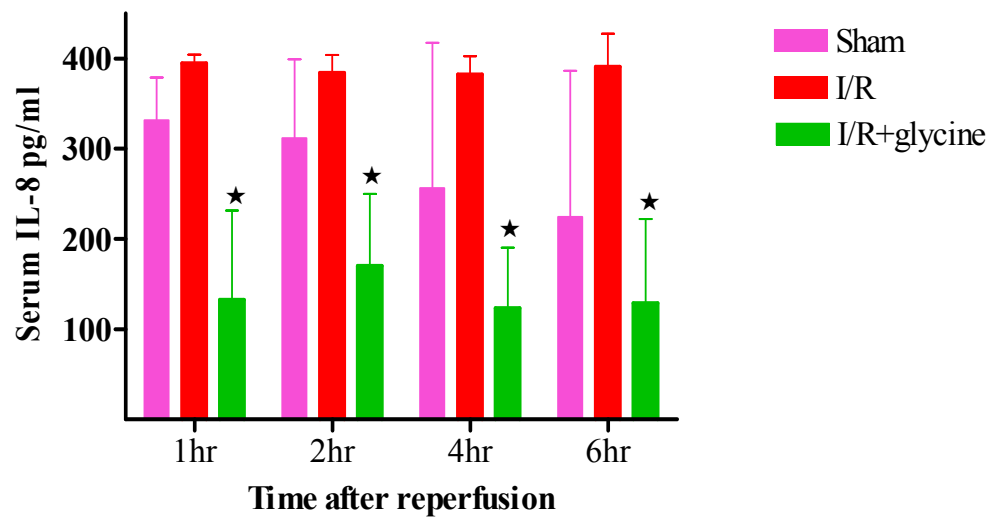


* = p<0.05; Glycine+I/R v/s I/R alone

6.3.3: Serum IL-8

Serum IL-8 levels gradually reduced in the sham operated group over the course of the experiment. IR alone significantly increased serum IL-8 levels over the entire reperfusion period as compared to sham operated group. In the glycine treated group a significant reduction in serum IL-8 levels was observed over the entire reperfusion period.(**fig 6.2**)

Figure 6.2: Serum IL-8

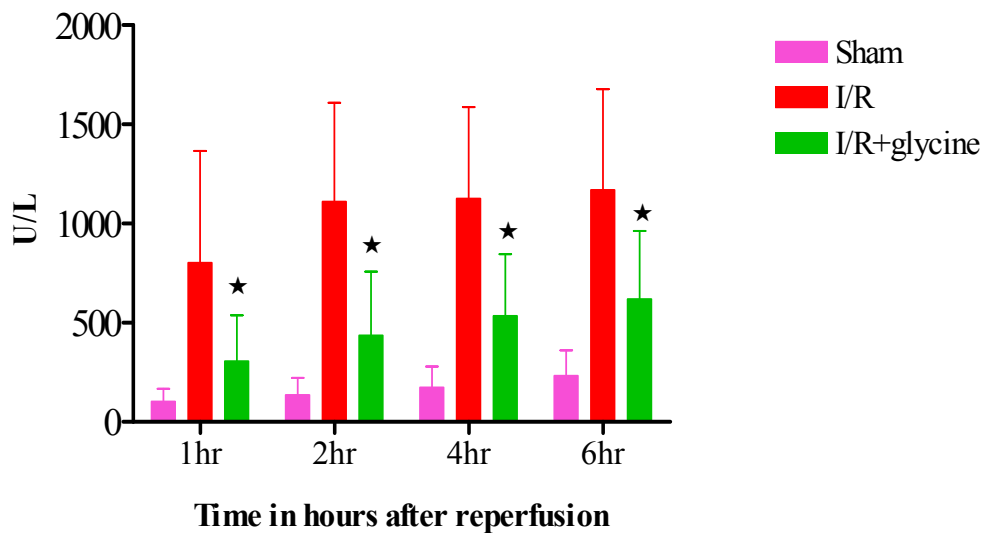


* = $p < 0.05$; Glycine+I/R v/s I/R alone

6.3.4 Serum AST and ALT

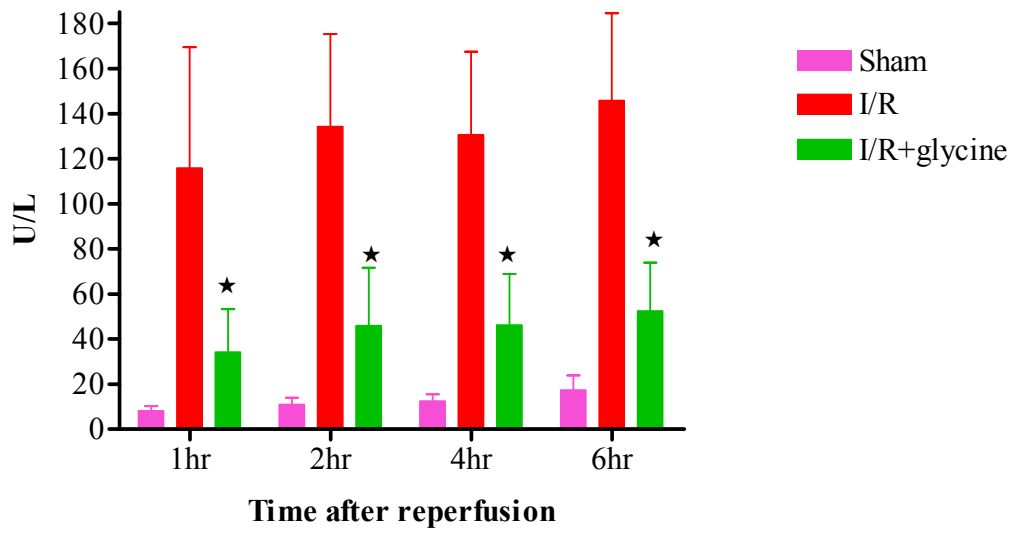
Serum AST and ALT levels steadily increased in the sham operated animals over the course of the experiment. IR alone significantly increased the transaminase levels as compared to sham operated group. In the Glycine treated group, a significant reduction in serum transaminases was observed throughout the entire reperfusion period. (fig 6.3 and 6.4)

Figure 6.3: Serum aspartate transaminase



* = $p < 0.05$; Glycine+I/R v/s I/R alone

Figure 6.4: Serum alanine transaminase



* = $p < 0.05$; Glycine+I/R v/s I/R alone

6.4 : Discussion

Glycine is a nonessential amino acid and an inhibitory neurotransmitter, which has been shown to modulate KC activity. Oral glycine has been shown to reduce warm IRI in different organs in a variety of experimental animal models (179;256;418;432;440).

In this study an established rabbit lobar warm IRI model (134;145) was used with a sham group to allow the effects of prolonged surgery and anaesthesia to be evaluated. The employment of a tracheostomy permitted a stable haemodynamic model which allowed assessment under prolonged anaesthesia(134;145). A time period of 6 hours of reperfusion was chosen in order to allow assessment of changes not only in the early phase but also during the beginning of the late phase of IRI(114;187). In the sham operated animals the systemic haemodynamic parameters remained stable throughout the duration of the operation.

TNF- α is one of the main cytokines released by KC and has a key role in early liver IRI (72;184;405). Glycine reduced the cytokine release following I/R suggesting direct KC inhibition as previously documented(210). IL-8 is another cytokine released by activated neutrophils and KC which is responsible for neutrophil activation and recruitment thereby promoting the second more sustained phase of cellular injury following liver IRI (114;187;191;351). In this study preconditioning with intravenous glycine prior to Ischaemia significantly reduced IL-8 levels, an effect of glycine not previously reported.

Serum AST and ALT are cytosolic enzymes which are markers of the severity of hepatocellular injury (134;145;160;291;292) . In this study the significant reduction in the serum levels of these enzymes in the glycine treated group confirms the cytoprotective effect of glycine in liver IRI.

6.5 : Conclusion

In conclusion, a single dose of intravenous glycine improved the early phase of hepatic IRI in this experimental model. There was sustained amelioration of two key cytokines involved in the early pathophysiology of liver IRI. The significant improvement in liver enzymes would be suggestive of hepatocellular protection conferred by the single intravenous dose of glycine.

Liver IRI significantly impacts on bile flow and bile composition. In fact bile flow has been used as a marker of liver function and recovery following ischaemia in experimental studies(31;142) and clinical liver transplantation(122;233) and persistently low bile output is considered to be a sign of primary non function(233). In the next chapter the next set of experiments elucidates the effect of glycine on bile flow and bile composition in the same animal model.

Chapter 7

Bile flow and bile (^1H NMR) spectroscopy in liver ischaemia reperfusion injury and the effect of glycine

7.1: Introduction

Bile formation is an active secretory process both of individual hepatocytes and of integrated biliary transport mechanisms (31) that is used as an indicator of liver function and recovery following ischaemia in experimental studies (31;142) and clinical liver transplantation (122;233).

IR injury has been shown to manifest by poor bile flow in vitro(167) and in vivo (31;142) and reduced bile acid secretion. However, gradual recovery occurs in reversibly damaged organs while persistently low bile output is considered to be a sign of primary non function (233). Little is known, however, about changes in bile composition during liver IRI and infact very little is known about the effect of therapeutic strategies to ameliorate IRI on bile composition, which will be the primary focus of this study. Bile salts are a major component of bile and significantly influence bile flow and secretion of biliary lipids such as phosphatidylcholine (PC, also known as lecithin) and cholesterol (107).

Bile salts are transported across the canalicular membrane in a primary active ATP dependent fashion (135;366) which is mediated by the bile salt export pump (BSEP) (287). BSEP is a genuine ATP-binding cassette (ABC) transporter according to the classic paradigm of 12 transmembrane spanning domains and ATP-binding folds, similar to ABC transporters expressed in many tissues and involved in active outward transport of molecules across the plasma membrane (107).

In recent years, high resolution proton nuclear magnetic resonance (¹HNMR) 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(356). This technique is robust, rapid, noninvasive and can detect metabolites present at millimolar concentrations and can be applied to detect unexpected compounds in biological fluids (141).

In bile, it has been used to confirm the hydrophobic association in micelles of conjugated bile acids and biliary lipids (355) and also to assess biliary lipid dynamics and the structure of micelle formation (362). It has also been used to assess the effects of cholesterol on the fluidity of human gallbladder bile (108;343). It has been also used to quantitatively determine the amount of total and taurine conjugated bile acids in bile obtained from rats (182) and to identify the lipid content of cellular or mitochondrial plasma membranes (304). ¹H NMR has also been used to assess hepatic glutamine metabolism (399), hepatic energetic status after warm ischaemia (323) and to study the effect of IR on liver cell membranes (165). In liver transplantation, an initial study of ¹H NMR analysis applied to T-tube 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 lactate and acetate (306). More recently there has been growing interest in attempting to predict graft function by analysing bile production immediately after transplantation (268)

Therefore, the aim of this study was to investigate the effect of liver warm IRI on bile flow and composition and the effect of glycine a KC modulant on bile composition and flow in an invivo established animal model of partial lobar liver IRI. We propose to study bile flow and bile acid levels as surrogate markers of synthetic function. To assess impact of IRI on hepatocellular injury we plan to assess levels of lactate and phosphatidylcholine (PC) in bile. Biliary acetate, pyruvate, glucose, and acetoacetate will serve as metabolic indicators of hepatocellular function.

7.2: Animal model and methods:

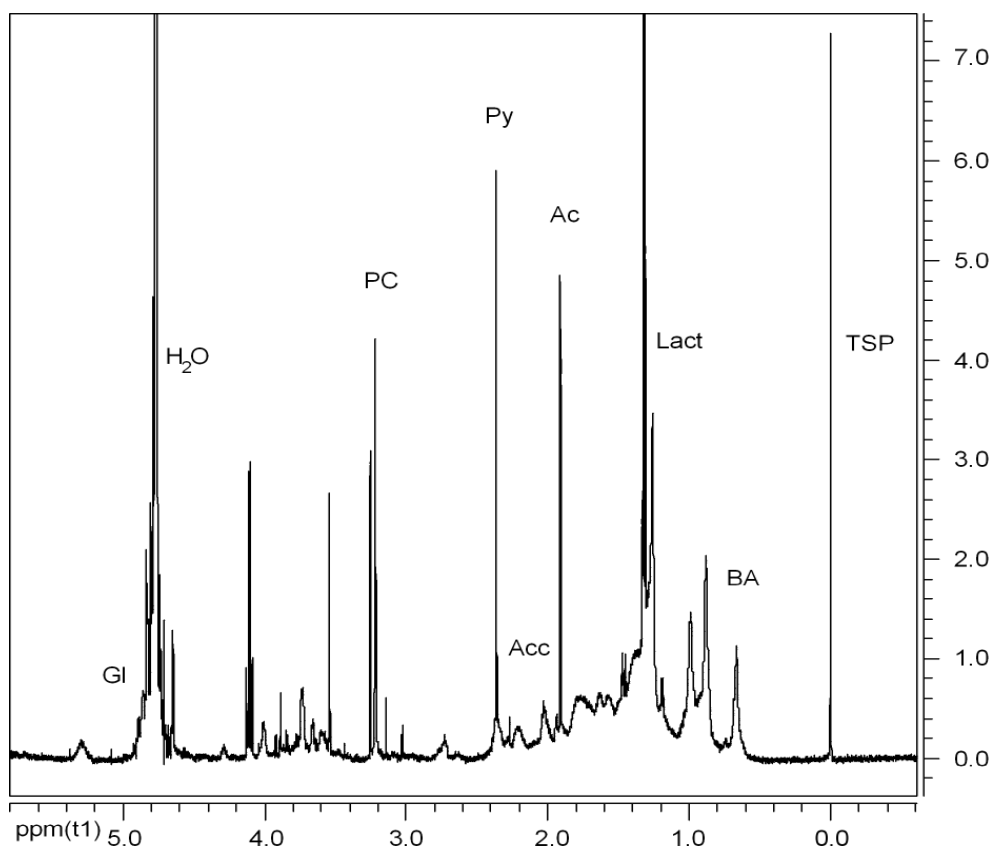
The animal model and groups including the various methods used in this study have been described in detail in chapter 4 section 4.1, 4.2, and 4.10.

7.2.1: Bile flow and proton nuclear magnetic resonance ^1H NMR spectroscopy

The bile duct was cannulated with a polyethylene catheter (PE-50, 0.58mm inner diameter, Portex, Kent, UK) for continuous measurement of bile flow. Bile samples were taken at baseline and each hour thereafter and stored at -80°C . Bile volume was expressed as $\mu\text{L}/\text{min}/100\text{g}$ of liver weight. ^1H NMR 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 [$^2\text{d}_4$] - trimethylsilylpropionate (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, 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 and 32 k data points in 64 scans were collected in both normal and spin echo mode. Pre saturation 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 Santiago de Compostela, Spain). The various markers studied included synthetic functional markers (bile flow and bile acid synthesis), injury markers (lactate and PC levels) and metabolic indicators (acetate, pyruvate, glucose and acetoacetate). All spectra were integrated

using a fixed range for each peak and published peak assignments (268). Baseline control bile spectra are shown in fig 7.1

Figure 7.1: ^1H NMR spectrum of baseline control bile



Key: TSP=sodium trimethylsilyl-[$^2\text{H}_4$] propionate standard, BA=bile acid, Lact=lactate, Ac=acetate, Acc=Acetoacetate, Py=pyruvate, PC=phosphatidylcholine head group, H₂O=water, Gl=glucose.

7.2.2: Data collection and statistical analysis

Results are presented as mean \pm standard deviation (SD). The statistical analysis was performed using a one-way analysis of variance (ANOVA) with multiple comparisons adjusted for by the Bonferroni test. Statistics were calculated using commercially available software (SPSS release 11.0.0; SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

7.3: Results

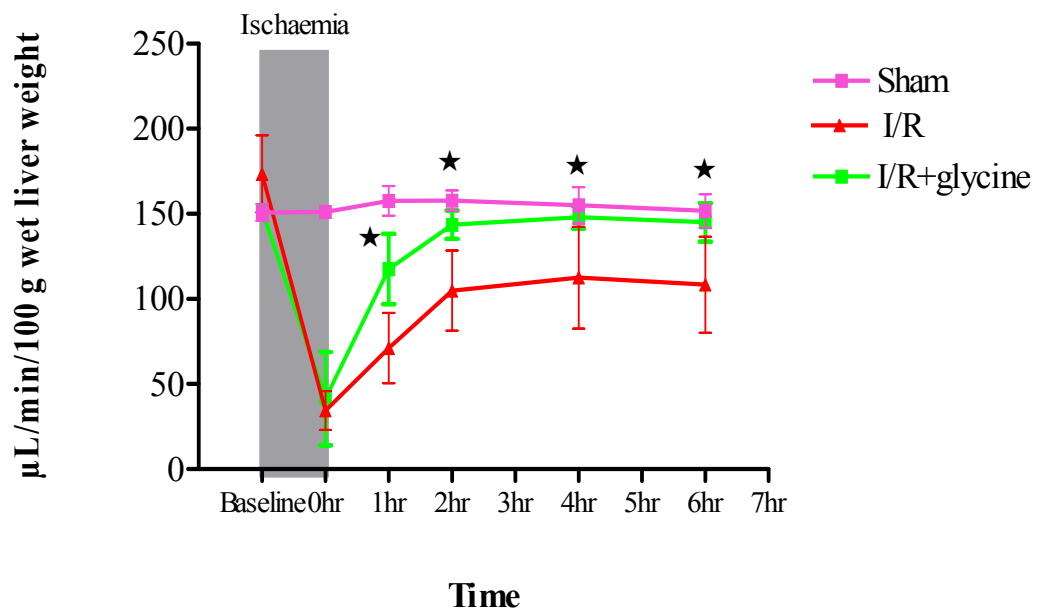
7.3.1: Systemic haemodynamic parameters

The results for systemic haemodynamics parameters are as described previously in chapter 5, section 5.3.1 and figures 5.1 and 5.2.

7.3.2: Bile flow

Baseline bile flow was similar in all groups (150 μ L/min/100g wet liver weight). Bile flow remained stable in the sham group throughout the duration of the experiment. Bile flow was reduced with ischaemia and improved during the reperfusion period. The glycine IR group showed a significantly increased bile flow throughout the reperfusion period as compared with IR alone group (Fig 7.2).

Figure 7.2: Bile flow

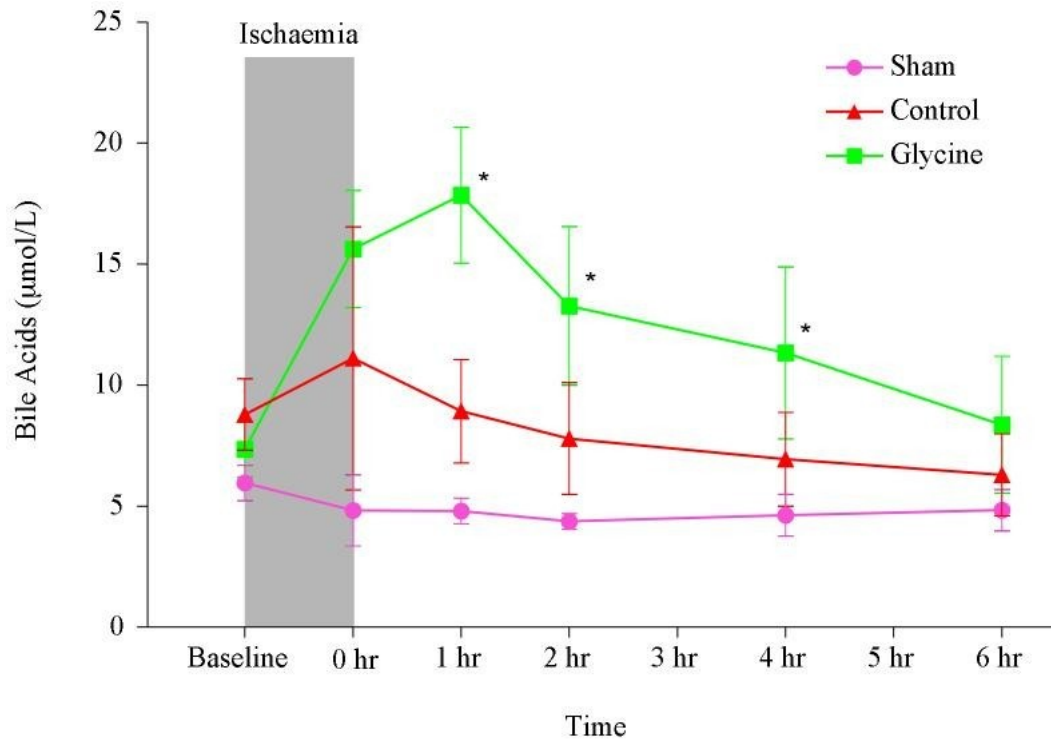


* = $p < 0.05$; Glycine+I/R v/s I/R alone

7.3.3: Bile Acids:

The results of the integration of the area under curve for the peaks assigned to conjugated bile acids in the sham group bile were relatively unchanged throughout the experiment at $4.91 \pm 0.95 \mu\text{mol/L}$ (fig. 7.3). Control group bile acid levels tended to be higher than shams throughout the experiment ($P=0.002$ when total group means were compared), however, they did not reach statistical significance when compared at each time point. In the glycine group bile acid levels rose significantly following Ischaemia to peak during the 1st hr of reperfusion, where they were significantly higher than both shams ($P<0.001$) and controls ($P=0.001$). They remained significantly elevated, compared to both shams and controls, during the 2nd and 4th hr of reperfusion ($P<0.05$).

Figure 7.3: Bile acids levels following warm liver I/R and glycine

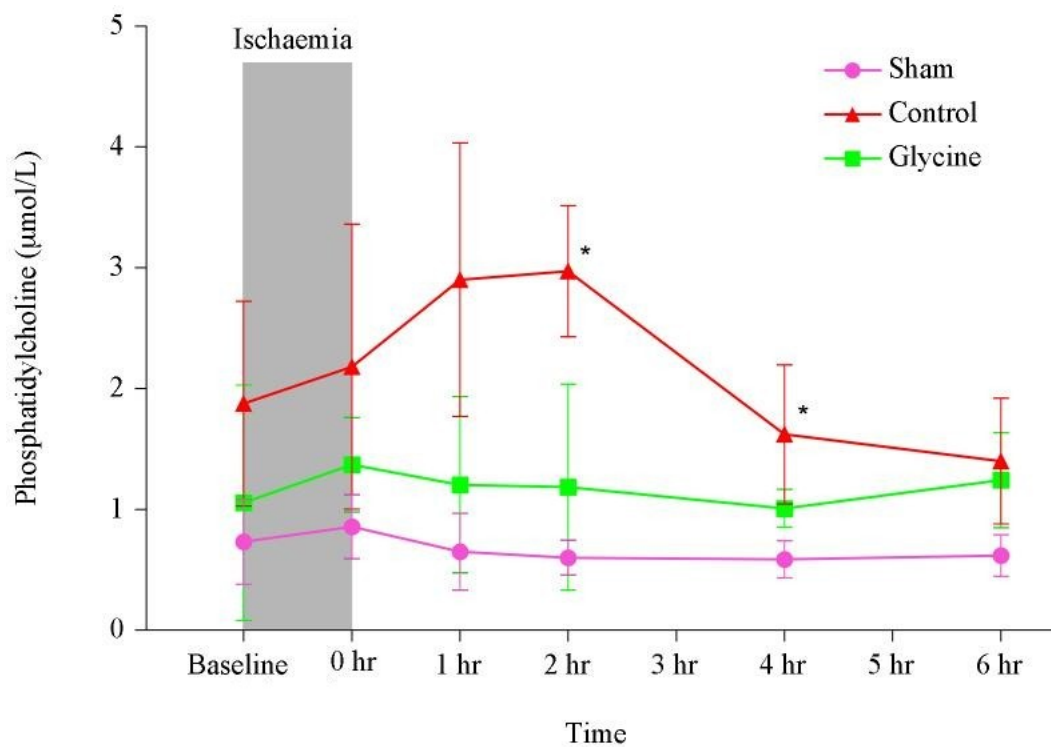


Bile acid levels were calculated by integration of peaks at 0.61-0.73 ppm and expressed as $\mu\text{mol/L}$; * = $p<0.05$; Glycine+I/R v/s I/R alone

7.3.4: Bile Phosphatidylcholine

Integration of peaks corresponding to phosphatidylcholine (PC) revealed no difference between glycine and sham groups throughout the experiment (**fig.7.4**). PC levels in the control group, however, increased gradually till the 2nd and 4th hr of reperfusion where they were significantly higher than controls and shams ($P<0.05$).

Figure 7.4: Bile phosphatidylcholine levels following warm liver I/R and glycine

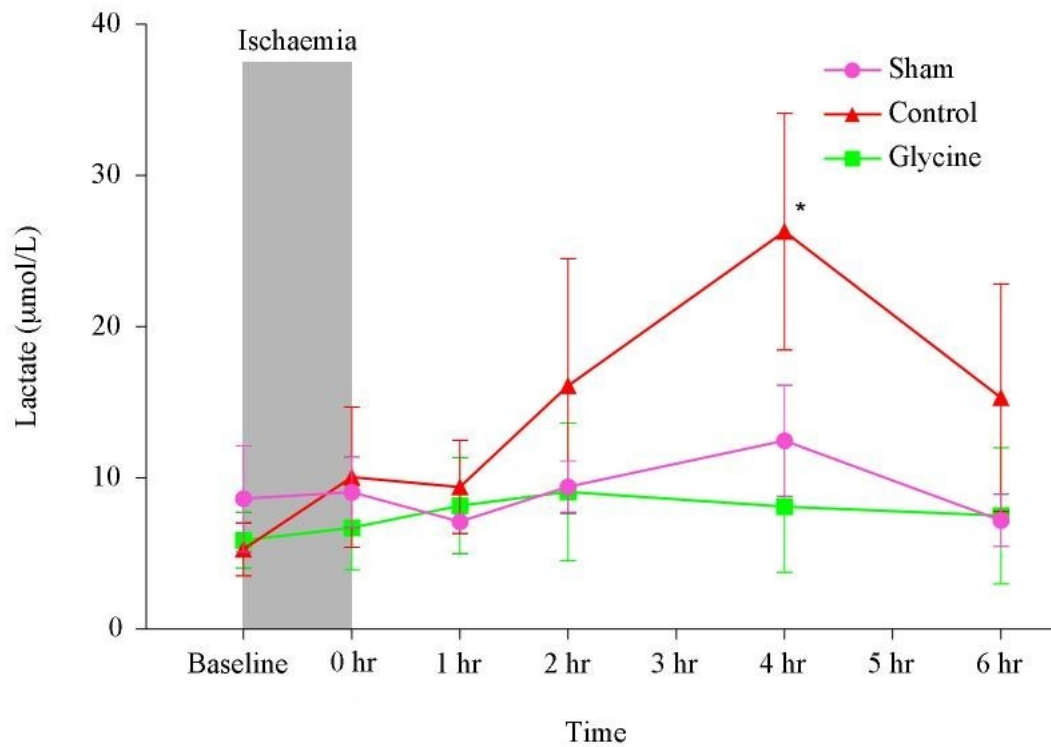


Phosphatidylcholine levels were calculated by integration of peaks at 3.2 ppm and expressed as µmol/L, * = $p<0.05$; Glycine+I/R v/s I/R alone

7.3.5: Bile Lactate

Similarly, there was no statistical difference in bile lactate levels between glycine and sham groups during the procedure (fig. 7.5); however, in the control group they increased, following reperfusion, to peak significantly at 4 hrs ($P < 0.001$ vs. controls).

Figure 7.5: Bile lactate levels following warm liver I/R and glycine

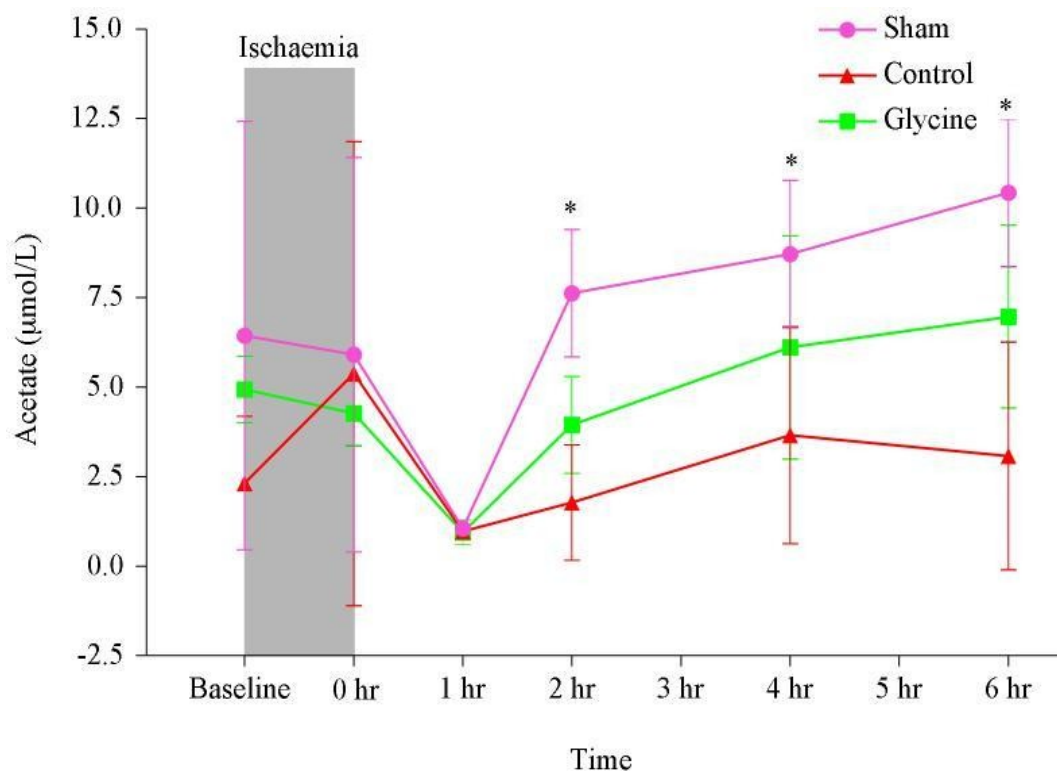


Lactate levels were calculated by integration of peaks at 1.33 ppm and expressed as $\mu\text{mol/L}$; * = $p < 0.05$; Glycine+I/R v/s I/R alone

7.3.6 Bile acetate

There was no statistical difference in bile acetate concentration between sham and glycine groups throughout the experiment (**fig. 7.6**). Acetate levels for all groups fell dramatically at 1 hr reperfusion. The sham and glycine groups regained their baseline values by the end of reperfusion and were not statistically different, while in the control group they remained depressed compared to glycine ($P < 0.01$).

Figure 7.6: Bile acetate levels following warm liver I/R and glycine

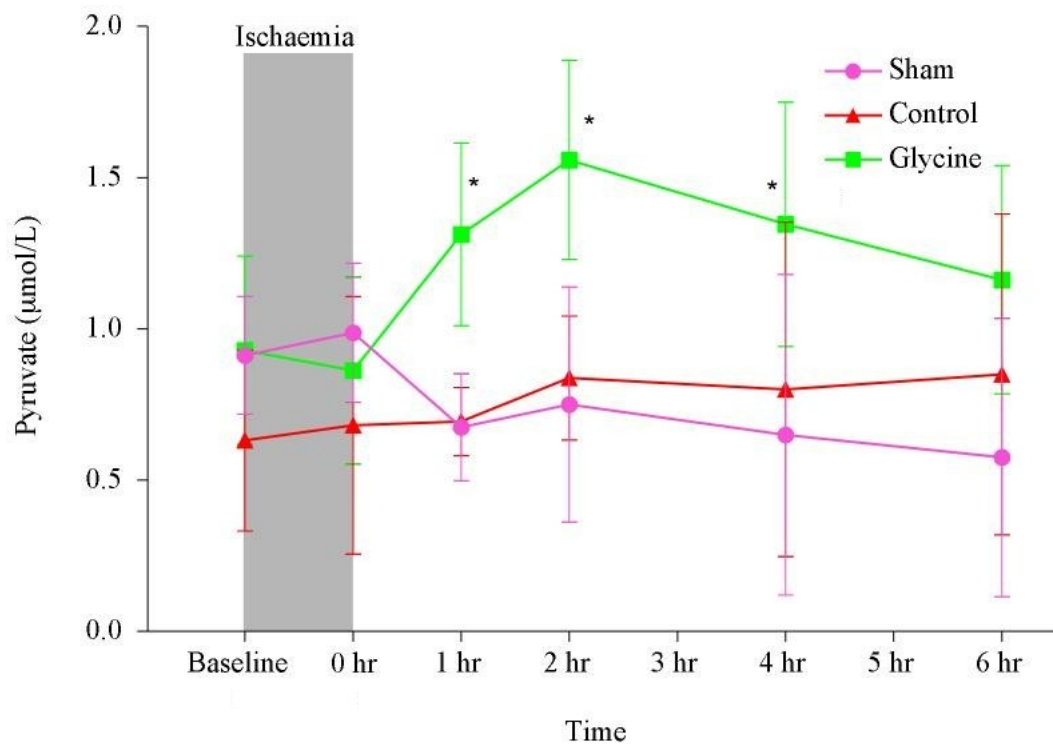


Acetate levels were calculated by integration of peaks at 1.9 ppm and expressed as $\mu\text{mol/L}$, * = $p < 0.05$; Glycine+I/R v/s I/R alone

7.3.7 Bile Pyruvate

Bile levels of pyruvate in control and sham groups were similar throughout the experiment (**fig. 7.7**). In the glycine group, however, they were significantly elevated during the 2nd and 4th hr of reperfusion compared to both sham and control groups ($P<0.05$).

Figure 7.7: Bile pyruvate levels following warm liver I/R and glycine

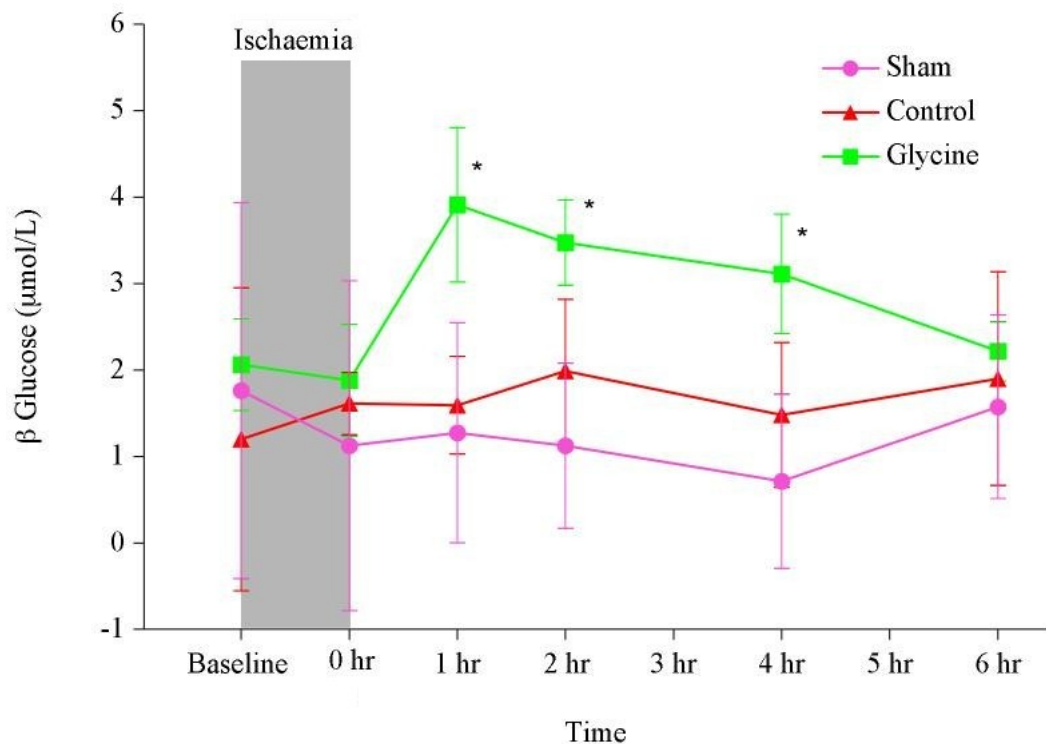


Pyruvate levels were calculated by integration of peaks at 2.3 ppm and expressed as µmol/L, * = $p<0.05$; Glycine+I/R v/s I/R alone

7.3.8 Bile Glucose

There was no statistical difference between bile β -glucose levels in sham and control groups throughout the experiment (**fig. 7.8**). However, there was a significant rise in β -glucose levels in the glycine group from the 1st hr of reperfusion until the 4th hr of reperfusion ($P<0.05$).

Figure 7.8: Bile glucose levels following warm liver I/R and glycine

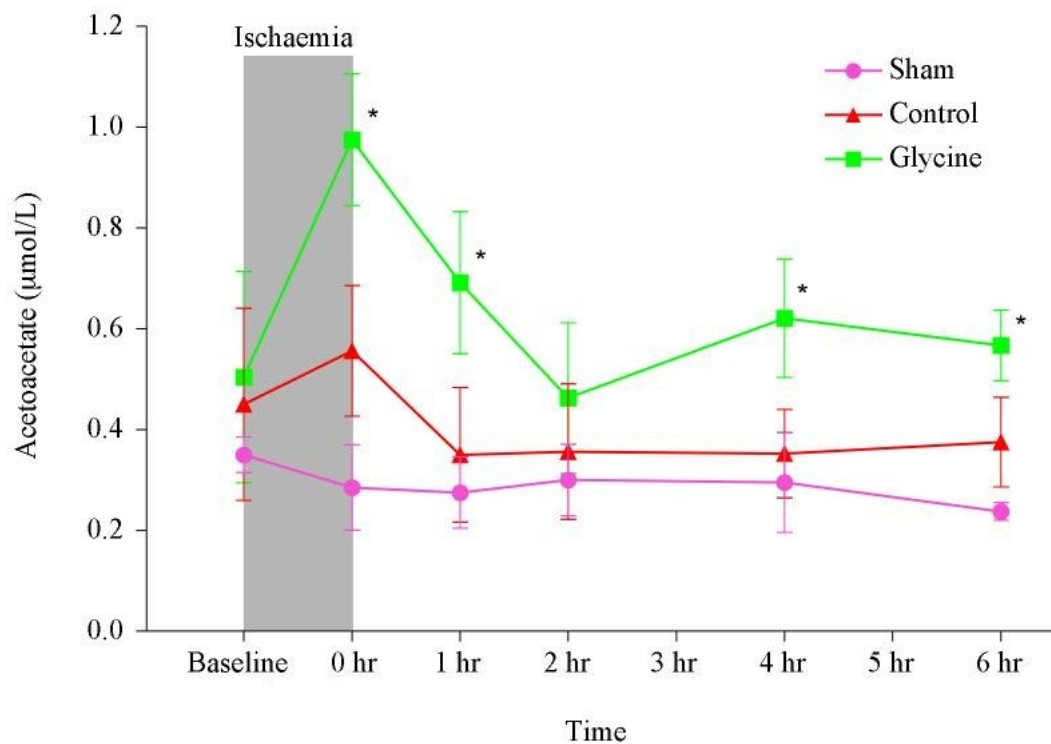


Glucose levels were calculated by integration of peaks at 5.2 ppm and expressed as $\mu\text{mol/L}$, * = $p<0.05$; Glycine+I/R v/s I/R alone

7.3.9 Bile Acetoacetate

Acetoacetate levels were not statistically different for sham and control groups during the experiment (**fig. 7.9**). The glycine group, however, was significantly elevated during the ischemic phase, 1st, 4th and 6th hr of reperfusion when compared to both sham and control groups ($P < 0.05$).

Figure 7.9: Bile Acetoacetate levels follow warm liver I/R and glycine



Acetoacetate levels were calculated by integration of peaks at 2.2 ppm and expressed as µmol/L; * = $p < 0.05$; Glycine+I/R v/s I/R alone.

7.4: Discussion

This is the first time that ¹H NMR analysis has been applied to study the effect of glycine on bile constituents in a controlled experimental model of liver I/R. The use of spin echo spectra in this study, allowed us to reduce the interference from broad lipid signals as well as identify and partially quantitate and compare bile constituents more accurately. Although the bile production in glycine and sham groups after reperfusion was similar, ¹H NMR spectroscopy of bile revealed differences in the levels of some bile constituents.

In the present study, a rabbit lobar I/R model was used with reperfusion period of six hours(101), which 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 was chosen, rather than one of total hepatic Ischaemia, as this avoids the production of acute portal hypertension and intestinal Ischaemia. In addition, lobar clamping avoids the complete occlusion of biliary drainage in the total hepatic Ischaemia model(196) and thus permitting the effects of I/R on biliary composition to be evaluated. Tracheostomy, ventilation and the use of Isoflurane, (which is metabolised in the lungs rather than in the liver) was useful in haemodynamically stabilising the model. and permitted bile constituents to be analysed into the late phase of I/R. This is supported by the relative stability of the sham findings.

Bile flow was maintained throughout the procedure in the sham group, while it fell during the ischemic phase in the control and glycine I/R groups. This is consistent with the results of previous studies(31;101) . However, in the glycine group, bile flow on reperfusion recovered to become similar to sham within 2hrs of reperfusion, while in the control group remained depressed until the end of the experiment. Moreover, a rise in bile acid secretion was also noted on reperfusion, in the glycine group, compared to controls. This suggests an early recovery of metabolic activity in

the glycine-treated livers, which is supported by observations that total serum bile acids were found to be early markers of liver function in porcine(170) and canine(131) liver transplants. The rise in bile acid observed during Ischaemia may be a result of the drop in bile flow.

In this study, lactate levels were higher in the control group than in the sham and glycine groups. ATP degradation during ischaemia leads to the activation of glycolysis, resulting in the net formation of lactate(318;424). It has also been shown that glycogen concentration decreased as a function of the length of the ischemic period. This suggests that serum lactate may be a product of the glycolytic/gluconeogenic pathway, which was verified by showing glucose-6-phosphate and fructose-6-phosphate to increase with I/R injury(301). However, the end product of this pathway is normally pyruvate, and lactate is not produced except during anaerobic situations(5). It is interesting that this rise in lactate is observed at 4hrs reperfusion, this corresponds to a large burst of neutrophil activity that constitutes the beginning of the late phase of Ischaemia-reperfusion injury(64). 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 (64) (194). Moreover, elements of neutrophil sequestration, sinusoidal narrowing and vasoconstriction combine to form a “no-reflow paradox” where by hepatocytes are subjected to a further perfusion deficit and persistent ischaemia (64;194) . 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 (283).

In this study, PC was observed to be similar in sham and glycine groups, while it was raised in the control group during the 2nd and 4th hr of reperfusion. The main source of phosphatidylcholine in cells is in the plasma membrane, which suggests that the

post-reperfusion increase may be associated with an increase in cellular breakdown and membrane lipid peroxidation (368). Increased PC has also been observed in association with poor graft function (268).

These results show an increase in post-reperfusion levels of biliary glucose and pyruvate on reperfusion in the glycine group compared to controls. This suggests an increased metabolic activity in the glycolytic/gluconeogenic pathway, which may be due to conserved cellular energetics. Hepatocytes are one of the few cells that can utilize fatty acids in the production of energy through the β -oxidation helix, however, this reaction requires ATP at its initiation(5). Ischaemia-induced reduction of ATP may result in a decreased ability to utilize β -oxidation causing acetate production to falter. The results show that glycine-treated livers possessed a greater ability to produce acetate than controls, which further suggests less injury to their metabolic energetics.

After reduction of hepatic blood flow, the mitochondrial redox potential is reduced. Consequently, there is accelerated production of ketone bodies to compensate for inhibited glucose utilization. Ketone body production is an alternative pathway to supply ATP when there is inhibition of glucose oxidation and the Krebs cycle. Recently, higher acetoacetate perfusate levels have been demonstrated in isolated-perfused rat livers preserved in University of Wisconsin versus Euro-Collins solution (141) which has been shown to correlate with NAD^+/NADH ratio in the hepatocyte mitochondria (421). The elevated levels of acetoacetate observed in this study, during ischaemia and reperfusion, suggests an early attempt by glycine-treated livers at utilising an alternative source of ATP. This finding further supports the suggestion of conserved cellular energetics in this group.

7.5: Conclusion

Bile spectroscopy has demonstrated significant changes in bile composition during glycine administration. These changes are evident despite a constant post-reperfusion rate of bile flow. Glycine treatment was associated with increased synthetic functional markers (bile flow and bile acid synthesis), decreased injury markers (lactate and PC levels) and enhanced metabolic indicators (acetate, pyruvate, glucose and acetoacetate).

The experimental data suggests that glycine ameliorates not only the haemodynamic changes and cytokine surge associated with IRI, but also significantly alters the bile composition and maintains bile flow during the early and the initial part of the late phase of hepatic IRI. The possible mechanism of action is maintenance of cellular energy production through amelioration of the systemic inflammatory response.

However, the early pathophysiology of IRI would suggest that along with KC; ROS also play a key role in modulating the subsequent cellular events as reviewed in chapter 1. In order to ameliorate IRI secondary to ROS production, we have assessed the effect of prophylactically administered N-acetylcysteine, a antioxidant on liver IRI in patients undergoing elective liver resection within a pilot randomised controlled clinical trial in the next chapter. NAC has been widely used in clinical medicine however; it has never been used in this setting before.

Chapter 8

Effect of N-acetylcysteine in hepatic resections:

A pilot prospectively randomised double blind

controlled clinical trial

8.1: Introduction

Surgical resection is the gold standard treatment for patients with cancers of the liver, with acceptable morbidity (20%-30%) and mortality (0.6%-5%) (21;125;325). Minimizing blood loss during surgery improves clinical outcome (100;275;325). Total hepatic inflow occlusion (Pringle's manoeuvre) can reduce operative blood loss, but leads to warm ischaemia-reperfusion (I/R) and pooling of portal blood. Warm I/R along with loss of liver volume leads to postoperative hepatocellular dysfunction (22;23;176). Warm I/R injury also occurs due to intraoperative mobilisation and compression of the liver due to retraction (327;331). Liver I/R injury is a significant cause of morbidity and mortality in these patients (92;250;427).

Liver ischaemia causes depletion of ATP with subsequent impairment of energy dependant intracellular homeostatic mechanism (67;236;340;386). Reperfusion leads to microcirculatory disturbances due to cellular oedema, accumulation of toxic oxidative radicals, cytokines, and up regulation of vascular adhesion molecules accompanied with depletion of intracellular glutathione stores which affects cellular metabolism and hepatocellular function (114;236;340;386). Reactive oxygen species (ROS) and adhesion molecules particularly ICAM-1 have a major role in the development of I/R injury (106;109;172;243;259).

Antioxidant agents have been shown to improve haemodynamic variables and outcome in early liver failure of varied aetiology (57;144;146;163). N-acetylcysteine(NAC) is one such agent which has been extensively used in clinical medicine for more than 25 years as a mucolytic agent, as a protective agent against the toxicity of drugs used for cancer and as a effective antidote in paracetamol poisoning (62;93;144;146). The main mechanisms of action are: a) conversion to cysteine, b) replenishing the depleted intrahepatic glutathione stores and c) direct scavenging of reactive oxygen and nitrogen species (76) .

Clinical studies in situations associated with oxidative stress such as cardiac I/R(76) and acute renal failure from contrast media(380) have shown protective effects with NAC administration.

In liver graft preservation for transplantation using NAC alone(408) or in combination with Prostaglandin E1 have shown reduced peak serum ALT levels with shorter hospital stay and reduced severity of rejection episodes in the treatment group as opposed to control group(39). Experimental work studying the effect of NAC in warm I/R injury has shown promising results(132;134;145;221;381). Experimental studies have shown that NAC inhibits the stimulated increase in ICAM-1 and VCAM-1 expression on endothelial cells in vitro(262;317) and leukocyte – endothelial interactions in rat liver transplantation(232;278). A clinical study by Weigand et al, in patients who underwent Orthotopic liver transplantation showed that NAC administration attenuated the increase in circulating ICAM-1 and VCAM-1 24 hrs after reperfusion of the donor liver(408).

In liver transplant patients, increased expression of ICAM-1 in the liver is implicated in allograft rejection(1;2) and it is possible that attenuation of the postoperative increase in ICAM -1 expression by NAC exerts protective effects with respect to longer –term graft survival.

There are no clinical studies published to evaluate this effect of NAC in patients undergoing liver resection. A pilot prospective randomised trial was designed to evaluate the effect of NAC on liver function following liver resection and on ICAM-1 expression. Sample size calculation was based on achieving a 50% reduction in ICAM-1 in NAC treated subjects. To demonstrate such a difference with 80% power at the 5% level, 15 subjects in each group were required.

ICAM-1 was used to power the study since;

- 1) This was a pilot study, hence the use of a very sensitive endpoint to reduce patient numbers required for the trial.
- 2) Elevated ICAM-1 expression has been shown to promote junctional and nonjunctional transendothelial migration across inflamed vascular endothelium (431) (423).
- 3) Previous experimental work has shown that NAC inhibits the stimulated increase in ICAM-1 and VCAM-1 expression on endothelial cells in vitro(262;317) and leukocyte – endothelial interactions in rat liver transplantation(232;278), and also attenuated the increase in circulating ICAM-1 and VCAM-1 24 hrs after reperfusion of the donor liver(408).

8.2: Patients & Methods:

8.2.1: Patients and Consent

Sequential patients with resectable liver cancers over a 2 year period were recruited into the trial. The study was approved by the local ethical committee and all patients gave written informed consent to participate in the study.

8.2.2: Randomisation issues

Randomisation was by computer generated list with treatment groups concealed in opaque sealed envelopes and the randomisation code was not known to patients or investigators (including the entire clinical team involved in patient care) until the study was finished. Randomisation was carried out by the Pharmacy department (Royal Free Hospital) and infusions were prepared on a named patient basis for the trial purposes and all infusions were labelled for NAC trial. Patients were randomized to receive NAC (Parvolex[®]; Medeva Pharma, Ashton-under-Lyne, Lancashire, UK), as an iv infusion (150mg/kg in 250mls of 5% dextrose loading dose at the beginning of the operation, followed by 50mg/kg in 500mls of 5% dextrose over 4 hours and 50mg/kg in 500ml of 5% dextrose over 8hours) or a placebo (equivalent volume of 5% dextrose) over the same time.

8.2.3: Resection Procedure:

All patients underwent resection under the care of one primary surgeon (BRD) with the same anaesthetic team. All patients received epidural infusion for analgesia, large bore central lines for IV access and arterial line for intraoperative monitoring. All patients received 1 litre of crystalloid at induction and intraoperative fluid administration was adjusted to maintain a urine output of $\geq 1\text{ml/kg/hr}$.

All resections were performed with complete liver mobilisation appropriate to the planned resection after assessment with intraoperative US scan and preoperative staging scans. Inflow to the proposed resection segments was divided prior to

parenchymal division. Parenchymal division was performed with CUSA[®] (Valleylab[®], TYCO Healthcare, Hampshire, UK) and meticulous haemostasis was achieved by suture ligation and diathermy of hepatic arterial & venous branches. All patients had low CVP (≤ 5 mm Hg) anaesthesia at time of parenchymal division and inflow occlusion was not routinely used during parenchymal division. Intraoperative transfusions were avoided unless the haematocrit was $\leq 30\%$ and blood products (i.e. FFP and Platelets) were transfused as guided by intraoperative assessment of coagulopathy by thromboelastography (TEG)(161).

8.2.4 Clinical end points

All patients had full blood count, renal function and liver function tests including coagulation profile prior to the surgery, just prior to parenchymal division, at the end of the operative procedure and daily up to 9th day postoperatively. Demographic data, blood loss, transfusion requirements, serum lactate levels (lactate-1= immediate preoperative, lactate-2=just prior to parenchymal resection, and lactate-3= at end of resection), and postoperative morbidity and mortality data were also recorded.

8.2.5: Immunohistochemistry for ICAM-1

Protocol liver biopsies were obtained with a trucut needle from the future liver remnant upon laparotomy prior to liver mobilisation or handling(biopsy-1) and the second protocol biopsy was procured at the end of the operation from the same lobe of the remnant liver (biopsy-2).

Biopsy samples were put on Optimum cutting temperature (OCTTM) (Tissue-Tek[®], Sakura Finetek, Berkshire, UK) covered cork, snap frozen in isopentane and then liquid nitrogen and stored at -80° until further analysis. Frozen sections of a thickness of 5 microns were cut using a standard cryostat.

Each section was placed on PLL coated slides, air-dried for 1 hour and then fixed in acetone at room temperature for 10 minutes. Blocking of endogenous alkaline

phosphatase (addition of levamisole to the Vector Red chromagen) and non-specific binding of protein was carried out by covering sections with normal rabbit serum diluted 1/10 in Phosphate buffered saline(PBS) for 20 minutes at room temperature. Sections were incubated with primary antibody (ICAM-1 (CD54) mouse monoclonal antibody, Novocastra, UK) for 1 hour at room temperature (dilution 1:800). Sections were washed with PBS and incubated with rabbit anti mouse antibodies (DAKO, Ely Cambridgeshire, UK) diluted 1:25 in PBS for 30 minutes in a humidity chamber. Sections were washed in buffer and covered with mouse APAAP (DAKO, Ely Cambridgeshire, UK) diluted 1:25 in PBS for 30minutes. Sections were developed with freshly prepared Vector red chromagen for 10 minutes, washed in PBS and then running water, counterstained with Mayer's haematoxylin, differentiated in 0.5% acid alcohol, dehydrated, and mounted in synthetic mountant.

Endothelial cells in capillaries of muscularis propria of intestinal wall were used as positive control. Sections stained without the primary antibody was used as negative controls. All samples were run in duplicate with positive and negative controls for each batch to reduce inter/intra assay variability. This method of ICAM-1 assessment has been reported previously and validated (104-106).

The histological examination was carried out by a pathologist (AQ) without knowledge of the treatment group or whether the individual biopsy was pre or post resection. The distribution of ICAM-1 staining was assessed in terms of: 1) staining of endothelial cells (sinusoids and portal vein endothelium), Kupffer cells, hepatocytes, bile duct epithelial cells and lymphocytes; 2) in terms of pattern of sinusoidal endothelial cells staining (i.e. patchy when sinusoids staining for ICAM-1 alternated with sinusoids not staining for ICAM-1 in the same lobule. Diffuse when all sinusoids in the same lobule stained for ICAM-1). Diffuse staining in one lobule with negative staining in an adjacent lobule was considered as a technical artefact

and staining was repeated. The intensity of sinusoidal staining was assessed using a semi quantitative grading system adapted from Kiuchi et al.(219), as shown in table 8.1.

Table 8.1: Grading system for ICAM-1 expression

Grade	Intensity of staining
Grade 0	No staining/expression on endothelium or hepatocytes
Grade 1	Weak immunostaining only identifiable at high magnification (40x)
Grade 2	Thin staining/expression on endothelium and scarce expression on hepatocytes
Grade 3	Thick staining/expression on endothelium and on hepatocytes membrane with or without cytoplasm (“blurred”/ “honey comb” pattern)

Endothelial ICAM-1 was regarded as up regulated in the post-resection biopsy if:

- 1) The pre-resection biopsy showed no staining, and the post-resection biopsy showed staining of any degree;
- 2) If the pre-resection biopsy showed grade 1 staining and the post-resection biopsy showed grade 3 staining;
- 3) If the pre-resection biopsy showed patchy staining and the post-resection biopsy showed diffuse staining.

Conversely, endothelial ICAM-1 was regarded as down regulated in the post-resection biopsy if:

- 1) The pre-resection biopsy showed staining of any degree, and the post-resection biopsy showed no staining;
- 2) If the pre-resection biopsy showed grade 3 staining and the post-resection biopsy showed grade 1 staining;
- 3) If the pre-resection biopsy showed diffuse staining and the post-resection biopsy showed patchy staining.

We chose a difference of two points in intensity of staining as evidence of up or down regulation, in order to avoid overlap between semi quantitative categories due to the subjective nature of this assessment.

8.2.6: Statistics

The non-parametric Mann-Witney U test was used for comparison between the groups at each time point. Also, the postoperative values at different time points for ALT and ALP were added together for intergroup comparison. Data are presented as median (range) and $p < 0.05$ was considered significant. Comparison of proportions was used to analyse ICAM-1 results.

8.3 Results

8.3.1 Clinical outcome:

43 patients were recruited to the trial; however 12 patients were excluded since they had inoperable tumours at laporotomy. Of the remaining 31 patients 16 were randomised to the placebo group and 15 were randomised to the NAC group. The case mix of the two groups and their demographic data is depicted in table 8.1. The largest diagnostic categories in both groups were colorectal liver metastases. All patients with synchronous liver metastases had preoperative adjuvant chemotherapy and these patients underwent liver resection at least 2 weeks following their last cycle of chemotherapy. The two groups were well matched and there were no differences in age, sex, or case distribution (Table8.2).

Table 8.2: Case Mix and demographic data of the patients in the two groups of the trial

Diagnosis	Placebo Group (n=number)	NAC Group (n=number)
Colorectal liver metastases	13 (n=6 synchronous metastases)	12 (n=8 synchronous metastases)
Hepatocellular carcinoma	2	2
GI stromal cell tumour	1	0
Cholangiocarcinoma	0	1
Total	16	15
Age (mean+/- SD)	59±14	64±14
M:F ratio	1.8: 1	1.5:1

There were no significant differences in morbidity or mortality between the groups. The overall morbidity was 25% and there were no deaths in the first 30 days postoperatively in either group (Table 8.3). There were no significant differences in blood loss, transfusion requirements and serum lactate levels between the groups (Table 8.3).

Table 8.3: Morbidity, Mortality, perioperative blood loss, transfusion requirements, and serum lactate levels within the 2 groups

	Placebo Group (n=16)	NAC-group (n=15)	p-value
Morbidity	25%	27%	ns
Mortality (30 day)	0%	0%	ns
Blood Loss (in mls)	3200+/-1700	2282+/-1387	ns
Blood (units transfused)	4.2+/-2.35	3.21+/-2.8	ns
Fresh frozen plasma (units transfused)	3.0+/-1.83	3.79+/-3.6	ns
Platelets (units transfused)	0.2+/-0.4	0.3+/-0.6	ns
Serum Lactate-1*	1.51+/-0.60	1.99+/-0.73	ns
Serum Lactate-2†	2.26+/-0.80	3.27+/-1.60	ns
Serum Lactate-3‡	2.62+/-0.9	3.66+/-1.26	ns

ns = not significant

*Serum Lactate-1= Lactate levels at start of operation prior to liver mobilisation

†Serum Lactate-2= Lactate levels just prior to parenchymal transection

‡Serum Lactate-3= Lactate levels at the end of operation

Similarly there were no significant differences between the 2 study groups as to the extent of liver parenchyma resected (Table 8.4). We did not routinely use the pringle's manoeuvre during parenchymal resection. There were only 2 patients in the NAC group and only 1 patient in the placebo group where we used Pringle's manoeuvre. The inflow occlusion was for a total duration of 40 minutes with intermittent reperfusion for 10 minutes for each patient respectively.

Table 8.4: Extent of liver resection within the 2 trial groups

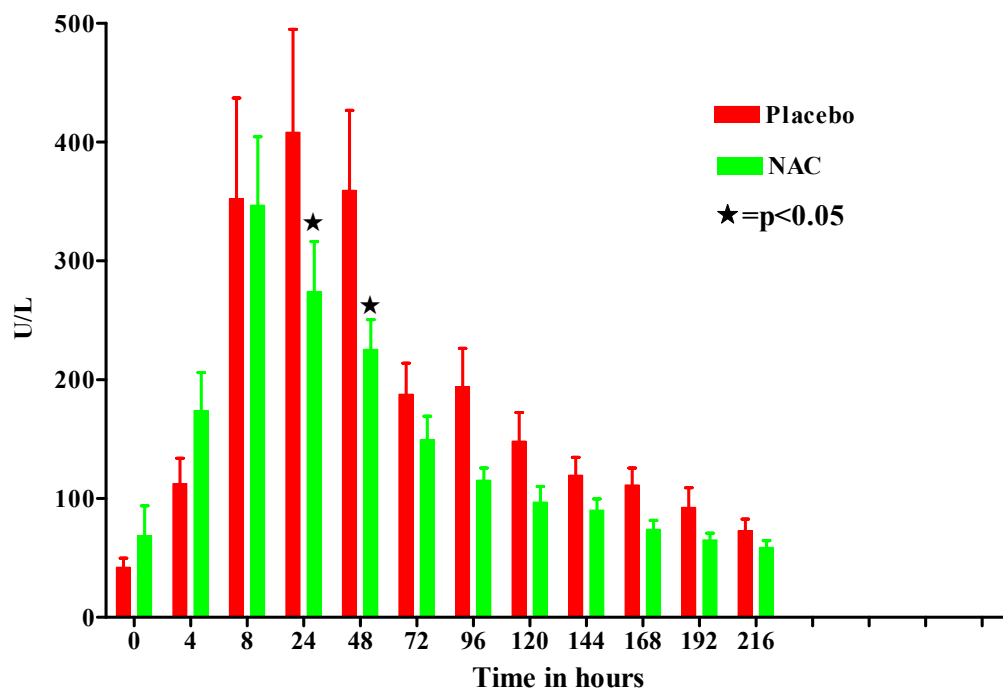
Extent of Liver Resection	Placebo group (n=number)	NAC group (n= number)
Right/left Hepatectomy	5	4
Extended Right Hepatectomy	2	4
Wedge resection (2≥ segments)	5	5
Wedge resection single segment	4	2
Total	16	15

8.3.2 Liver Function

Serum Alanine amino transferase (ALT):

ALT values in the NAC group were lower compared to the placebo group for the whole postoperative period (**Fig 8.1**). This difference was significant at 24 hours ($p=0.04$) and 48 hours postoperatively ($p=0.027$). The added ALT values of all postoperative days were also significantly lower in the NAC group ($p=0.001$).

Figure 8.1: Serum alanine transaminase

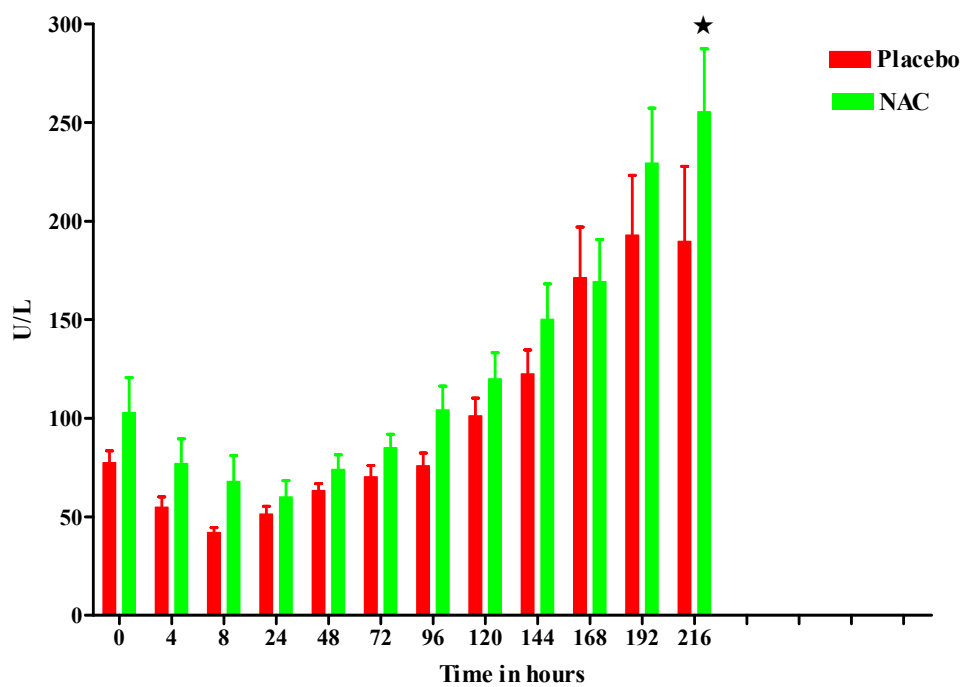


* = $p < 0.05$; NACG v/s Placebo

Serum Alkaline Phosphatase (ALP)

ALP values were consistently higher in the NAC group for the whole postoperative period studied (**Fig 8.2**). This difference reached significance in the 9th postoperative day ($p=0.05$). The overall serum ALP values for the entire postoperative period was significantly higher in the NAC group ($p=0.003$).

Figure 8.2: Serum alkaline phosphatase



* = $p < 0.05$; NACG v/s Placebo

8.3.3 Immunohistochemistry for ICAM-1

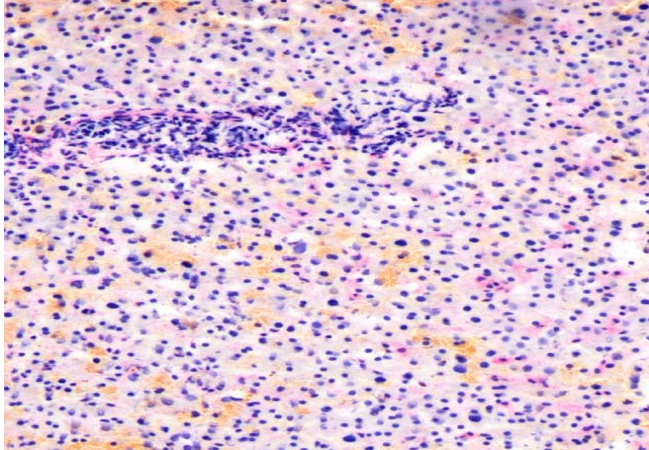
ICAM-1 was expressed predominantly in sinusoidal endothelial cells and Kupffer cells. The different grades of ICAM-1 expression are depicted in **fig 8.3**. There was no change in ICAM-1 expression, between the pre & post resection biopsies in 10/16 patients in the Placebo group and 11/15 patients in the NAC group (using the semi quantitative criteria described in methods)

ICAM-1 up regulation was observed in 6/16 patients (38%) in the placebo group and in 3/15 patients (20%) in the NAC group. ICAM-1 down regulation was observed in 1/15 patients (7%) in the NAC group and none in the placebo group. These differences were not statistically significant ($p>0.05$).

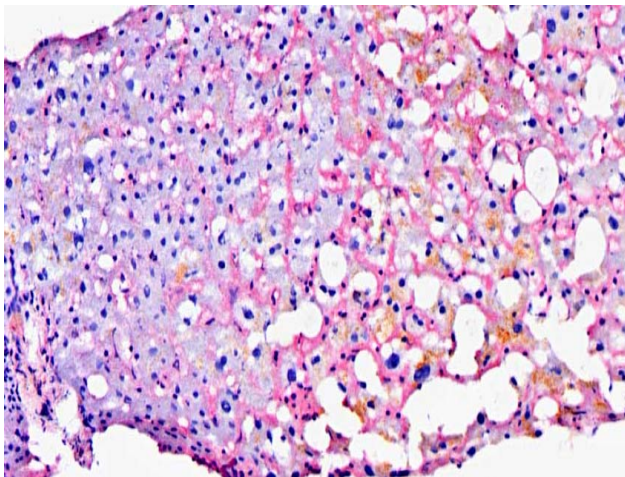
Fig.8.3: Immunohistochemistry showing ICAM-1 Expression

Light microscopy (400x) stained with Meyer's haematoxylin (blue background counter stain) and immunohistochemistry for ICAM-1 (bright pink stain) showing no ICAM-1 (a), Patchy ICAM-1(b) and Diffuse ICAM-1 (c).

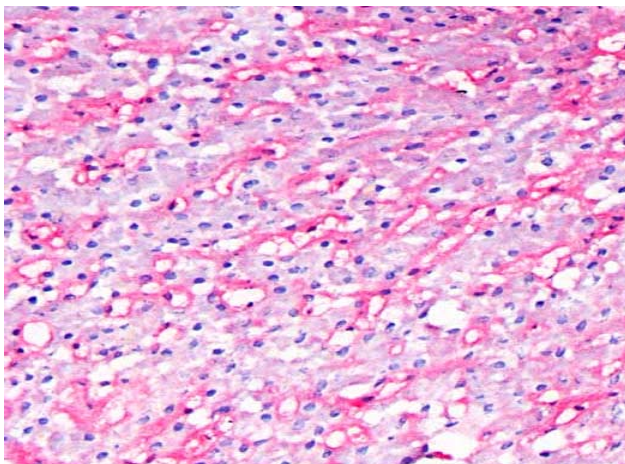
a) No ICAM-1 expression



b) Patchy ICAM-1



c) Diffuse ICAM-1



8.4 Discussion

The present study has evaluated the use of NAC in preventing I/R injury during liver resection surgery. Treatment with NAC was shown to reduce I/R injury, with a reduction in hepatocellular injury being evident by 24-48 hours post operatively. This is the first time that such observation has been made in humans in this clinical setting. However, there was no reduction in any clinically relevant end points such as morbidity, mortality, intraoperative or postoperative transfusion requirements. NAC infusion was associated with reduced serum ALT levels, higher ALP levels and with a trend towards less up regulation of ICAM-1 during the immediate postoperative period.

NAC is a thiol containing compound which scavenges free radicals by nonenzymatic reactions. In high concentration it can protect cells by its direct antioxidant action and also by replenishing the intracellular glutathione stores(76;408). It is ideally suited for clinical use due to its ease of availability, low cost, ease of administration and limited side effect profile.

8.4.1: Serum ALT

Serum ALT is a cytosolic enzyme and a marker of the severity of hepatocellular injury (134;145;160;291;292). ALT is more liver specific, reflects the loss of hepatocyte membrane integrity and release of cytoplasmic content into the circulation and is a useful marker of global liver function(149;198;242). In a prospective clinical trial with patients who underwent Orthotopic liver transplantation, NAC administration significantly reduced the rise in α -glutathione S-transferase and decreased peak ALT levels after reperfusion of the donor liver (408). We observed a similar reduction in peak serum ALT at 24- 48 hours postoperatively in patients who received NAC.

8.4.2: Serum ALP

Serum ALP levels dropped in both groups during the first 24-48 hours postoperatively with a gradual increase afterwards. Similar pattern has been observed in other studies following liver resections, where persistent increased levels have been found 8-12 weeks after the operation(396;397) (367). Serum ALP levels were higher in the NAC group compared to placebo group for the whole postoperative period. This difference reached significance in the 9th postoperative day. Serum ALP levels are surrogate markers of liver regeneration (112;367;396;397) and hence the significant difference found in our study suggests that NAC may enhance liver regeneration.

8.4.3: Effect of NAC on ICAM-1 expression.

In our study, although the differences found in ICAM-1 expression were not statistically significant, there was still a suggestion that NAC subjects showed less up regulation. The lack of statistical significance could be due to the relatively small number of patients investigated and the semi-quantitative nature of the technique used. Hence, these results should be validated by an additional quantitative method of ICAM-1 measurement such as PCR from tissue biopsies. Using a two point difference in ICAM-1 staining intensity as evidence of up or down regulation, no difference was noted between the NACG & placebo group. This could be explained by the constitutional expression of ICAM-1 by sinusoidal cells in normal conditions and it's further induction in acute or chronic liver disease(219).

All patients in our study were resected for either primary (one patient with HCV infection) or secondary liver tumours. The largest group included colorectal cancer with liver metastases and 6/16 and 8/15 patients in placebo and NAC group received preoperative chemotherapy. Although the biopsies were taken from "normal appearing" future liver remnant, preoperative chemotherapy may have induced

ICAM-1 in the baseline biopsies making it more difficult to assess ICAM-1 expression in response to I/R injury due to surgical intervention alone (202;406).

8.5 Conclusion

The results of the present study suggest a trend towards reduced liver injury when NAC is administered prophylactically during liver resection. In order to see if this effect can be translated to clinically relevant endpoints a large scale randomised trial would be required.

The pathophysiology of hepatic I/R injury is multimodal and complex(247). Although free radical induced injury is an important damaging mechanism in liver I/R, it is unlikely to be the primary or sole mechanism of injury(320;340). Evidence of beneficial effect of NAC from related but essentially different clinical situations should be carefully extrapolated to liver resection surgery(357;380).

Chapter 9

Summary and Conclusion

This thesis examines the pathophysiological changes related to Liver IRI in two different settings;

- 1) The role of Glycine a non essential amino acid in an in vivo established experimental animal model of partial lobar liver IRI and
- 2) Role of antioxidant NAC within a prospectively randomised clinical trial.

9.1: Summary of main findings

- 1) Administration of glycine did not affect the systemic haemodynamic parameters in this particular animal model.
- 2) A single dose of intravenous glycine produced sustained amelioration of liver IRI up to 6 hours post reperfusion.
- 3) Glycine administration improved portal blood flow and hepatic microcirculation as evidenced by the LDF data.
- 4) Intracellular tissue oxygenation and mitochondrial activity were preserved by the single intravenous dose of glycine and this effect was maintained up to 6 hours post reperfusion.
- 5) Glycine ameliorated the cytokine surge following reperfusion and this effect was maintained up to 4 hours post reperfusion on TNF- α and up to 6 hours post reperfusion on IL-8 expression. TNF- α is one of the key cytokines released by activated KC's which plays a central role in liver IRI. Reduction in levels of TNF- α would be suggestive of direct KC inhibition. Suppression of IL-8 surge is even more interesting since it would down regulate the subsequent neutrophils recruitment and activation and hence further reducing cellular injury.
- 6) Serum AST and ALT are cytosolic markers of severity of hepatocellular injury and the sustained reduction of these enzymes post reperfusion, lends further credence to the cytoprotective effect of glycine in liver IRI.

- 7) Glycine administration significantly improved the bile flow post reperfusion and this effect was sustained throughout the reperfusion period studied in this experimental model. Bile secretion is a useful marker of early liver function following transplantation and is considered to be a primary indicator of liver function and recovery following IRI in both experimental and clinical studies of liver IRI.
- 8) This is the first time that ¹HNMR analysis has been applied to study bile composition following glycine administration in a controlled experimental model of liver IRI. A rise in bile acid secretion in the glycine treated animals is suggestive of early recovery of metabolic activity. Along with this reduced lactate levels and phosphatidylcholine levels following reperfusion in the glycine treated animals would be suggestive of hepatocellular protection. These changes are mirrored by increase in post reperfusion levels of biliary glucose and pyruvate suggesting an increased metabolic activity due to conserved cellular energetics in the glycine treated animals. The increase in acetate and acetoacetate levels of bile in the glycine treated animals is again suggestive of early attempt at utilising alternative sources of ATP and conserved cellular bioenergetics in this group.

9.2: Methodological considerations

9.2.1: The animal model

The experimental model used in this study allows the assessment of both the haemodynamic and functional parameters in the rabbit liver, both under normal conditions and when subjected to ischaemia and reperfusion. The sham group simulates the effect of prolonged anaesthesia on the liver and other haemodynamic

parameters while the control group (I/R alone) simulates ischaemia reperfusion as encountered during liver transplantation/resectional surgery.

The rabbit was primarily selected for its size, to allow us to study the reperfusion phenomenon in a continuous fashion, within the same animal model and allow multiple samples at sequential time intervals without significantly compromising the haemodynamic stability of the model and simulating the clinical scenario of resectional surgery/transplantation. The other advantages being resistance to surgical trauma and infection, advantage of size and liver anatomy which makes the technical procedure possible and the availability and relative economy of maintenance.

The anatomy of the rabbit liver with separate lobes makes it easy to identify the vascular elements thus allowing for selective interruption of the blood supply. This model offers a well defined volume of liver tissue that can be rendered ischaemic with little or no alteration of systemic haemodynamics. In this model 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 ischemic insult without inducing mesenteric venous hypertension(134;145). During the occlusion period the right lateral and caudate lobes are not subjected to ischaemia thereby accommodating the splanchnic blood flow without major changes in portal pressure. This allows the study of small changes in liver vascular bed due to ischaemia reperfusion. Since the portal blood flow is not totally interrupted haemodynamic stability of the model is maintained. The time points studied in these experiments were selected on the basis of previous experimental evidence which has shown consistent time related changes in the post reperfusion blood flow and thus provide a sensitive indicator of any beneficial or adverse drug effects. The model is easy to establish and reproducible; the initial ischaemic changes in the liver are evident and leave no doubt as to the correct positioning of the clamp.

An important criticism of this particular animal model has been that it does not simulate the clinical conditions of total liver ischaemia, which occurs in liver transplantation or during periods of inflow occlusion (Pringle's manoeuvre) encountered during liver resections. Previously Kanoria et al have demonstrated that total hepatic ischaemia is poorly tolerated by rabbits, resulting in severe metabolic acidosis and cardiac arrest due to severe hypotension(203). Besides acute portal venous occlusion causes mesenteric venous congestion leading to compromise of the intestinal mucosa with bacterial translocation predisposing to systemic inflammatory response syndrome(225). In the rabbit model of partial liver IR used in these experiments only the blood flow to the medial and left lobes was interrupted leaving the right and caudate lobes with normal circulation. The partial lobar ischaemia allows splanchnic blood flow, preventing the haemodynamic instability due to mesenteric venous congestion and portal bacteraemia found in the total inflow occlusion model(225).

The normothermic lobar ischaemia simulates to an extent the circulatory and metabolic conditions of transplantation, which is simple, robust and reproducible with low mortality. The animal liver transplant model as opposed to the in vivo model used in this study, involves organ harvest and preservation at low temperatures with the technical difficulty of re-establishing portal and arterial blood flow, organ denervation and artefacts due to organ cooling with the possibility of immunological involvement which renders significant complexity to the liver transplant model. One of the major limitations of the model is that it is not appropriate for recovery and thus cannot assess the effect of glycine during the latter phases of liver IRI.

9.2.2: Laser Doppler Flowmetry (LDF)

Study of the hepatic microcirculation is of major pathophysiological significance in any strategy to reduce/ameliorate IRI. Microcirculatory changes correlates well with the degree of liver IRI(402). LDF used in the present study assessed the microcirculation directly and the depth of penetration was adequate since the maximum thickness was less than 10mm. LDF however, is a reflection of the local changes in the liver and does not measure microcirculatory blood flow of the whole liver. The LDF probe was applied to a fixed point throughout the experiments allowing continuous record of the local changes in microcirculation. Previously Seifalian et al have reported a coefficient of variation of 4% at intersite LDF measurements(336;337). This would suggest that measurements of local microcirculatory changes would be reflective of the entire affected lobe and surface LDF measurements would be representative of deep parenchymal perfusion(337).

Motion artefact due to vibration or probe movement was attempted to be minimised by deeper anaesthesia, with good liver mobilisation and placement of the probe such that it allowed the whole liver and probe to move simultaneously as one unit.

The value of blood flow measured was expressed in flux units. A baseline recording of flow as a standard (100%) was used in each individual experiment. The amount of back scattered light varies from one organ to another depending on factors like light absorption and red cell fraction of different tissues. It is not possible therefore to translate the values into a unit of flux which can be utilised as standard for different organs. However, when applied to the same organ the signal is reproducible within a narrow range of variation. LDF is now a well recognised and extensively validated technique for estimation of liver blood flow.

9.2.3 Near Infra red Spectroscopy (NIRS)

NIRS is a non-invasive technique to assess mitochondrial activity and cellular energy production (101;145). Seifalian and El-desoky et al have validated the measurement of cytochrome oxidase(CytOx) against other techniques(101;102) such as nuclear magnetic resonance spectroscopy and partial oxygen pressure. Bright operating lights interfered with the measurements and hence the probes were shielded with lightproof black cloth. Variations in measurements with reapplication even on the same site meant that the probes must be applied and maintained in the same site during the whole procedure which is impractical in a clinical scenario. In the present study recording of CytOx data was unaffected from the reapplication of probes and accurately represented the redox state of the enzyme which correlated with the LDF data, cytokine and transaminase measurements as well as changes in bile composition, this has been shown previously by Glantzounis et al (145).

9.2.4 Bile Proton Nuclear Magnetic Resonance (¹HNMR) spectroscopy

Bile flow and changes in bile composition demonstrated by preconditioning with glycine provides evidence to the cytoprotective effects of glycine against liver IRI. These changes in bile composition by preconditioning with glycine have not been reported previously. The quantitative data provides robust data compared to the other endpoints wherein the measurement of change was relative to the baseline. This technique may be helpful in the postoperative assessment of early liver graft dysfunction. In the animal model used bile was specifically collected for ¹HNMR spectroscopy measurements, however in clinical setting of liver transplantation unless a T-tube is used for biliary anastomosis or alternatively bile is collected by other means, this technique may be difficult to apply clinically routinely.

9.2.5 Cytokine measurements

KC activation appears to be the primary event which sets the liver IRI cascade into motion. Hepatic I/R injury is initiated by activation of KC in the liver during Ischaemia (192;194;348). However it is the cytokine release by these activated KC which plays a pivotal role in the inflammatory response during hepatic IRI (72;192;251). Tumour necrosis factor alpha (TNF α) and interleukin-1 (IL1) are the key cytokines which are produced by activated KC and are most commonly implicated in hepatic IRI (15;71;369). These two cytokines in turn induce IL-8 synthesis, which initiates the late phase of liver IRI (71;192). Since glycine directly prevents KC activation measurement of serum TNF- α would be a direct reflection of the extent of amelioration of the IRI by the administered glycine. The ELISA provides robust quantitative data and a technique which is easily reproducible within a short period of time. This technique can be easily extended into clinical practise since ELISA kits for various cytokines are readily available for clinical use. The increase in serum TNF- α levels in the I/R+Glycine group at the 6th hour following the ischaemic episode would suggest that the protection conferred by a single intravenous dose would last for up to 4 hours. These results are consistent with previously reported studies on half life of glycine in circulation (155). The only difficulty with the TNF- α ELISA was the primary and detecting antibody concentrations had to be optimised prior to use.

IL-8 ELISA was straightforward and easily reproducible since it was a commercially available kit with pre optimised paired monoclonal antibodies. Sustained significant reductions in IL-8 levels would lend credence to the cytoprotective effects of glycine and this has not been reported before. Since IL-8 is responsible for neutrophil

recruitment and the late phase of liver IRI amelioration of this key cytokine would suggest further reduction in the severity of the liver IRI.

9.2.6 Liver transaminases

The quantitative assessment of liver function remains a problem because of the complexity of the multiple metabolic and excretory functions of the liver. Liver has a significant functional reserve which allows it to sustain significant damage without evidence of metabolic derangement.

Indocyanine green clearance and rate of urea synthesis have been proposed as quantitative function test. Serum levels of bilirubin, transaminases and alkaline phosphatase are measured easily and available widely. Alanine aminotransferase (ALT) occurs virtually in the liver while aspartate amino transferase (AST) occurs partly in the liver. These enzymes are present in the hepatocyte cytoplasm and thus provide valuable information regarding the extent of hepatocyte damage. The amount of intracellular enzyme released into the plasma is an index of the integrity of the cell membrane and hence indirectly of damage to hepatocytes. These enzymes and their levels however, cannot give information about the function of viable living liver cells.

9.3 N-acetylcysteine in patients undergoing liver resection

The use of NAC within a clinical trial allows us to study and translate the experimental benefits of an antioxidant to a clinically safe and relevant scenario. A double blind randomised trial incorporating a placebo arm is the gold standard for studying the effects of a drug in a clinical setting. All relevant permissions and consents were obtained prior to recruitment. Both the patient and the observers were blinded using computer generated non sequential numbers. The blinding was removed only after all endpoints were assessed and the trial completed.

Randomisation was done by the department of inpatient pharmacy who had no clinical contact with the patients recruited for the study. Recruitment to the trial was voluntary and the trial protocol was approved by the local ethics committee.

The power calculations were done by the hospital statistician (Dr Morris). Due to ethical constraints trucut liver biopsies were only performed during the actual operation, since the risk associated with elective liver biopsies postoperatively following major liver resection was considered too high.

The following difficulties were encountered during the trial. First of all the primary endpoint was ICAM-1 expression following surgery in trucut liver biopsies which were snap frozen in liquid nitrogen. Although all the biopsies were considered to be adequate there were artefacts encountered due to air bubbles incorporated in the cryo glue used for snap freezing. This was tackled by careful technique and improved handling of the biopsy specimen.

The other confounding factor was semi quantitative nature of the ICAM-1 as evaluated by immunohistochemistry. In some specimens the baseline ICAM-1 was upregulated which might be due to the preoperative chemotherapy or concomitant viral infections (hepatitis B/C) thus making it difficult to adequately assess up regulation following liver resection. The first biopsy and the second biopsy were both performed from the same remnant liver, but for more robust evaluation the second liver biopsies should be obtained 12-24 hours post resection. These reasons could explain the lack of statistical significance with regards to ICAM-1 expression. Despite these shortcomings there was a trend towards reduced ICAM-1 up regulation in the NAC treated group. Recruiting larger numbers of patients might also help in elimination of this problem. Both the patient groups were well matched for age, sex, number of segments resected and type of resections performed with comparable morbidity and mortality. Serum ALT levels were significantly attenuated in NAC

treated group while the serum alkaline phosphatase a marker of bile duct regeneration post liver resection was significantly higher in the NAC treated group. This would suggest that NAC did confer protection within these groups of patients. This is the first trial of its kind using NAC in patients undergoing elective liver resections with a trend towards support for use of NAC in complex liver resections.

9.4 Future Studies

- 1) There is enough experimental evidence both in this thesis and in the literature to support the prophylactic use of glycine in liver resectional surgery and transplantation to prevent IRI. It would be very interesting to see the effect of this molecule in a randomised clinical trial involving liver resection/transplantation. In liver transplantation it should be given to donors prior to organ procurement as a single intravenous dose and to organ recipients it can be administered twice a day initially till patients are resumed on oral feeds and can then be administered orally for the first week following surgery.
- 2) NAC is a very useful antioxidant and although the clinical trial in this thesis showed a trend towards improved liver function, a large scale multicentre randomised controlled trial would be required to achieve a significant difference in the clinically relevant outcomes.
- 3) Since KC and ROS are the two key factors involved in initiation and propagation of liver IRI combining NAC and glycine may prove to be pivotal in ameliorating IRI almost completely. This hypothesis has not been investigated before.

Presentations and publications from the thesis

Presentations:

- 1) **A randomised double blind controlled clinical trial to assess the effects of prophylactic N-Acetylcysteine on liver injury during liver resection, H Sheth, G Glantzounis, T Hafez, A Quaglia, J Duncan, B R Davidson,** presented as a free paper at the **Society of Academic and Research Surgery (SARS)** held in January 2005 at Newcastle, UK.
- 2) **Glycine suppresses cytokine expression and improves bile flow in a rabbit model of liver warm ischaemia-reperfusion injury, H Sheth, T Hafez, G Glantzounis, K M Sales, A Seifalian , B Fuller, B R Davidson,** presented as a free paper at the **Society of Academic and Research Surgery (SARS)** held in January 2005 at Newcastle, UK.
- 3) **Bile flow and composition are modulated by intravenous glycine in an in-vivo warm ischaemia-reperfusion injury model: T Hafez, H Sheth, G Glantzounis, H G Parkes, B Fuller, B R Davidson.** presented as a free paper in the young investigator's prize session, at the 6th World Congress of the International Hepatopancreatobiliary association, held in June 2004 at Washington DC.
- 4) **Intravenous glycine ameliorates ischaemia reperfusion injury in the rabbit liver lobar ischaemia reperfusion model: H Sheth, T Hafez, G Glantzounis, A Seifalian, B Fuller, B R Davidson,** presented as a **free paper** at the 6th World Congress of the International Hepatopancreatobiliary association, held in June 2004 at Washington DC.
- 5) **Glycine suppresses cytokine expression and improves bile flow in a rabbit model of liver warm ischaemia-reperfusion injury: H Sheth, Hafez T, G Glantzounis, K Sales, B Fuller, B R Davidson,** presented as a poster at the 6th World Congress of the International Hepatopancreatobiliary association, held in June 2004 at Washington DC.
- 6) **A randomised double blind controlled clinical trial to assess the effects of prophylactic N-acetylcysteine on liver injury during liver resection: H Sheth, G Glantzounis, Hafez T , A Quaglia, B Fuller, J Duncan, B R Davidson,** presented as a poster at the 6th World Congress of the International Hepatopancreatobiliary association, held in June 2004 at Washington DC.

- 7) **Intravenous glycine ameliorates the early phase of warm ischaemia-reperfusion injury in a rabbit lobar liver ischaemia reperfusion injury model:** H Sheth, T Hafez, G Glantzounis, A Seifalian, B Fuller, B R Davidson. Presented as a poster at the Society of Academic and Research Surgery (SARS) held in January 2004 at Belfast.
- 8) **Bile flow and composition are modulated by intravenous glycine in an in vivo warm ischaemia reperfusion injury model:** T Hafez, H Sheth, G Glantzounis, W Yang, H G Parks, A Seifalian, B Fuller, B R Davidson, presented as a plenary session poster at the British Society of Gastroenterology annual meeting held in March 2003 at Glasgow, UK.
- 9) **Glycine ameliorates the early phase of liver warm ischaemia-reperfusion injury in a rabbit model:** H Sheth, T Hafez, G Glantzounis, K M Sales, A Seifalian, B Fuller, B R Davidson, presented as a poster at the British Society of Gastroenterology annual meeting held in March 2003 at Glasgow, UK.

Publications

- 1) **Glycine reduces liver warm ischaemia reperfusion injury by improving the hepatic microcirculation and enhancing mitochondrial activity :** H Sheth, T Hafez, G Glantzounis, K Sales, A Seifalian, B Fuller, B R Davidson. Accepted for publication by **Journal of Hepatology and Gastroenterology** March 2010.
- 2) **Acute limb Ischaemia caused by femoral arterial line induces remote liver injury in a rabbit model of liver Ischaemia/reperfusion injury:** G Glantzounis, H Sheth, C Thompson C, T Hafez, S Kanoria, V Pamecha, S Davies, D P Mikhailidis, A Seifalian, B R Davidson. *Angiology*. 2009 Oct-Nov;60(5):554-61. Epub 2009 Jul 21.
- 3) **Glycine protects bile physiology and biliary-specific liver cell metabolism from Ischaemia-reperfusion injury; a ¹H NMR study:** T Hafez, H Sheth, G Glantzounis, H Parkes, A Seifalian, B Fuller, and B R Davidson; *Cell Preservation Technology* 6:173–180 (2008).

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