Study of the effect of Bucillamine on the early and late phase of hepatic ischaemia reperfusion injury.

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DECLARATION CONCERNING THESIS PRESENTED FOR THE DEGREE OF MD (Res)

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solemnly and sincerely declare, in relation to the thesis entitled:

Study of the effect of Bucillamine on the early and late phase of the hepatic IR.

(a) That work was done by me personally

and (b) The material has not previously been accepted in whole, or in part, for any other degree or diploma.

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Dedication

I dedicate this work to my late grand mother- Heerabai, my mother- Smita, my wife- Vrushali and daughter-Neha.

Abstract

Ischaemia of the liver followed by reperfusion results in endothelial and parenchymal injury through a complex cascade of events. This often occurs in human liver transplantation as well as with major liver resections and is referred to as Ischaemia Reperfusion (IR) Injury. Bucillamine is a low molecular weight thiol antioxidant that is capable of rapidly entering cells.

This thesis evaluates the effect of Bucillamine on both the early and late phases of liver warm IR injury with the hypothesis that beneficial effects are induced could be due to its action as a free radical scavenger. The drug was evaluated in an *in vivo* lobar liver ischemia reperfusion model as previously described. Male Sprague –Dawley rats were subjected to 45 mins of partial hepatic (70 %) ischaemia followed by 3 hrs of reperfusion to investigate the early phase of hepatic IR and 24 hrs of reperfusion to study the late phase of hepatic IR. Changes to the microcirculation, leucocyte adherence and apoptosis were assessed by intra-vital microscopy. Hepatocellular injury was assessed by standard liver function tests. Expression of pro and antiapoptotic gene expression was studied by RT-PCR. Oxidative stress was assessed by measuring plasma and hepatic F_2 isoprostane levels and tissue glutathione levels. Cytokine response was assessed by measuring serum CINC-1 levels. Bucillamine improved liver sinusoidal perfusion, reduced leukocyte adherence and apoptosis in both the early and late phases of IR injury. Hepatocellular injury

was reduced. There was no difference in the level of tissue glutathione or tissue and plasma F_2 isoprostane levels.

This study shows that the hepato protective effect of Bucillamine in warm Liver ischemia reperfusion injury is not by direct replenishment of Glutathione level; however, it is through decreased neutrophil activation and recruitment. A clinical trial could hence be undertaken in the future to study its efficacy.

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Thesis Description

Chapter 1

Introduction, Pathophysiology of Liver Ischaemia reperfusion injury, the role of reactive oxygen species, nitric oxide in liver IR injury. Oxidative stress and antioxidant system, Intracellular and extracellular antioxidant defenses and the role of Haemoxygenase in Liver IR.

Chapter 2

Bucillamine: A Thiol Antioxidant

Chapter 3

Materials and Methods- Model, Intravital microscopy: An description of animals and surgical preparation, experimental Protocols, tissue and blood collection. The experimental model is described and the methodology used within the experiments is elaborately described.

Chapter 4

Intravital microscopy results: This chapter describes in an experimental rat model of warm hepatic IR, microvascular, neutrophil endothelial interactions, biochemical changes & hepatocellular death seen in hepatic IR and the impact of Bucillamine on these changes.

Chapter 5

This chapter discusses the histological changes and the effect on IR on Bax and Bcl-2 gene expression and the effect of Bucillamine infusion on these changes.

Chapter 6

Effect of Bucillamine infusion in late phase of Liver ischaemia reperfusion injury

Chapter 7

Effect of Bucillamine on Oxidant stress: is mediated by the replenishment of intracellular glutathione levels?

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Summary and discussion of thesis.

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Abbreviations:

ALT	Alanine amino transferase
AST	Aspartate amino transferase
CINC	Cytokine induced neutrophil chemoattractant
CO	Carbon Monoxide
GSH	Reduced Glutathione
GSSG	Oxidised Glutathione
HO	Haemoxygenase
IR	Ischaemia reperfusion
I/R	Ischaemia reperfusion
IRI	Ischaemia reperfusion injury
IVM	Intra vital Microscopy
NAC	N-Acetyl cysteine
NO	Nitic oxide
NOS	Nitric oxide synthase

Publications and Presentations arising from this thesis

Publications:

Bucillamine improves hepatic microcirculation and reduces hepatocellular injury following liver warm ischaemia reperfusion injury. *SP Junnarkar*, N Tapuria, N Dutt, B Fuller, AM Seifalian, BR Davidson. *HPB* 2009; **11(3)**: 264-273.

Papers Accepted for Publication:

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Chapter 1

Introduction

Introduction

Liver Transplantation and liver resection surgery have increased dramatically due to the excellent outcomes they offer in patients with chronic liver disease and liver cancers. Both procedures involve a period of ischaemia and reperfusion to the liver which initiates an inflammatory cascade resulting in liver and remote organ injury. When severe these changes can be fatal. Reactive oxygen species (ROS) have a central role to play in Ischaemia –Reperfusion injury (IR).

ROS activate cytokines, macrophages and other components of the inflammatory pathway(Entman *et al.* 1991; Jaeschke and Farhood 1991a; Le *et al.* 1997). When generated in large numbers they can also cause direct oxidative damage to the cells through iron mediated reactions(Horwitz *et al.* 1998). Thiol donors are antioxidants which can interrupt redox signalling pathway and thereby reduce cytokine and macrophage activation(Sano *et al.* 2001). In addition, thiol donors can protect against oxidative injury by replenishing intracellular glutathione and other endogenous thiol compounds(Ceconi *et al.* 1988a).

1.1 Pathophysiology of Liver I/R

1.1.1 Effects of ischaemia in the Liver:

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 (Chazouilleres

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

Intracellular calcium accumulation is thought to be a crucial step in the transition to irreversible damage with ischaemic injury(Dhar *et al.* 1996). 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. The increased cytosolic calcium causes activation of cell membrane phospholipases resulting in phospholipid degradation and cell membrane disruption(Farber 1981). Calcium also activates tissue proteases such as xanthine oxidase (XO) which play a role in oxygen free radical production and reperfusion injury(Ishii *et al.* 1990). Calpains are proteases that are capable of degrading cytoskeletal proteins such as spectrin. There is experimental evidence, that calpains are mediators of both warm and cold ischemic injury in

the rat liver(Kohli *et al.* 1999a). Calpains seem to be activated by raised levels of free cytosolic Ca^{+2} that accumulates in the liver during ischemia(Arnould *et al.* 1992).

Although the basic mechanisms of I/R injury after warm and cold liver ischaemia are similar, there are also significant differences. In liver transplantation the liver undergoes cold ischemic storage followed by rewarming ischaemia and reperfusion. Cold ischemia is associated with marked ATP depletion and increased glycolysis (Churchill *et al.* 1994). Cold ischaemia causes Kupffer cell stimulation, while warm ischemia leads to oxidative stress and mitochondrial dysfunction (Baumann *et al.* 1989; Mochida *et al.* 1994). It is the hepatocytes which are most susceptible to warm ischaemia(Gujral *et al.* 2001; Kohli *et al.* 1999c), whereas, nonparenchymal cells (Kupffer, endothelial cells, and Ito cells) are more susceptible to cold I/R than hepatocytes (Ikeda *et al.* 1992).. It has been shown in the 1980s that cold ischaemia specifically caused injury in the sinusoidal endothelial cells (EC)(Caldwell-Kenkel *et al.* 1988; Otto *et al.* 1984; McKeown *et al.* 1988; Momii and Koga 1990). On exposure to cold ischaemia the EC get detached, lose cytoplasmic processes, become rounded as a result of alteration of the extracellular matrix and cytoskeleton and sloughed into the sinusoidal lumen(Caldwell-Kenkel *et al.* 1988; Holloway *et al.* 1990; McKeown *et al.* 1988). The degree of EC detachment has been shown to correlate with duration of cold ischaemia(Clavien *et al.* 1991; Caldwell-Kenkel *et al.* 1988; Holloway *et al.* 1990). Despite these structural changes most ECs remain viable

during the cold ischaemic period but rapidly die on reperfusion(Imamura *et al.* 1997; Miyagawa *et al.* 2002). The resultant disruption of endothelium results in leukocyte(Clavien *et al.* 1991; Clavien *et al.* 1993; Jaeschke *et al.* 1990; Jaeschke and Farhood 1991a; Takei *et al.* 1991) and platelet adhesion(Cywes *et al.* 1993; Sindram *et al.* 2000), which induces microcirculatory disturbances(Marzi *et al.* 1991) in the reperfusion phase. Leukocytes and platelets synergistically exacerbate EC injury by induction of apoptosis(Sindram *et al.* 2001). Kupffer cells play a contributive role in the EC injury along with platelets and leukocytes(Sindram *et al.* 2001).

Increased activities of non-lysosomal proteases preferentially in anoxic hepatocytes may play a causal role. Inhibition of non-lysosomal proteolysis by acidosis or glycine protects against anoxic hepatocyte death (Nichols *et al.* 1994).

1.1.2 Reperfusion Injury

Although ischemia causes significant injury to tissue and cells, reperfusion results in an escalation of organ and cellular damage. Reperfusion injury occurs in a biphasic manner(Jaeschke and Farhood 1991a).

1.1.2.1 Early phase: This occurs within 1-4 hours following reperfusion and is characterized by Kupffer cell (KC) and polymorphonuclear(PMN)(Cutrin *et al.* 1998) cell activation, increased production of NO by liver mitochondria and KCs(Kurose *et al.* 1996; Stephenson *et al.* 1997), activation of complement

cascade and production of C5a by proteolytic cleavage(Jaeschke *et al.* 1994) and generation of reactive oxygen species (ROS)(Jaeschke and Farhood 1991a; Bailey and Reinke 2000; Liu *et al.* 1995; Shiratori *et al.* 1994). This early phase injury is mediated by ROS generation(Fan *et al.* 1999a; Jaeschke 1998; Lichtman and Lemasters 1999; Muller *et al.* 1996). During this phase the main event is the activation of Kupffer cells(Jaeschke and Farhood 1991b).The activation starts during ischemia but becomes more evident during the onset of reperfusion. This effect occurs after no-flow ischemia (major liver resections with Pringle maneuver, transplantation) but not after low flow ischaemia (haemorrhagic shock)(Jaeschke and Farhood 2002). Complement activation, recruitment and activation of $CD4^+$ T-cells are the factors responsible for the activation of Kupffer cells(Fondevila *et al.* 2003; Jaeschke 2003a). Kupffer cell activation and the subsequent vascular inflammation can be enhanced by extrahepatic mechanisms. The most important event is the priming of Kupffer cells by endotoxin(McCuskey *et al.* 1996; van Goor *et al.* 1994). Endotoxin translocates across the gut, most likely as a consequence of intestinal congestion due to portal vein clamping at the time of surgery. Kupffer cells are the main source of formation of vascular reactive oxygen species (ROS) during the initial reperfusion period. Other sources of ROS formation are xanthine oxidase(Jaeschke 2002) and mitochondria(Jassem *et al.* 2002). Activation of Kupffer cells leads to activation of neutrophils and production of cytokines (like TNFα, IL-1 and IL-12)(Lentsch *et al.* 2000). The production of TNF-α induces the expression of adhesion molecules on vascular endothelial cells and stimulates

the production and release of neutrophil-attracting chemokines (like CINC-1)(Hisama *et al.* 1996). The final result is the recruitment of neutrophils within the late phase.

1.1.2.2 Late phase: This occurs 4-24 hours after onset of reperfusion and is characterized by PMN influx into the post ischaemic liver and organ injury(Fan *et al.* 1999a; Jaeschke 1998; Jaeschke and Smith 1997a; Simpson *et al.* 1997). PMNs accumulate in the liver vasculature in response to the exposure to inflammatory mediators such as TNF-α, IL-1, CXC chemokines [IL-8, CINC-1, macrophage inflammatory protein-2 (MIP-2)], activated complement factors and platelet activating factors (PAF)(Jaeschke 2003a; Jaeschke and Smith 1997b; Okaya and Lentsch 2003). These mediators increase the expression of CD11b/CD18, a member of the β_2-integrin family of adhesion molecules, and other receptors on the surface of neutrophils by causing fusion of the secretory vesicles within the neutrophils to cell membrane(Jaeschke 2006). ICAM-1 is also transcriptionally induced in endothelial cells and hepatocytes(Farhood *et al.* 1995; Bell *et al.* 1997). Selectins(Sawaya, Jr. *et al.* 1999) and β_2-integrin-ICAM-1(Rentsch *et al.* 2000; Vollmar *et al.* 1995a) interactions are involved in neutrophil rolling and adhesion, respectively, in post sinusoidal venules. β_1-integrin have also been implicated in leukocyte rolling/adhesion(Fox-Robichaud and Kubes 2000). There is however, little evidence of transmigration of neutrophils from the venules(Vollmar *et al.* 1994b). In contrast sinusoids are identified as a site of major extravasation(Chosay *et al.* 1997). Extravasation is

considered a prerequisite for hepatocyte damage by neutrophils(Chosay *et al.* 1997). There is no experimental evidence for involvement of adhesion molecules in neutrophil accumulation in sinusoids(Rentsch *et al.* 2000; Vollmar *et al.* 1995a; Fox-Robichaud and Kubes 2000). However, the extensive vascular injury during reperfusion damages the sinusoidal endothelial barrier and allows direct access for the neutrophils to the hepatocytes(McKeown *et al.* 1988; Caldwell-Kenkel *et al.* 1991). Once extravasated the neutrophils adhere to hepatocyte by β_2-integrin-ICAM-1 interactions with the ICAM-1 expressed on hepatocytes)(Nagendra *et al.* 1997). The adherence to the target induces degranulation of neutrophils with release of proteases and formation of ROS. Some of the ROS can diffuse into the hepatocytes and can cause intracellular oxidant stress resulting in mitochondrial dysfunction and cell death(Jaeschke and Smith 1997b). Intracellular oxidant stress leads to oxidation of pyridine nucleotides, accumulation of calcium in mitochondria, and superoxide formation by mitochondria, which ultimately leads to opening of membrane permeability transition pores and breakdown of mitochondrial membrane potential(Jaeschke and Smith 1997b; Nieminen *et al.* 1997a). Some of the proteases (cathepsin G, elastase) increase the hepatocyte damage, whereas others generate more pro inflammatory mediators(Jaeschke and Smith 1997b).

1.1.3 The role of reactive oxygen species in I/R injury

A radical is any atom or biomolecule that contains unpaired electrons(Halliwell B and Gutteridge JMC 1999). These unpaired electrons alter the chemical reactivity

by making the radical more reactive than the corresponding non-radical. The most biologically relevant radicals are the superoxide anion ($O_2^{\cdot -}$) and hydroxyl ($HO^{\cdot}$) (Halliwell 1994). Under normal conditions around 1-3 % of the oxygen that is metabolized in the body is converted to superoxide (Nohl *et al.* 2003). Another important radical is nitric oxide ($NO^{\cdot}$). Some other species are intermediate in the metabolism of O_2 or $NO^{\cdot}$ but are not radicals as they do not contain unpaired electrons. These intermediate species along with radical species are called reactive oxygen species and reactive nitrogen species (RNS) respectively. The most representative examples of non radical ROS are hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl). The most representative of the RNS is peroxynitrite ($ONOO^-$)(Ischiropoulos *et al.* 1992). Peroxynitrite is a toxic substance that is formed when there is simultaneous production of nitric oxide with superoxide anion:

$$NO^{\cdot} + O_2^{\cdot -} \longrightarrow ONOO^{\cdot} \quad (1)$$

Superoxides damage cells by their direct reactivity with numerous biological molecules including lipids, DNA, RNA, catecholamines and steroids, and from its dismutation to form H_2O_2(Cuzzocrea *et al.* 2001). Trace amounts of metals ions (principally iron or Copper) can react with H_2O_2 in what is known as the Fenton reaction to produce the toxic hydroxyl radical (Sutton and Winterbourn 1989). This radical can cleave covalent bonds in proteins and carbohydrates and destroy cell membranes.

The burst of ROS generated after reperfusion may contribute to the initiation of postischemic liver injury and to the subsequent inflammatory activation. Although the exact subcellular sources of ROS generation in I/R are still under investigation, both the xanthine /xanthine oxidase system and mitochondria have been suggested to play important roles (Fan *et al.* 1999b). Although xanthine oxidase was regarded as the principal source of post ischemic oxidant stress in the liver, recent evidence suggests that xanthine oxidase plays a minor role compared to mitochondria(Jaeschke 2002). Mitochondria are the site of the production of large amounts of superoxide, under conditions of oxidative stress. It is this stress that finally leads to the formation of membrane permeability transition pores and the breakdown of the mitochondrial membrane potential that can cause cellular death (Nieminen *et al.* 1997b).

Intracellular production of ROS can activate signal transduction pathways and regulate gene expression. Two of the systems that are believed to be affected by ROS are the nuclear factor-kappa B (NF-kB) and activator protein 1 (AP-1) pathways. Both of these affect cell growth and apoptosis(Palmer and Paulson 1997). There is evidence that extracellular ROS, such as superoxide radicals produced by activated neutrophils, may act on redox-sensitive membrane receptors to initiate intracellular ROS production. Through this mechanism extracellular ROS lead to necrotic cell death.

Although ROS have a significant role in hepatic I/R injury, they also have an essential role in the defence function of phagocytes and are involved in the production of mediators regulating liver blood flow and regeneration(Nakatani *et al.* 1997; Pannen 2002). In summary, ROS are important cytotoxic and signalling mediators in the pathophysiology of liver I/R injury. A list of ROS properties and roles in liver I/R injury is given in table 1.

1.1.4 The role of nitric oxide in liver I/R injury

$NO^{\cdot}$ is a radical synthesized via the oxidation of L-arginine by $NO^{\cdot}$ synthetase (NOS)(Moncada and Higgs 1993). There are two major isoforms of NOS in the liver, endothelial NOS (eNOS) and inducible NOS (iNOS). eNOS is expressed constitutively and its activity is dependent on Ca^{++} and Calmoduline(Vasquez-Vivar *et al.* 1998). eNOS is expressed only in sinusoidal endothelial cells, whilst iNOS is induced by extracellular stimuli such as cytokines and lipopolysacharide (LPS), leading to the production of much higher levels of $NO^{\cdot}$. iNOS is produced by endothelial cells, hepatocytes and Kupffer cells and its activity is Ca^{++} independent. All NOS isoforms can be inhibited to varying degrees with N-substituted L-arginine analogs. Many of the biological actions of $NO^{\cdot}$ are mediated through the guanylyl cyclase/cyclic GMP system. $NO^{\cdot}$ is a lipophilic biomolecule that diffuses to adjacent cells and enters the cytosol, where it activates soluble guanylyl cyclase by binding to the iron in the heme center resulting in an intracellular increase of cGMP levels(Schmidt *et al.* 1993). Triggering the cyclic GMP cascade has different effects in different cells that it is

produced(Cottart *et al.* 2003). In the sinusoidal endothelial cells it offers cytoprotection as well as inhibits platelet aggregation and infiltration of PMNs(Cottart *et al.* 2003). In hepatocytes, through mechanism regulated by p38 mitogen activated kinase (p38 MAPK) it causes cytoprotection by preservation of pH, Na+ and Ca++ homeostasis, preservation of mitochondrial functions, reduced production of ROS and preservation of cytoskeleton(Carini and Albano 2003). NO can also bind to non-heme iron in the iron-sulphur centers of a variety of enzymes, thus altering their biological activity (Nathan 1992).

Under physiologic conditions only constitutive eNOS is present in the liver and the low level of $NO^{\cdot}$ produced regulates hepatic perfusion, preventing platelet adhesion, thrombosis and polymorphonuclear cell (PMN) accumulation(Mittal *et al.* 1994). $NO^{\cdot}$ also induces vasodilation at the level of the sinusoid and at presinusoid sites(McCuskey 2000; Ming *et al.* 1999); and plays an important part in keeping a balance with vasoconstrictors such as endothelins (Pannen 2002). It has also been reported that $NO^{\cdot}$ inhibits leukocyte-endothelial cell adhesion and prevent leukocyte secretion of inflammatory mediators(Gauthier *et al.* 1994).

Induction of iNOS may have either toxic or protective effects. The effects are dependent on the type of insult to the tissue, the tissue type, the level and duration of iNOS expression and the simultaneous production of superoxide anion (Cuzzocrea *et al.* 2001).

In liver I/R iNOS is expressed 5 hours after the ischaemic insult(Hur *et al.* 1999). The literature concerning the effect of iNOS in liver I/R injury is still ambivalent. Some studies suggest that iNOS expression can have detrimental effects(Meguro *et al.* 2002; Serracino-Inglott *et al.* 2003) in liver function whilst others that it is beneficial(Hsu *et al.* 2002; Wang *et al.* 1998) and yet in others no effect(Hines *et al.* 2002; Rivera-Chavez *et al.* 2001). One study with mice deficient in iNOS showed a moderate reduction in reperfusion injury.

The toxic effects are linked with the production of peroxynitrite which can cause cell injury, either by inhibiting mitochondrial enzymes and thus mitochondrial respiration or through the formation of nitrotyrosine and nitrosylation which can cause DNA damage.

1.1.5 Oxidative stress and antioxidant system

The body has developed major antioxidant defense mechanisms to protect it from free radicals. The definition of an antioxidant is quite difficult. A broad definition is that an antioxidant is any substance that when present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of the substrate(Gutteridge 1995). These defenses can be classified according to the site of action as: intracellular; membrane and extracellular.

Another classification is according to the mode of action. There are four mechanisms:

a. Catalytical removal of ROS. Enzymes such as superoxide dismutase, catalase, peroxidases and thiol-specific antioxidants act through this mechanism.
b. Chelation of prooxidants such as iron ions, copper ions and haem. Proteins like transferrins, haptoglobins, haemopexin, metallothionein and caeroloplasmin are examples.
c. Protection of biomolecules against oxidative damage. In this category are the heat shock proteins.
d. Scavenging of ROS and RNS. Examples are low-molecular-mass antioxidants such as glutathione, a-tocopherol, ascorbic acid, bilirubin, uric acid.

1.1.5.1 Intracellular antioxidant defences

Intracellular antioxidant defences include: the superoxide dismutase (SOD); catalase; glutathione peroxidase and reductase enzymes, thioredoxine and other peroxidases such as Cytochrome c, NADH, horseradish peroxidase.

Superoxide dismutases catalyse the dismutation of superoxide to hydrogen peroxide and oxygen:

$$2O_2^{\cdot -} + 2H^+ \longrightarrow H_2O_2 + O_2 \quad \textbf{(2)}$$

Three forms of SOD exist with different subcellular localizations. Those containing copper and zinc (Cu/ZnSOD) are located in cytosol, manganese

(MnSOD) in the mitochondria (Ho and Crapo 1988) and the third form is secreted into the extracellular environment.

The product of reaction **(2)**, hydrogen peroxide, is a weak oxidant and is relatively stable. However unlike superoxide, H_2O_2 can rapidly diffuse across cell membranes and in the presence of transition metal ions it can be converted to hydroxyl radicals via Fenton chemistry:

$$Fe^{+2} + H_2O_2 \longrightarrow Fe^{+3} + {}^{\cdot}OH + OH^- \quad \textbf{(3)}$$

Two enzymes can break down H_2O_2 .

One of them is the hemoprotein catalase. Catalase is present in all major body organs being especially concentrated in liver. It catalyzes the breakdown of hydrogen peroxide in oxygen and water:

$$2\,H_2O_2 \longrightarrow O_2 + 2H_2O \quad \textbf{(4)}$$

The second is the system consists of glutathione peroxidases. This group includes four different isoforms(Dufaure *et al.* 1996). They are located in cytosol and mitochondria and have a major role in removing hydrogen peroxide generated by superoxide dismutase with the oxidation of glutathione (GSH):

$$2\,GSH + H_2O_2 \longrightarrow GSSG + 2H_2O \quad \textbf{(5)}$$

Glutathione reductase is also an important enzyme in this system. It expresses its action through the regeneration of GSH from GSSG using NADPH(Meister 1988).

GSH scavenges $^{\cdot}OH$, OHCl, peroxynitrite, carbon centred radicals and singlet oxygen ($^1O_2$). GSH is involved in many other metabolic processes, including chelation of Copper ions, and prevention of oxidation of protein –SH groups.

Thioredoxin is a polypeptide being especially concentrated in the endoplasmic reticulum but some is also found on the cell surface. Thioredoxin contains two adjacent –SH groups in its reduced form that are converted to a disulphide in oxidized thioredoxin. It can undergo redox reactions with multiple proteins.

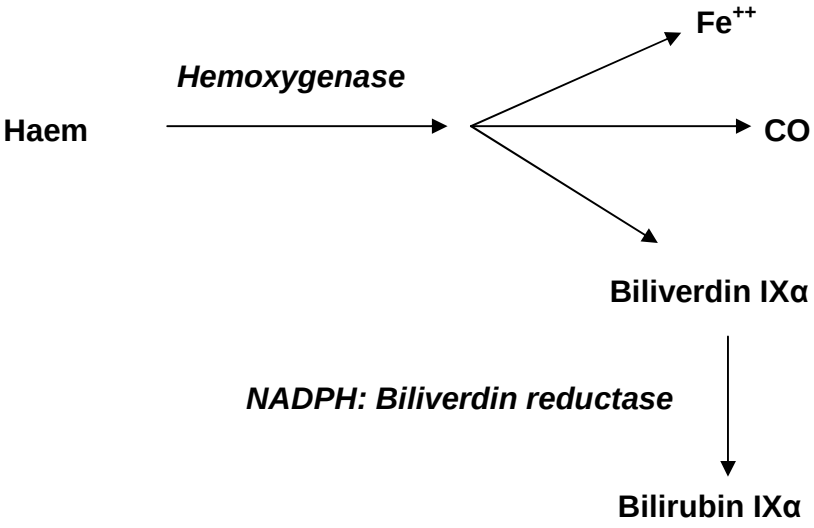
1.1.5.2 Extracellular antioxidant defences

Major extracellular antioxidant defences include the metal-binding proteins(Betteridge 2000). It is well known that the free metals iron and copper can promote free radical damage, accelerating lipid peroxidation and catalyzing hydroxyl radical formation. The body is protected against these potentially adverse effects by binding proteins (transferrin, lactoferrin and ceruloplasmin) which ensure that these metals are maintained in a nonreactive state(Halliwell and Gutteridge 1990). Similarly haptoglobins, hemopexin and albumin bind haemoglobin and haem. Haemoglobin and myoglobin are normally intracellular proteins. When these proteins are exposed to a large amount of oxidative stress (e.g. large amounts of H_2O_2) they are degraded releasing both haem and iron ions that can stimulate lipid peroxidation.

1.1.6 Role of Hemoxygenase in IR

Hemoxygenase is an enzyme found in the endoplasmic reticulum that catalyses the first and rate limiting step of degradation of haem to yield equimolar amounts of biliverdin, carbon monoxide (CO) and free divalent iron(Wunder and Potter 2003). Biliverdin is subsequently reduced to bilirubin while the iron is sequestered by ferritin.

Figure 1.1



Three isoforms of HO have been identified. HO-1 also known as heat shock protein 32, is highly inducible in all cells. HO-2 is a constitutively expressed 36kDa protein which is unresponsive to stimuli increasing HO-1 expression. HO-3 is a 33 kDA protein which is a weak catalyst for haem degradation and is non inducible(Maines 1988; McCoubrey, Jr. *et al.* 1992; McCoubrey, Jr. *et al.* 1997). HO-2 is most abundant in hepatocytes, sinusoidal endothelial cells, hepatic stellate cells and Kupffer cells(Bauer *et al.* 1998). In normal physiological states,

Kupffer cells are the only cells which constitutively express HO-1(Bauer *et al.* 1998). In conditions of stress such as hypoxia, I/R, hyperthermia, oxidative or cytotoxic stress HO-1 is upregulated primarily in the parenchymal cells(Wunder and Potter 2003; Bauer *et al.* 1998). Endogenous HO activity within the liver was shown to preserve microcirculatory dysfunction and prevent cell injury following I/R injury(Kobayashi *et al.* 2002; Coito *et al.* 2002; Kato *et al.* 2001; Redaelli *et al.* 2002). The exact mechanism of the protective role although not known, CO, biliverdin and ferritin are thought to be responsible for it(Wunder and Potter 2003).

1.1.6.1 Carbon Monoxide (CO)

CO released during haem oxidation by HO functions as a second messenger in a fashion similar to NO(Verma *et al.* 1993; Maines 1997). CO has a stimulatory effect on soluble guanylate cyclase thereby increasing cyclic GMP, which in turn through effect on smooth muscle contractility leads to vasodilatation(Pannen *et al.* 1998; Morita *et al.* 1995; Sammut *et al.* 1998). CO has also been shown to cause smooth muscle relaxation via activation of calcium dependent potassium channels(Wang *et al.* 1997). CO also influences vascular resistance by inhibiting cytochrome P450 mediated production of endothelin-1(Coceani *et al.* 1996; Coceani *et al.* 1997). It also acts through mitogen activated protein kinase (MAPK) in reducing the expression of TNFα, IL-1β, and macrophage inflammatory protein (MIP-1)(Otterbein *et al.* 2000). The use of water soluble

carbon monoxide releasing molecule intravenously in reperfusion phase of I/R has been shown to reduce the infarct size in hearts of mice(Guo *et al.* 2004).

1.1.6.2 Biliverdin and Bilirubin

Both bilirubin and biliverdin have antioxidant properties(Stocker *et al.* 1987; Stocker and Ames 1987) and are known to prevent oxidation of poly unsaturated fatty acids(Neuzil and Stocker 1994). Bilirubin protects cells from oxidative stress by scavenging ROS(Snyder and Baranano 2001). Administration of bilirubin to rats has been shown to modulate the expression of P-selectin and E-selectins which suggests that bilirubin has anti inflammatory properties(Vachharajani *et al.* 2000).

In addition to the major protective role of the metal-binding proteins, various low-molecular-weight molecules that are synthesised in vivo have antioxidant properties(Ames *et al.* 1981; Frei *et al.* 1988; Layton *et al.* 1996). The most important of these substances are bilirubin, melatonin, lipoic acid, Coenzyme Q, Uric acid and Melanin.

In clinical settings where liver I/R injury occur, the endogenous antioxidant system can be depleted and a serious imbalance between production of ROS/RNS and antioxidant defence can happen. This imbalance called oxidative stress leads to cell, tissue and organ injury. In these situations the replacement of the antioxidants that have been depleted could have a therapeutic role.

Figure 1.2
Schematic Representation of Pathophysiology of Liver I/R injury

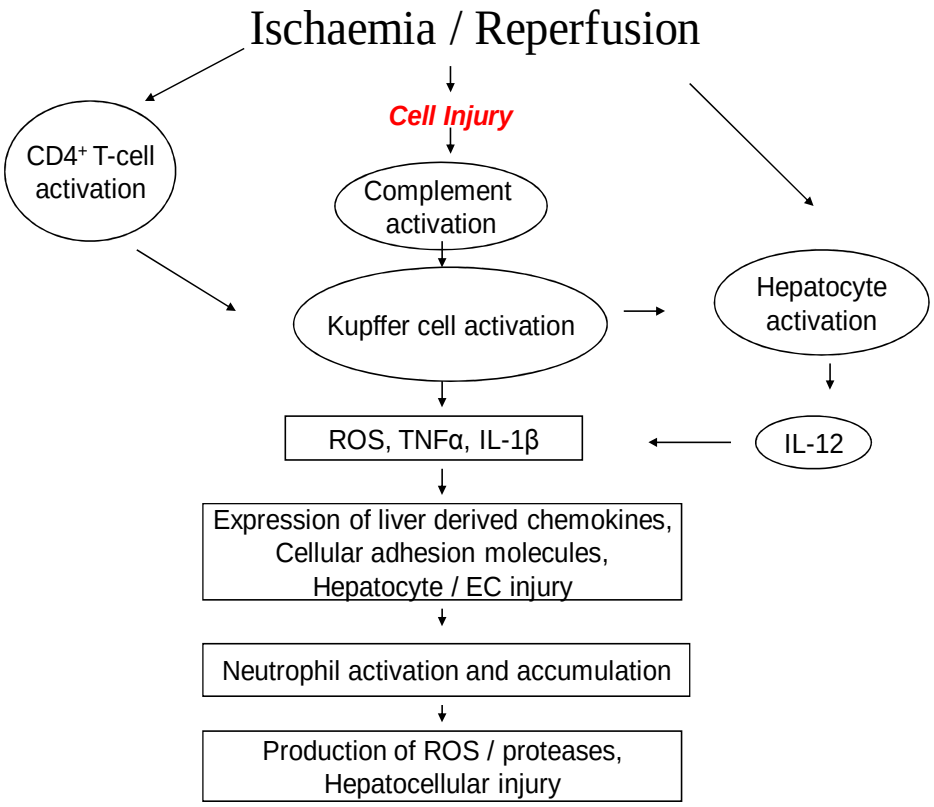


Figure 1.3 Antioxidant defences against Ischaemia reperfusion injury

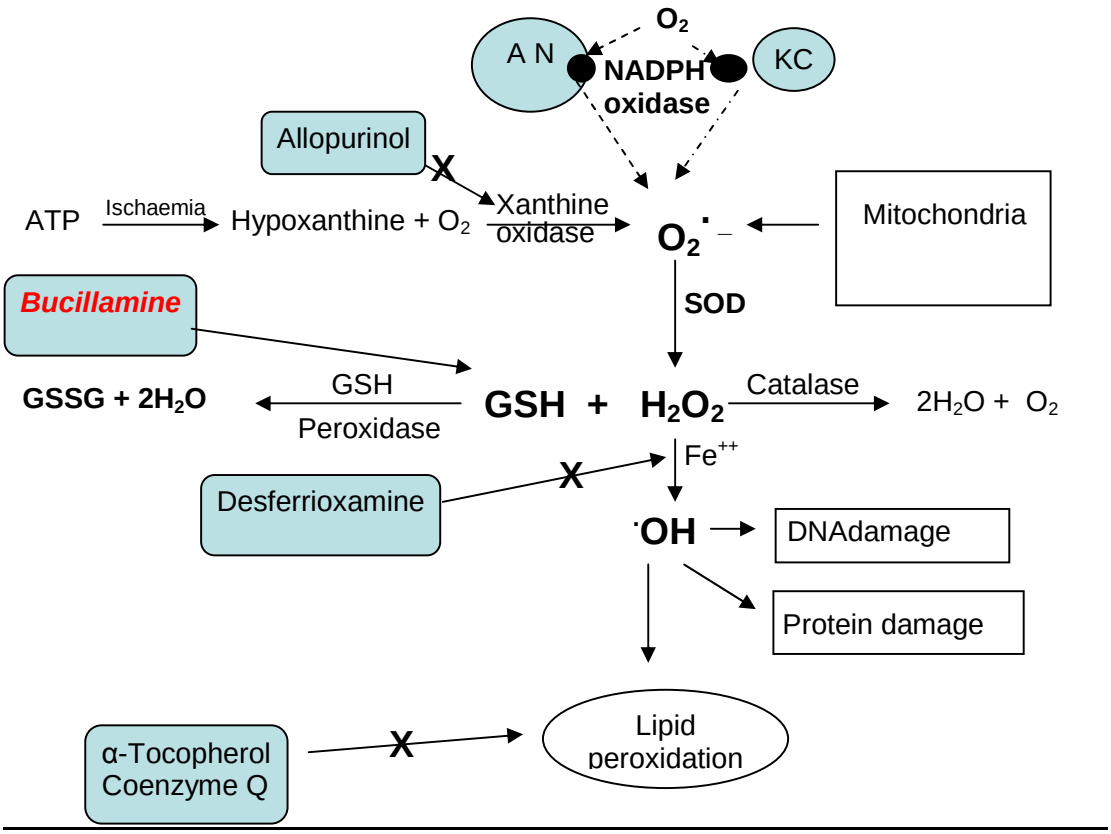


Table 1.1

Role of ROS in liver IR injury

1. Enhance pro-inflammatory gene expression (TNF-a, IL-1, IL-8, cellular adhesion molecules)
2. Induce expression of the transcription factors NF-kB and activator protein-1
3. Direct cellular damage through protein oxidation and degradation, lipid peroxidation and DNA damage
4. Direct induction and regulation of apoptotic and necrotic cell death
5. Inactivation of antiproteases
6. Induction of protective stress genes in hepatocytes
7. Formation of mediators involved in regulating sinusoidal blood flow and liver regeneration

Chapter 2

Bucillamine: A thiol antioxidant

2.1 Bucillamine: Introduction

Bucillamine [N- (2-mercapto-2- methylpropinyl- L- cysteine)] (previously called SA96), is structurally analogous to cysteine. The compound contains two donatable thiol groups and is more potent than cysteine and other cysteine derivatives containing only one thiol grouping ameliorating the effects of IR(Amersi *et al.* 2002; Amersi *et al.* 2002; Horwitz and Sherman 2001). Bucillamine is fourfold more potent than NAC in *in vitro* studies(Horwitz and Sherman 2001) and in mice 20 mg /kg i.p. of bucillamine had similar effects to a 16-fold greater dose of NAC (320 mg/kg i.p.) in models of cardiac I/R(Whitekus *et al.* 2002). . As an oral formulation it is marketed in Japan and Korea for the treatment of rheumatoid arthritis(Matsuno *et al.* 1998).

2.2 Chemistry

Bucillamine [Molecular Weight 223.3]

Cysteine is the rate limiting intracellular precursor of glutathione(De *et al.* 2001). Reduced glutathione (GSH) cofactored by glutathione peroxidase gets converted to the oxidised form (GSSG) in the presence of peroxides(Ceconi *et al.* 1988b). It serves as a major endogenous defence against oxidative stress. Bucillamine can be rapidly transported into the cells by transport protein utilised by cysteine and can restore intracellular glutathione levels. Bucillamine is available as a lyophilized powder which is highly soluble. It forms an acidic solution with normal saline which can be neutralised with sodium hydroxide for intravenous use in experimental animals.

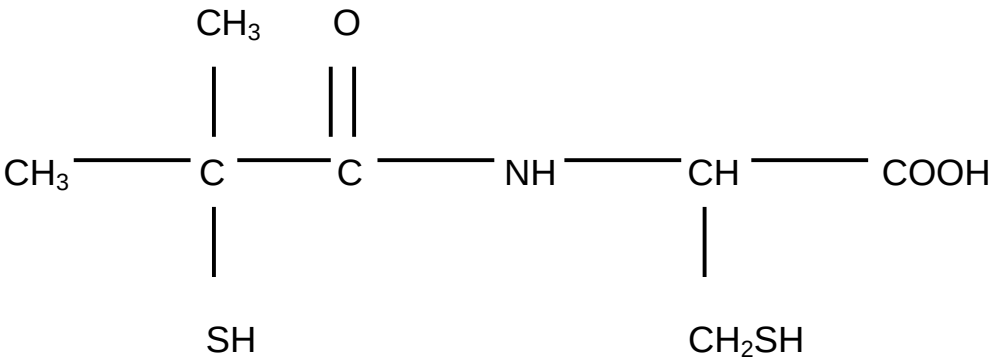


Figure2.2.1: Chemical structure of Bucillamine

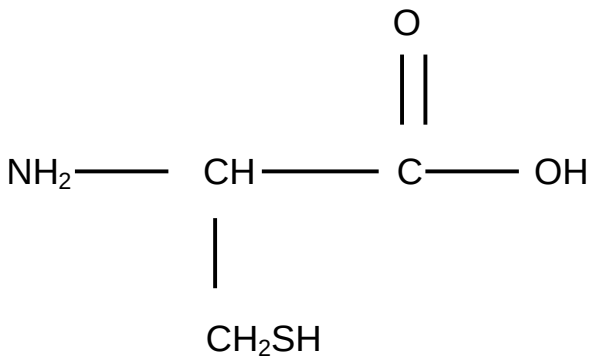


Figure2.2.2: Chemical structure of Cysteine

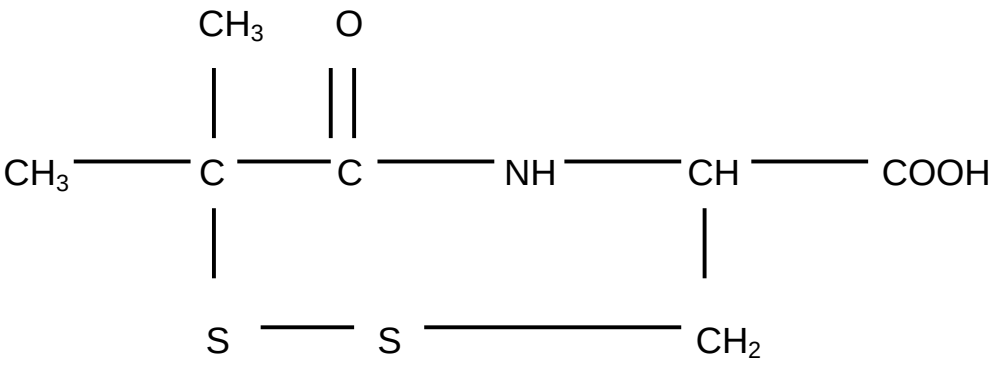


Figure2.2.3: Chemical structure of Bucillamine metabolite SA 981 which has a structural similarity to D- Penicillamine

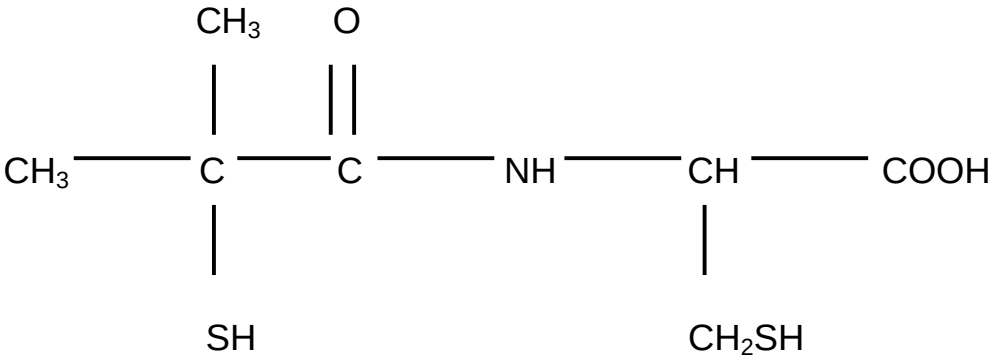


Figure2.2.4: Chemical structure of Bucillamine metabolite SA672 which has one donatable thiol group

2.3 Pharmacology

The blood concentration of Bucillamine reaches a peak of 20µg/ml after oral administration of 50mg/kg with a half life of just under 1hour in rats. The half life after intravenous administration is about 24 minutes **(Figure 2.3.1)**. Bucillamine enters erythrocytes rapidly and hence precise measurement of bioavailability requires whole blood measurements. This can be done using high performance-liquid chromatography- mass spectrometry technique(Beaudry *et al.* 2004). In humans the bioavailability and half life is almost similar to animal models(Sugawara *et al.* 1985a; Sugawara *et al.* 1985b; Horwitz 2003).

After absorption Bucillamine is metabolised in the liver into three metabolites SA 981, SA 679 and SA 672, of which, SA 981 has a disulfide compound. These can be detected in serum and urine in humans, dogs and rats(Horiuchi *et al.* 1985; Takashina *et al.* 1985; Matsuno *et al.* 1998).

In Phase I human studies in normal volunteers, bucillamine at a dose up to 25mg/kg/h i.v. for 3 hours elicited no serious toxicity(Horwitz 2003). On the basis of the pharmacokinetic assessment of blood levels during these studies it was concluded that bucillamine infused i.v. at doses ≥ 10 mg/kg/h for 3 hours in humans could be therapeutically beneficial in I/R injury(Horwitz 2003).

Figure 2.3.1

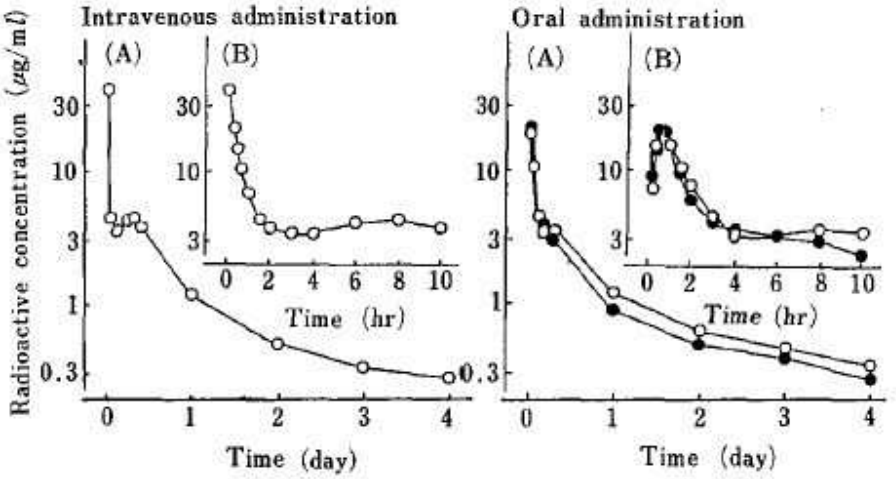


Fig. 2 Blood levels of radioactivity after intravenous administration (left) of ^{35}S-SA96 to male rats (n=5), and after oral administrations (right) of ^{35}S-SA96 to male (n=5, ○) and female (n=4, ●) rats[dose; 50 mg/kg]. Each point represents the mean±S. E. of 4 or 5 animals.
(A): 0~4 day (B): 0~10 hr

(Taken From: Sugawara, S, Ishigama, M, and Kageyama, T. Phase I study of N-(Mercapt-2-methlpropionyl)-L-cysteine (SA96). (I) Single administration study. Rinsho Yakuri 16(3), 611-620. 1985.)

2.4 Bucillamine in Rheumatoid arthritis

Rheumatoid arthritis is a chronic inflammatory process associated with inflammatory synovial hyperplasia. Cytokines (including TNF- α, IL-1, IL-6, IL-8, interferon-γ and GM-CSF) are involved in the pathogenesis of Rheumatoid arthritis(Arend and Dayer 1995). TNF-α and IL-1 stimulate the gene expression of these cytokines and cell adhesion molecules (CAM) through a signal transduction pathway leading to NF-κB activation in human synovial cells (Arend and Dayer 1995; Ledebur and Parks 1995). Bucillamine has been shown to block

the NF-κB activation cascade, leading to decrease in cytokine production and attenuation of Rheumatoid arthritis(Aono *et al.* 1996; Fujisawa *et al.* 1996; Tsuji *et al.* 1999). Bucillamine is a more potent inhibitor of NF-κB than NAC(Tsuji *et al.* 1999). Treatment with Bucillamine for long duration either on its own or in combination with other drugs has shown clinical benefit in patients with rheumatoid arthritis along with lowering of IgG, IgA and rheumatoid factor(Nagashima *et al.* 2006; Goto *et al.* 1992; Isozaki *et al.* 1992; Kashiwazaki and Shiokawa 1987; Kim *et al.* 1996).

2.5 Adverse Effects

Incidence of adverse effects with Bucillamine is very low with most adverse effects being reported as case reports in patients who were receiving treatment for a few months. The common side effects with Bucillamine treatment are pruritus rash, stomatitis, proteinuria, nausea, anorexia and abnormal liver function tests(Goto *et al.* 1992; Isozaki *et al.* 1992; Yoshida *et al.* 1991; Nagahama *et al.* 2002). Most of these developed on chronic usage and disappeared on stopping the drug. Sakai et al have also reported giant mammary hyperplasia in a young women(Sakai *et al.* 2002). Other adverse effects reported are interstitial pneumonia, myasthenia gravis and agranulocytosis(Nakashima *et al.* 1995; Sawa *et al.* 1999; Miwa *et al.* 2002).

2.6 Studies Involving use of Bucillamine in IR injury:

These have been tabulated below (Table 2.1):

Model	Organ	Ischaemia Type	Duration	Route of administration	Bucillamine Dose	Outcome Measures	First author (Year)
Tissue	Cardiac Myocytes	Oxidative stress - H_2O_2	44 hours	Incubation	0, 125, 250, 500μM	Dose dependent ↓ in LDF levels	Horwitz LD (2001)
Canine	Cardiac	Warm ischaemia	90 min ischaemia 48 hour reperfusion	I. V. during 3hr reperfusion	11 mg/kg/hr, 22mg/kg/hr	Dose dependent improvement in (Infarct size)/(region at risk) ratio	Horwitz LD (2001)
Rat	Normal Liver *ex vivo*	Cold ischaemia	24 hour	Intraportal	10mg at harvest, 90mg in perfusate	↑ portal venous flow, ↑ bile production, ↓SGOT, ↑ Liver GSH, ↓ Liver GSSG, ↑ Blood perfusate GSH, ↓ Blood perfusate GSSG, Histology	Amersi F (2002)
Rat	Steatotic Liver *ex vivo*	Cold ischaemia	24 hour	Intraportal	10mg at harvest, 90mg in perfusate	↑ portal venous flow, ↑bile production, ↓SGOT, ↑ Liver GSH, ↓ Liver GSSG, ↑ Blood perfusate GSH, ↓ Blood perfusate GSSG, Histology	Amersi F (2002)
Rat	Liver *in Vivo* OLT	Cold ischaemia	24 hour	Intraportal Intravenous	15mg/kg before reperfusion 10mg/kg 30min after reperfusion	Improved survival, ↓serum GOT levels, histology	Amersi F (2002)

Table2.1

2.7 Probable mechanism of action in IR

1. Bucillamine has been shown to preserve high concentrations of glutathione in an *ex vivo* liver cold ischaemia model(Amersi *et al.* 2002; Matsuno *et al.* 1998). Its anti oxidant activity could hence be primarily related to replenishment of endogenous reduced glutathione by the donated thiol groups.
2. The fully oxidised metabolite of Bucillamine SA 981 has immunomodulating effect. It is more potent in suppressing IL-6 production in cultured synovial cells than bucillamine itself(Matsuno *et al.* 1998).
3. Bucillamine and SA 981 increase glutathione biosynthesis in both human and murine hepatoma cell lines(Wielandt *et al.* 2006).However, the increase in biosynthesis was seen after 24 hours and is unlikely to be the mechanism of action in early phase of I/R injury.
4. Bucillamine is a chelating agent which may retard iron mediated generation of free radicals(Mazor *et al.* 2006).

Bucillamine has also been found to act as a pro- oxidant at low concentrations(Kladna *et al.* 2006) and induces apoptosis through enhanced generation of ROS in the presence of copper ions(Sawada *et al.* 1997). High doses of Bucillamine (150mg/kg- 400mg/kg) given intraperitoneally is known to deplete liver GSH in mice and cause oxidative damage(Yeung 1991).

2.8 Hypothesis

The effect of bucillamine in warm ischaemia reperfusion model in liver has not been studied as yet. We hypothesize that bucillamine would protect against warm ischaemia reperfusion injury through its antioxidant and anti-inflammatory effects.

2.9 Aim

To use a well described model of Liver ischaemia reperfusion to determine the effect of Bucillamine administration on liver function, liver microcirculation and apoptosis.

Chapter 3
Materials and Methods

3.1 Animals and surgical preparation for lobar IR model

The drug was evaluated in an *in vivo* lobar liver ischaemia reperfusion model as previously described(Koo *et al.* 1992; Koti *et al.* 2005). The study was conducted under a project license from the Home Office in accordance with the Animals (Scientific Procedures) Act 1986. Male Sprague –Dawley rats, weighing 270-330gms were used. Animals were kept in a temperature controlled environment with a 12 hour light-dark cycle and allowed tap water as well as standard rat chow pellets *ad libitum*. Animals were anaesthetised with 4% Isoflurane and maintained with 2.0% Isoflurane. They were allowed to breathe spontaneously through a concentric mask connected to an oxygen regulator and monitored with a pulse oximeter (Ohmeda biox 3740 pulse oximeter, Ohmeda, Louisville, USA).

Polyethylene catheters (Portex 2 Fr) were inserted into the carotid artery (right or left) for monitoring of mean arterial blood pressure and the right jugular vein for administering normal saline (1ml /100gm body weight/hour) to compensate for intraoperative fluid loss.

Laparotomy was carried out through a midline incision. The ligamentous attachments of the liver were cut and the liver exposed. All animals were given heparin 20 units /kg body weight and partial hepatic ischaemia of the left lateral and median lobes of (70% of liver) was induced by clamping the corresponding vascular pedicle with an atraumatic microvascular clamp for 45 minutes in the control and Bucillamine groups. This model prevents splanchnic congestion by

allowing flow through the remaining liver(Koo *et al.* 1992; Koti *et al.* 2005). Animals in the Sham group did not have any vascular clamping. Animals were monitored for 3 hours in the reperfusion period. Animals were randomly allocated to the following groups:

3.1.1 Experimental groups (n=6 in each group)

Group1 - (Sham) – laparotomy and mobilisation of liver but no occlusion of the vascular pedicle.

Group2- IR- 45 minutes of partial hepatic ischaemia followed by 3 hrs of reperfusion.

Group3- Bucillamine 15mg/ kg/hr intravenously + IR followed by 3 hrs of reperfusion.

Group4- (SB) - Sham + Bucillamine infusion 15mg/kg/hr (SB)

Group5- IR24- 45 minutes ischaemia followed by 24 hour reperfusion

Group6- B24- 45 minutes ischaemia + Bucillamine 15mg/ kg/hr intravenously for 3 hours followed by 24 hour reperfusion

Subjects in the bucillamine group were administered an infusion of bucillamine (15mg/kg/hr) over the operative period (preischaemia, ischaemia and 3 hour post reperfusion). Bucillamine was supplied by Santen pharmaceuticals, Osaka, Japan. Animals in the Sham, SB and I/R groups were given an equivalent volume of saline up to 3 hours of reperfusion (or equivalent period in case of sham operated animals). Temperature of the animals was monitored and maintained at 36-38^{o}C by means of a heated platform (Harvard Apparatus, Kent, UK). The

experiments were terminated by exsanguination and serum and plasma samples were collected by spinning the blood at 3000rpm for 10 minutes and stored in aliquots of 0.2 mls at -80^{o} C. Liver tissue was stored in formalin for histopathology and immunohistochemistry. Liver tissue was also frozen immediately on termination of the experiment in liquid nitrogen and stored at -80^{o} C for further analysis.

3.1.2 Recovery experiments

In the recovery experiments (Group 5 and 6) the animals were given Bucillamine 15 mg/kg/hr or equivalent volume of saline for 3 hours intravenously following the ischaemia, the abdominal wound was closed with 4-0 vicryl and the neck line was removed after the period of infusion and the neck wound closed using 4-0 Vicryl continuous suture. The animals were recovered, administered adequate analgesia (Buprenorphine 0.15mg/kg subcutaneously) and kept in a temperature controlled environment with a 12 hour light-dark cycle and allowed tap water as well as standard rat chow pellets *ad libitum*. After 24 hours the animals were re-anaesthetised with 4% Isoflurane and maintained with 2.0% Isoflurane (Abbott Laboratories Ltd., Kent, UK). They were allowed to breathe spontaneously through a concentric mask connected to an oxygen regulator and monitored with a pulse oximeter (Ohmeda biox 3740 pulse oximeter, Ohmeda, Louisville, USA). The right carotid artery was cannulated with a polyethylene catheter (0.40-mm inner diameter, Portex, Kent, UK) for administering fluorochromes. The abdomen was re-opened through the previous incision. The left lobe of liver was mobilised

gently and placed under the intravital microscope for visualisation of the hepatic microcirculation. Following this experiments were terminated by exsanguination and samples were collected as above.

3.1.3 Preparation of Bucillamine

Bucillamine was kindly provided by Santen Pharmaceutical, Osaka, Japan, as a lyophilized powder. This dissolves in normal saline readily to form an acidic solution. Bucillamine solution was freshly prepared just before each experiment (1.5 mg/ml in normal saline [concentration adjusted in dosing experiments]). The pH of the solution was then adjusted to 7.4 by titrating with NaOH solution.

3.1.4 Blood collection in lobar IR model

Blood samples were collected from the IVC at the end of reperfusion. Samples were heparinised and centrifuged at 3000 RPM for 10 minutes at room temperature to sediment the erythrocytes. The plasma supernatant was removed and stored at –80 °C until required for assay of plasma F_2 isoprostanes. Sample was also collected in serum gel tube (BD Vacutainer Systems, Plymouth, UK) and allowed to stand for 2 hours, following which sample was spun down at 3000RPM for 10 minutes and supernatant serum removed and stored in -80^{o} C until required for assay for Alanine transaminase and Aspartate transaminase and serum CINC-1 levels.

3.1.5 Tissue collection

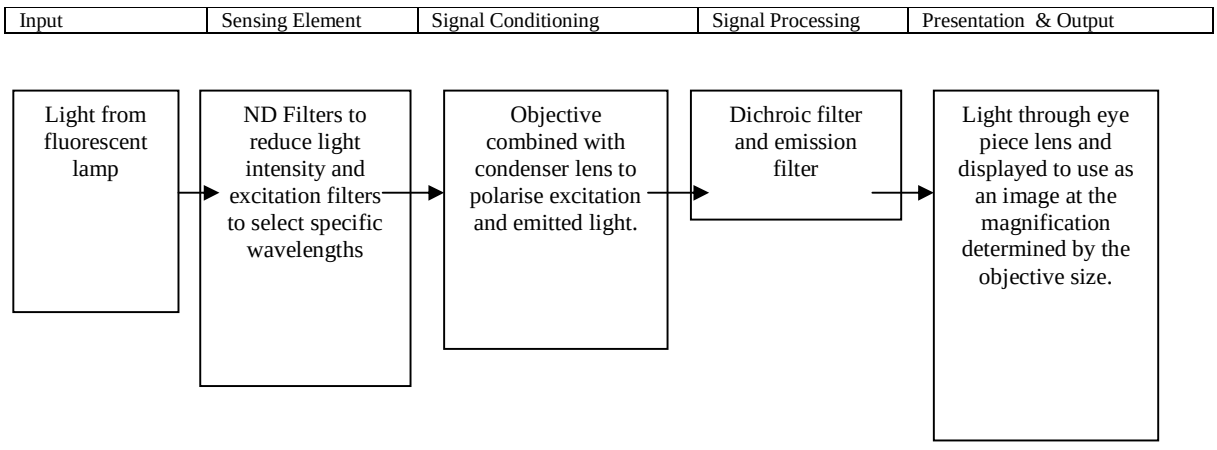
At termination of reperfusion phase, sections of liver were (1) freeze clamped in liquid nitrogen (for determination of tissue glutathione, tissue F_2 isoprostanes and immediately stored [at -80^0 C] for western blotting), and (2) fixed in 10% formalin for histological study.

3.2 Intravital microscopy

3.2.1 Introduction

The first intravital fluorescent microscope (IVFM) was made by Ellinger and Hirt in Germany , which was a modified version of the fluorescence microscope (developed in the early 20th century [1929]) to allow tissues to be examined(Kasten 1993). The IVFM can be divided into two separate measuring systems. The first represents the microscope alone, with the light source, lenses, filters and fluorescence acting as the functional elements (Fig 3.2.1).

Figure 3.2.1. Intra-vital fluorescence microscope – instrumental components.



The second system begins with the image seen from the microscope being converted via the CCD camera, passing through the frame grabber and onto the computer monitor display (Figure 3.2.2)

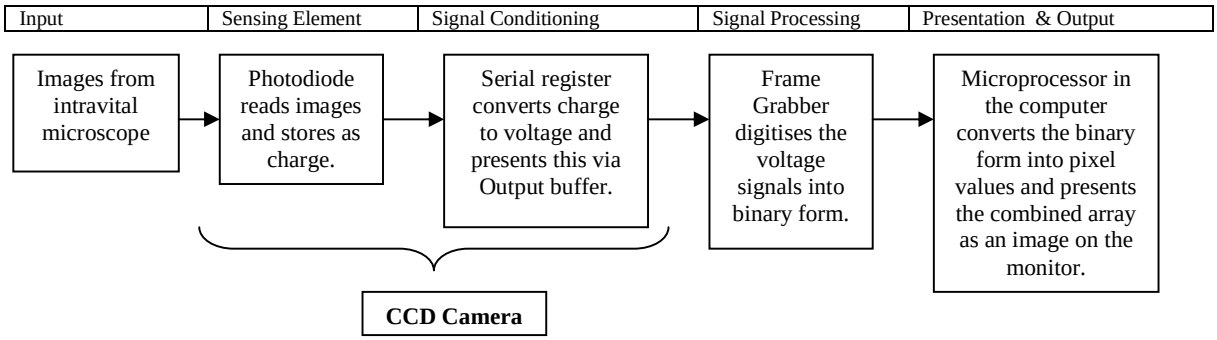


Figure3.2.2. Image recording system in the intravital microscope

The IVFM used for this study was a custom built Nikon Epi-illumination system with filter block set suitable for Texas Red, FITC and DAPI dyes as detailed in table 3.2.1

Table 3.2.1.The filter set details for the Nikon Epi-illumination system.

Fluorochrome	**Excitation (nm)**	**Emission (nm)**	**Colour**	**Type of label**
DAPI	360	450	Blue	Nuclear Stain
FITC	495	525	Green	Protein Conjugation
Texas Red	596	620	Red	Protein Conjugation

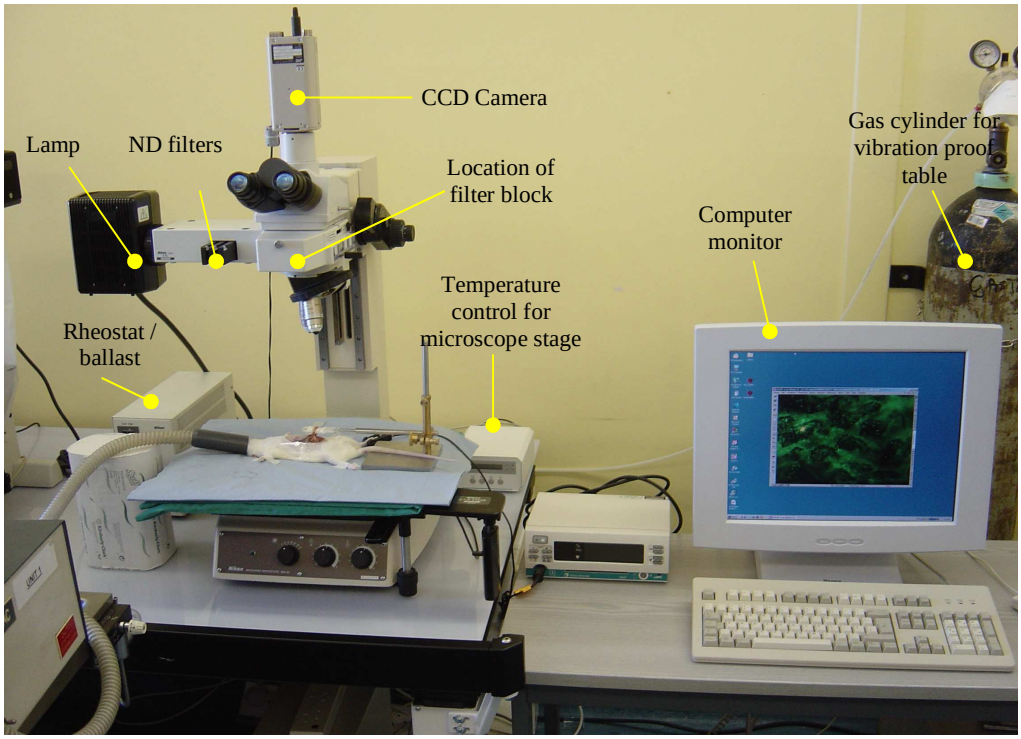


Figure 3.2.3.The complete intravital microscopy setup.

3.2.2 Principles

Fluorescence is the inherent property of some atoms and molecules to absorb light of a particular wavelength and, after a brief interval (the fluorescent lifetime) re-emit light at a longer wavelength.

These atoms and molecules (chromophores) are excited by an external light source. They absorb the light energy and pass into an excited energy state. After entering the higher energy state, the molecules undergo internal changes. The electrons in certain molecules instead of returning to the ground state enter a metastable state. When the molecules pass down to the ground state they emit

the excess energy as electromagnetic radiation (Sykes *et al.* 1991) which is seen as fluorescence. The energy between the metastable and ground states is less than the energy absorbed during excitation, so the emission wavelength will be of longer wavelength than the absorbed or excitation light(Sykes *et al.* 1991).

The light from the epi-illumination light source (which is usually a mercury arc or xenon lamp in standard IVFM setups) first passes through an excitation or short-pass (SP) filter allowing only the excitation waves through. These are then passed onto a chromatic beam splitter (dichromatic mirror) where wavelengths below a certain value are reflected onto the specimen while any above the wavelength value are passed through and dissipated. The light hitting the specimen then activates the chromophore which emits light of a particular wavelength.

A dichromatic mirror allows passage of certain values of excitation and emission wavelengths. The dichromatic mirror is designed to allow the transmission of longer *emission* wavelengths and reflect the shorter *excitation* wavelengths. The three filters are usually contained within a filter box as illustrated in figure 3.2.4.

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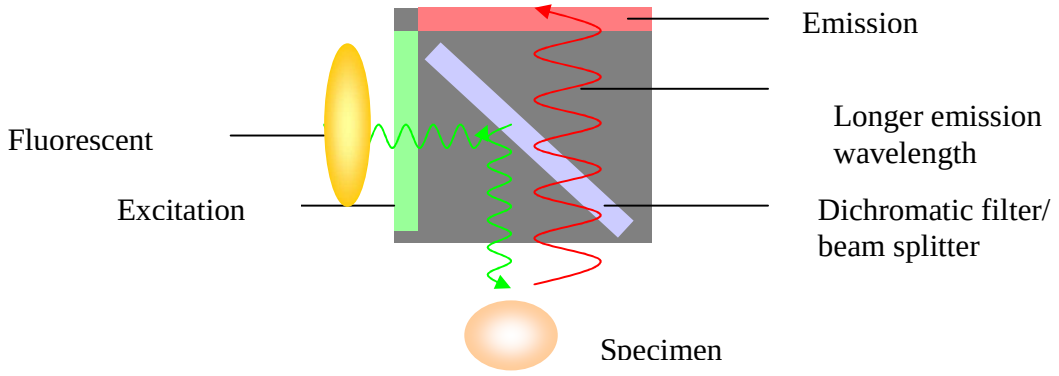

Figure 3.2.4 Schema of Filter Block

After passing through the dichromatic beam splitter the excitation wavelength then passes through a barrier filter that removes any light of shorter wavelength that may have passed through the dichromatic beam splitter. This emission wavelength then passes into a detector that sends the signals to the eye pieces for operator observation or into a video camera for image recording.

3.2.3 Components

3.2.3.1 Light source

The light of the IVFM is a fluorescent mercury arc lamp, which is protected in a lamp-house. Since the development of epi-illumination, microscope optics has developed with the incorporation of laser light sources increasing the resolution of the image and improving the signal-to-noise ratio. A rheostat or ballast alters the current applied to the lamp to control the intensity of the light produced. In a gas discharge, such as a fluorescent lamp, current causes resistance to

decrease. This is because as more electrons and ions flow through a particular area, they bump into more atoms, which frees up electrons, creating more charged particles.

3.2.3.2 Objective lenses

IVFMs usually use water immersion objectives as these reduce the refractive index in living in vivo imaging .The water immersion objective delivers contrast and resolution values nearly equivalent to the theoretical limits, and maintains its performance when water layers of 80 and 153 μm are added between the target specimen and cover slip, a simulation of the situation encountered in imaging deep within aqueous material such as living cells or tissue.

There are four types of filters in the intra-vital fluorescence microscope system, neutral density (ND) filters, an excitation filter, an emission filter and a multi-band pass filter.

Special neutral density (ND) filters are built in the light path to block 90-95% of the incidence light passing into the microscope to prevent damage to the eye as well as reduce overexposure of fluorescence in specimens(Nikon 2004). There are three neutral density filters which are placed in front of the optical light path to reduce illumination without altering the colour balance.

The excitation filter passes only a selected range of wavelengths of light to cause the fluorescently labelled specimen to fluoresce and filters the rest. The bandwidth of a filter determines the brightness of the fluorescent image. If the bandwidth is narrow then the image appears dark but minimal auto-fluorescence and photo-bleaching occur. With a wide bandwidth, although the image appears bright, autofluorescence may also be detected with the added disadvantage of photo-bleaching(Nikon 2004).

The emission or barrier filter allows only light wavelengths that have been emitted from the specimen. These are usually longer according to Stoke's law. In older epi-illumination microscopes only a dichromatic beam splitter is present allowing use of one fluorescent dye at a time. However newer developed models have a multi-band pass filter or a polychromatic beam splitter that can allow visualisation of three different coloured fluorescent dyes.

3.2.3.3 CCD Camera

The image obtained from the IVFM is then recorded by a charged coupled device (CCD) camera. For this study a JVC TK-C1360B colour video camera was used (JVC video camera, JVC, Osaka, Japan). CCD sensors are light integrating devices that accumulate photo charges until image readout. The CCD chip in the camera contains an array of pixels that transform light (wavelength 400nm to 1000nm) into a charge, which during readout is transformed into a voltage.

Once photo charges are shifted to the storage area, images are erased from the CCD light sensing area(Cinelli 1998). New photo charges can not pile up on top of the previous images.

The photo charges are shifted in block from the sensing area to the storage area, then each line is individually read to the serial registers and finally photo charges are transferred to the output buffers. Each readout cycle is initiated by a vertical sync pulse (vertical blanking sync) which activates the parallel driver and triggers the shift block of photo charges accumulated in the sensing area to the CCD storage area.

Exposure time is the main factor that determines the sensitivity of CCD cameras. Long exposure times improve camera sensitivity and reduce the noise levels of the images, since the accumulation of the photo charge in the CCD sensor is proportional to the duration of the exposure period .The analogue video images are digitised at varying resolutions typically around 8 bits resolution by a frame grabber board.

3.2.3.4 Frame Grabber

The Matrix Meteor II/Standard frame grabber used in this study allows image acquisition at 25 frames per second. The main parts of the frame grabber are the low-pass filter, the decoder, the trigger and the image coding components. The low pass filter reduces the high frequency noise and aliasing effects from the analogue CCD signals and passes the refined signals to the video decoder. This

is the component of the frame grabber that performs the actual analogue to digital conversion of the component (Y/C) analogue video signals.

3.2.3.5 Image Analysis Software

Adjusting the output image can enhance the resolution and contrast of the image. Light intensity and colour are represented in numbers between 0 (black) and 255 (white). Converting the image into binary form means it is converted into a black and white image. This is done by assigning threshold values which determine the distribution of the pixels into two populations either of value 0 or 255.

An image can be transformed into binary form using many different types of software packages.

Laboratory Universal Computer Image Analysis (LUCIA) is a multi-spectral image analysis software developed specifically for image processing independently on red, green and blue components and then combining them together into the RGB image at the same time. Most Nikon microscopes are supplied with Lucia software and there are many versions available denoted by the letter following Lucia. Lucia G is the top of the range package that allows 24-bit colour image analysis with the function to allow user to create specialized macros which was used in this study.

3.2.4 Preparation of Fluorescent Dyes

In order to measure the parameters RBCs were labelled with fluorescein isothiocyanate (FITC) and leukocytes were labelled with Rhodamine 6G. At the end of each experiment the amount of cellular damage was assessed by labelling with propidium iodide.

3.2.4.1 Labelling of RBCs

An animal was sacrificed by exsanguination and RBCs were labelled using the following protocol(Zimmerhackl *et al.* 1983):

Protocol for Fluorescence Labelling Of RBC

Stock Solution 1 (adjust to pH 7.4)
Barbital Sodium 2.55 g
1M HCl 10 ml
NaCl 6.8 g
Bring to 0.5 L

Stock Solution 2:-
$MgSO_4.7H_2O$ 24.6 g
100ml distilled water makes 1M solution

Stock Solution 3(0.03M $CaCl_2$**):**
$CaCl_2.2H_2O$ 4.41 g
100ml distilled water makes solution.

Note: *Stock solutions can be kept for around a month in a fridge at* 4^0C.

Working Solution (glucose saline buffers)
Stock 1 50 ml
Stock 2 0.1 ml
Stock 3 0.1 ml
Glucose 4.2 g
Make up to 200ml with distilled water

FITC for red blood cells:-
40mg of FITC
2ml of glucose saline buffer

Labelling cells:
1. Collect approx 8ml blood in heparinised tube or as much as you need.
2. Centrifuge blood at 400g or 2000r.p.m for 10mins
3. Remove plasma + buffy coat
4. Wash cells with glucose saline buffer 5 times
5. 1ml of washed red cells are added to 1ml of buffer and 0.4ml of FITC
6. Leave for 1.5 to 2hrs at room temp
7. Labelled cells are washed 3 times until no colouring is left in supernatant
8. Suspend in glucose saline buffer in 1:2 dilution.

RBCs which were labelled could be stored at 4^0 C for 3-4 days.

3.2.4.2 Leukocyte labelling

Leukocyte were labelled *in vivo* with rhodamine 6G-(Sigma, Rodermark, Germany) 0.3 mg/kg by administering the dye intra- arterially(Wunder *et al.* 2002) through the carotid artery at 150 minutes post reperfusion. Rhodamine 6G accumulates in the mitochondria of the leukocytes. Intrarterial or intravenous injection results in labelling of circulating leukocytes(Dunn *et al.* 2002). *In Vivo* labelling of leukocytes requires using freshly prepared rhodamine solution dissolved in normal saline.

3.2.4.3 Detection of hepatocellular death in vivo*.*

Irreversible injury in anoxic hepatocytes results in abrupt increase in plasma permeability which results in uptake of Propidium Iodide fluorescent dye stains nuclei of cells that are lethally damaged(Herman *et al.* 1988). Propidium iodide solution 0.05mg per ml of saline was prepared and 0.05 mg/kg was injected at the end of 3 hours of reperfusion.

3.2.5 Measurement of Parameters by Intravital microscopy

Pictures taken at Intravital microscopy were recorded on computer (25 frames per second) and off line microcirculatory analysis performed at a later time.

3.2.5.1 Measurement of RBC velocity

RBC velocity was determined by using 40x magnification. Ten sinusoids were randomly selected at each time point in each animal and RBC velocity was calculated by frame by frame analysis as previously described by Kelly *et al*(Kelly *et al.* 1998). RBC velocity was calculated by measuring the distance travelled by a RBC by frame by frame analysis as shown in figure 3.2.5. RBC velocity was then calculated as:

RBC Velocity = (distance travelled/ n-1) x 25 in µ/sec

Where n is the number of frames.

Mean value was calculated for each time point.

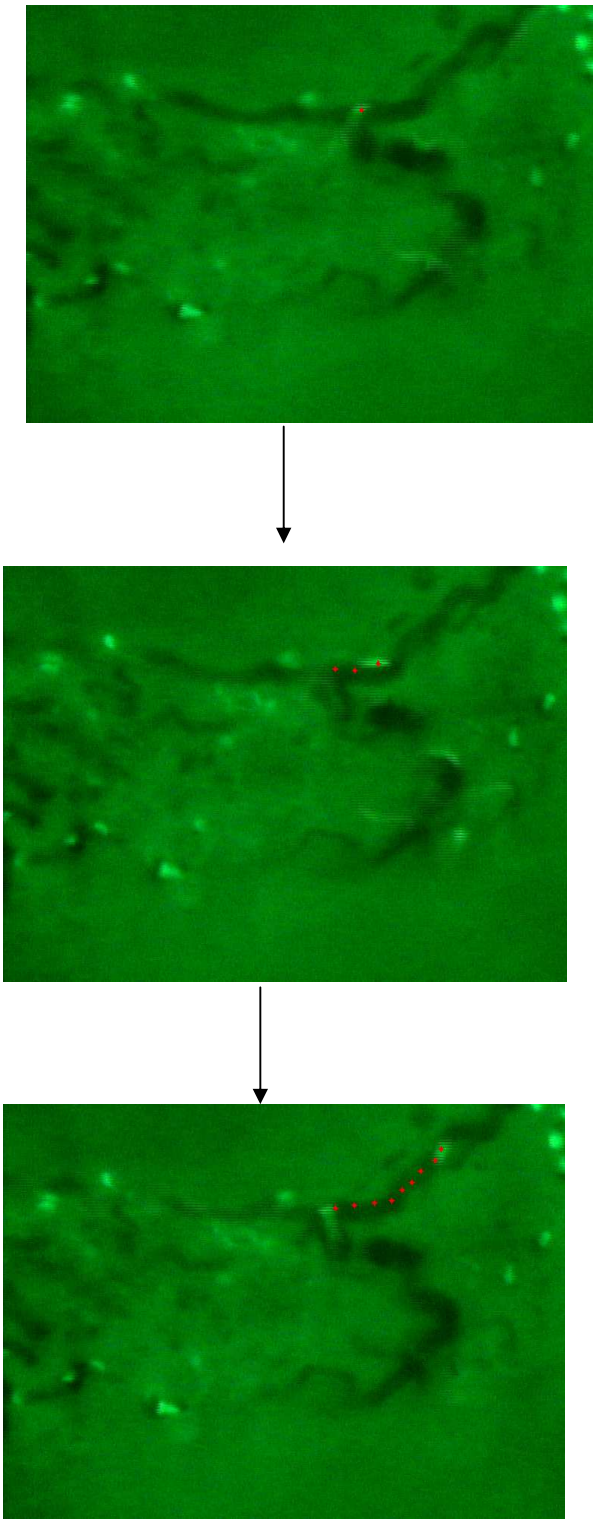

Figure 3.2.5 Frame by Frame analysis of measuring red blood cell velocity.

3.2.5.2 Sinusoidal diameter

Sinusoidal diameter was measured in ten randomly selected sinusoids at each time point in each animal and mean was calculated.

3.2.5.3 Sinusoidal perfusion

Five randomly chosen non overlapping Rappaport acini were observed for more than a minute with recording from representative areas for 2 seconds with the JVC video camera (25 frames per second) on the computer for each time point. Perfusion was established by studying the ratio of perfused to total visible sinusoids after administration of FITC labelled RBC. Perfused sinusoids described as continuously perfused (continuous perfusion for > 1 min) (N_c) or interrupted (intermittent perfusion for 1 min. period) (N_i) or non perfused (N_n). Sinusoidal perfusion index was calculated as:

Perfusion index= (N_c + 0.5 N_i) / (N_c + N_i + N_n)(Post *et al.* 1993) and expressed as %.

3.2.5.4 Sinusoidal Blood Flow

Sinusoidal blood flow was calculated using formula for capillary blood flow as previously described by Wunder *et al* in 2002(Wunder *et al.* 2002).

Sinusoidal blood Flow= Velocity x 0 **x $(d/2)^2$** (expressed as pl/sec)

3.2.5.5 Leukocyte endothelial interactions

Ten randomly chosen post sinusoidal venules were visualized for at least 20 seconds each at 40x magnification, under green filtered light. The number of sticking leukocytes were counted and expressed as cells per mm^2 of endothelial surface (length of observed vessel length x diameter x 0 = stickers per mm^2) as previously described(Croner *et al.* 2006; Wunder *et al.* 2002). Adherent leukocytes were calculated as adherent cells/ mm^2 of liver tissue.

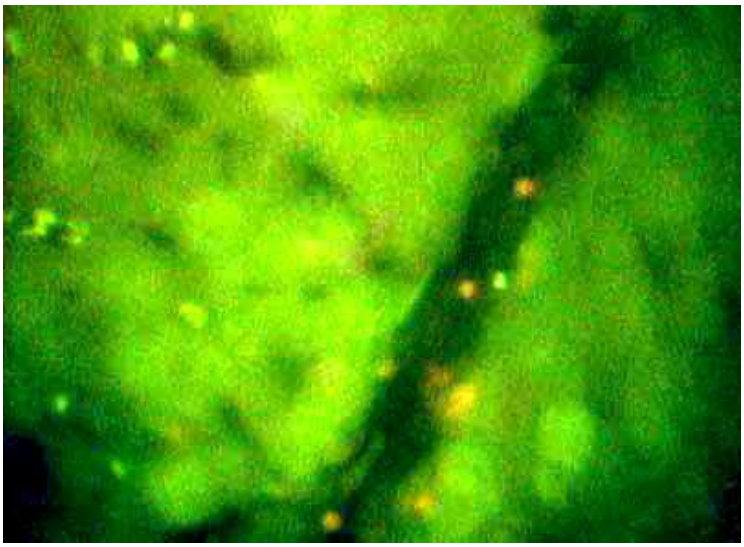

Figure 3.2.6 Venules showing adherent Leukocytes (stained yellow)

3.2.5.6 Detection of hepatocellular death in vivo.

Dead nuclei stained with propidium iodide and were expressed as number of cells per mm^2. An average value was taken for each subject after studying at least 5 high power fields.

Figure 3.2.7. Nonviable nuclei stained with propidium iodide.

3.3 Histological assessment

Samples of liver were taken, at the end of the experiment from the left lobe. These were fixed in 10 per cent neutral buffered formalin and embedded in paraffin; paraffin section 4μm thick were cut using a microtome and mounted on slides for haematoxylin and eosin staining. Assessment of liver injury was performed with light microscopy by Consultant pathologist who was blinded from the study groups, by a scoring system devised by Suzuki *et al*(Suzuki and Toledo-Pereyra 1993) as shown in the table 3.3.1. In this system, sinusoidal congestion, hepatocyte ballooning or vacuolation, and necrosis are graded from 0 to 4. No congestion, vacuolation or necrosis is given a score of 0, while severe congestion, vacuolation and necrosis is given a score of 4, as shown in the table.

Table 3.3.1

Suzuki's Criteria for Liver I/R

Numerical assessment	**Congestion**	**Vacuolation**	**Necrosis**
0	None	None	None
1	Minimal	Minimal	Single cell
2	Mild	Mild	<30%
3	Moderate	Moderate	30 - 60%
4	Severe	Severe	>60%

3.4 Liver Injury Assay

Blood was sampled from the IVC 3 hours post perfusion and centrifuged at 3000 rpm for 10 minutes. Serum was analysed on an autoanalyzer (Hitachi 747, Hitachi, Tokyo, Japan) using commercially available kits (Boehringer Mannheim, Lewes, East Sussex, UK) for serum aspartate transaminase and serum alanine transaminase.

3.5 Measurement of Hepatic F_2-Isoprostanes

Approximately 250 mg whole liver tissue was homogenized in a mixture of saline and chloroform/methanol solution containing butylated hydroxytoluene (5%) (to inhibit *ex vivo* lipid peroxidation), and centrifuged at 3,000g for 10 minutes. This process results in three phases, an upper aqueous phase, separated from the lower lipid containing phase by a ring of protein precipitate. The lower lipid layer was aspirated and, following the addition of 500 pg of [^{2}]H_4-iso-PGFα (Cayman Co., Ann Arbor, MI) as internal standards, was dried down under nitrogen, and hydrolyzed in methanolic 15% potassium hydroxide solution (1 hour, 37℃). To extract the F_2-isoprostanes, the pH was adjusted to 3.0, and the samples were extracted on a C_{18} solid-phase extraction cartridge (Elstree, Hertsfordshire, Waters, UK) as described(Morrow and Roberts 1999), converted to the pentaflurobenzyl ester, purified by thin-layer chromatography and analyzed as the tri-methysilyl ether. F_2 isoprostane levels were quantified by selected ion monitoring gas chromatography negative ion chemical ionization/mass spectrometry with monitoring of ions at *m/z 569* and *573(Morrow and Roberts*

1999). The levels of F_2-isoprostanes were expressed as the ratio of F_2-isoprostanes to mg dried protein in liver tissue homogenates.

3.6 Measurement of Plasma F_2-Isoprostanes

Following the addition of 500 pg of [^{2}]H4-iso-PGF (as internal standard) to 500 µl of rat plasma, the samples were hydrolyzed in methanolic 15% potassium hydroxide solution (1 hour, 37℃). To extract the F 2-isoprostanes, the pH was adjusted to 3.0, and the samples were extracted on a C18 solid-phase extraction cartridge (Elstree, Hertsfordshire, Waters, UK) as described(Morrow and Roberts 1999), converted to the pentaflurobenzyl ester, purified by thin-layer chromatography and analyzed as the tri-methysilyl ether. Detection was performed by selected ion monitoring gas chromatography negative ion chemical ionization/mass spectrometry with monitoring of ions at *m/z 569* and *573.*

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3.7 Liver Tissue Glutathione Assay

Liver tissue Glutathione was measured by modified Tietze method(Baker *et al.* 1990; Eyer and Podhradsky 1986; Tietze 1969) using a commercially available kit (Cayman Chemical Company, Ann Arbor, USA). This utilizes a carefully optimized enzymatic recycling method, using glutathione reductase, for the quantification of GSH. The sulfhydryl group of GSH reacts with 5,5' dithiobis-2-nitrobenzoic acid (DTNB) and produces a yellow coloured compound 5-thio-2-

nitrobenzoic acid (TNB). The mixed disulfide that is concomitantly produced is reduced by glutathione reductase to recycle the GSH and produce more TNB. The rate of production of TNB is directly proportional to this cycling reaction which is proportional to the concentration of GSH. GSH and GSSG were measured according to manufacturer's protocol. GSH concentrations of the sample, was measured by the kinetic method as described in the protocol to avoid interference by other thiol groups.

3.8 Serum CINC-1 assay

Serum samples were assayed by using CINC-1 assay kit (Quantikine rat CINC-1 immunoassay, R & D systems, Inc., Minneapolis, USA) using the manufacturers recommended protocol. This assay employs the quantitative sandwich enzyme immunoassay technique. A polyclonal antibody specific for rat CINC-1 is precoated onto a microplate. Standards, controls and samples are micropipetted into the wells and any rat CINC-1 gets bound by the antibody. After washing away unbound substances, an enzyme linked polyclonal antibody specific for rat CINC-1 is added to the wells. The enzyme reaction produces yellow colour the intensity of which is measured colorimetrically.

Chapter 4

Study of microvascular and biochemical changes in the early phase of Liver IR (3 hrs) and the effect of Bucillamine on early phase Liver IR

4.1 Introduction:

Liver I/R is known to cause microcirculatory perfusion failure, activate polymorphonuclear leukocytes and increase leukocyte-endothelial cell interaction which in turn contribute to hepatocellular damage and liver dysfunction(Menger *et al.* 1999; Vollmar *et al.* 1994a; Vollmar *et al.* 1996).

The effect of bucillamine in the treatment of liver warm I/R injury has not been investigated, furthermore its effect on liver microcirculation are not known. The aim of the present study was to use a well described model of Liver ischaemia reperfusion injury to determine the effect of Bucillamine administration on liver function, liver microcirculation and hepatocyte apoptosis/ necrosis.

4.2 Materials and Methods

4.2.1 Animals and surgical preparation

The study was conducted under a project license from the Home Office in accordance with the Animals (Scientific Procedures) Act 1986. Male Sprague – Dawley rats, weighing 270-330gms were used. Animals were kept in a temperature controlled environment with a 12 hour light-dark cycle and allowed tap water as well as standard rat chow pellets *ad libitum*. Animals were anaesthetized with 4% Isoflurane and maintained with 2.0% Isoflurane (Abbott Laboratories Ltd., Kent, UK). They were allowed to breathe spontaneously through a concentric mask connected to an oxygen regulator and monitored with a pulse oximeter (Ohmeda biox 3740 pulse oximeter, Ohmeda, Louisville, USA).

Polyethylene catheters (Portex 2 Fr, Portex, Kent, UK) were inserted into the carotid artery (right or left) for monitoring of mean arterial blood pressure and the right jugular vein for administering normal saline to compensate for intraoperative fluid loss (1ml /100gm body weight/hour).

Laparotomy was carried out through a midline incision. The ligamentous attachments of the liver were cut and the liver exposed. All animals were administered heparin 20 units /kg. Partial hepatic ischaemia of the left lateral and median lobes (70% of liver) was induced by clamping the corresponding vascular pedicle with an atraumatic microvascular clamp for 45 minutes. This model

prevents splanchnic congestion by allowing flow through the remaining liver(Koo *et al.* 1992; Koti *et al.* 2005). Animals were randomly allocated to the following groups:

4.2.2 Experimental groups (n=6 in each group)

Group1 – Sham

These animals underwent laparotomy and liver mobilisation under general anaesthesia but without clamping of the liver vascular pedicle.

Group2- IR

45 minutes of partial hepatic ischemia followed by 3 hrs of reperfusion.

Group3- Bucillamine (15mg/ kg/hr intravenously) + IR

Group4- (SB)- Sham + Bucillamine (15mg/kg/hr i.v.)

The Bucillamine group were administered Bucillamine infusion (15mg/kg/hr) over the operative period [For 10 minutes prior to ischaemia, during the period of ischaemia and for 3 hours in the reperfusion period]. Bucillamine was supplied by Santen pharmaceuticals, Osaka, Japan. Animals in the sham and I/R groups were given equivalent volume of saline. Temperature of the animal was monitored and maintained at 36-38^{o}C by means of a heating pad (Harvard Apparatus Ltd., Kent, UK).

4.2.3 Intravital Microscopy

Intra vital microscopy (IVM) was used to assess the perfusional changes associated with I/R and possible alterations with Bucillamine administration. The left lobe of the liver was gently mobilised and placed over a specially designed platform (Nikon microscope, Nikon, Tokyo, Japan). The surface of the liver was moistened with normal saline and visualised through a coverslip. Images (25 pictures /sec) from the microscope were recorded by camera (JVC video camera, JVC, Osaka, Japan) directly on to a computer for further analysis. Off line microcirculatory analysis was performed from recorded images to measure RBC velocity and sinusoidal diameter using Lucia G software (Laboratory Universal Computer Image Analysis, Nikon, Tokyo, Japan).

Red blood cells (from a previously bled rat) were labelled with fluorescein isothiocyanate (FITC) using a technique previously described(Zimmerhackl *et al.* 1983) and labelled RBCs (0.5ml) were administered via the jugular vein at 30 minutes reperfusion. The hepatic microcirculation was evaluated after 30, 60,120 and 180 minutes following reperfusion. Microcirculatory changes were studied in sinusoids (periportal, midzonal and pericentral sinusoids) within randomly selected acini. The following parameters were studied.

4.2.3.1 Mean RBC velocity

Mean RBC velocity was determined at 40x magnification. Ten sinusoids were randomly selected at each time point in each animal and RBC velocity was

calculated from frame by frame analysis as previously described(Kelly *et al.* 1998). Mean value was calculated for each time point.

4.2.3.2 Sinusoidal diameter

Sinusoidal diameter was measured in ten randomly selected sinusoids at each time point in each animal and the mean was calculated.

4.2.3.3 Sinusoidal perfusion

Five randomly chosen non overlapping rappaport acini were observed for a minute and recording made from representative areas for 2 seconds with a JVC video camera (JVC TK-C1360B colour video camera, JVC, Osaka, Japan) (25 frames per second) and stored on the computer for each time point. Perfusion was established by studying the ratio of perfused to total visible sinusoids after administration of FITC labelled RBC. Sinusoidal perfusion was graded as continuously perfused (continuous perfusion for > 1 min) (N_c) or interrupted (intermittent perfusion for 1 min. period) (N_i) or non perfused (N_n). Sinusoidal perfusion index was calculated as previously described(Post *et al.* 1993):

Perfusion index= (N_c + 0.5 N_i) / (N_c + N_i + N_n) and expressed as %.

4.2.3.4 Sinusoidal Blood Flow

Sinusoidal blood flow was calculated as previously decribed(Wunder *et al.* 2002).

Sinusoidal blood Flow= Velocity x 0 **x $(d/2)^2$** (expressed as pl/sec)

4.2.3.5 Leukocyte parameters

Leukocyte endothelial interactions were studied by labelling leukocytes in vivo by administering rhodamine 6G-(Sigma, Rodermark, Germany) 0.3 mg/kg intra-arterially(Wunder *et al.* 2002) at 150 minutes post reperfusion. Ten randomly chosen postsinusoidal venules were visualized for 20 seconds each under green filtered light. The number of adherent leukocytes were counted and expressed as cells per mm^2 of endothelial surface (length of observed vessel length x diameter x 0 = adherent cells per mm^2) as previously described(Croner *et al.* 2006; Wunder *et al.* 2002). Leukocytes adherence in sinusoids was measured as adherent leukocytes per mm^2 of liver tissue.

4.2.3.6 Detection of hepatocellular death in vivo.

Propidium Iodide fluorescent dye stains the nuclei of cells that are lethally damaged(Herman *et al.* 1988). Propidium iodide (0.05 mg/kg) was injected after 3 hours of reperfusion. The dead nuclei were identified as those stained with propidium iodide. These were counted in each high power field and an average value was taken for each subject after studying at least 5 high power fields (studying all regions of the acini). The area in each high power field was calculated using Lucia G software (Laboratory Universal Computer Image Analysis, Nikon, Tokyo, Japan) and results were expressed as number of dead cells per mm^2.

At the end of the experiment animals were killed by exsanguinations. Serum and Plasma samples were collected by spinning the blood at 3000rpm for 10 minutes and stored in aliquots of 0.2 mls at -80^{o} C. A sample of liver tissue was taken from the left lobe was stored in formalin for histopathology.

4.2.4 Liver Injury assay

Blood was sampled at the end of the procedure from the IVC 3 hours post reperfusion and centrifuged at 3000 rpm for 10 minutes. Serum was analysed on an autoanalyzer (Hitachi 747, Hitachi, Tokyo, Japan) using commercially available kits (Boehringer Mannheim, Lewes, East Sussex, UK) for serum aspartate transaminase and serum alanine transaminase.

4.3 Data collection and statistics

Data was continuously collected for oxygen saturation, blood pressure and mean arterial pressure (MAP). Averages for one minute were calculated at 30, 60, 120 and 180 minutes post reperfusion. Data is expressed as mean± standard error of mean (SEM). Analysis of data was done using SPSS 14.0 (SPSS Inc., Chicago, Illinois, USA). Differences in data between groups were assessed by using one way ANOVA with Bonferroni's post hoc test. Data was considered statistically significant if $p< 0.05$.

4.4 Results:

There were no procedure related deaths in either group. The model was haemodynamically stable (Figure 4.4.1a, 4.4.1b). There was a statistically significant transient fall in oxygen saturation immediately after reperfusion which was reduced by Bucillamine therapy. (Figure 4.4.1c)

Figure 4.4.1a:

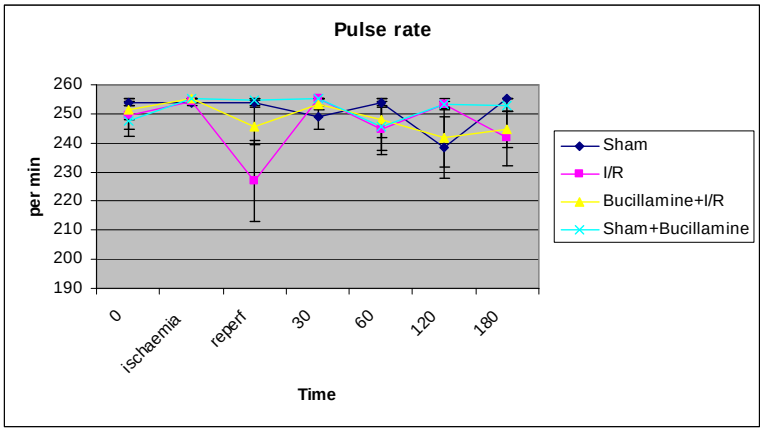


Figure 4.4.1b:

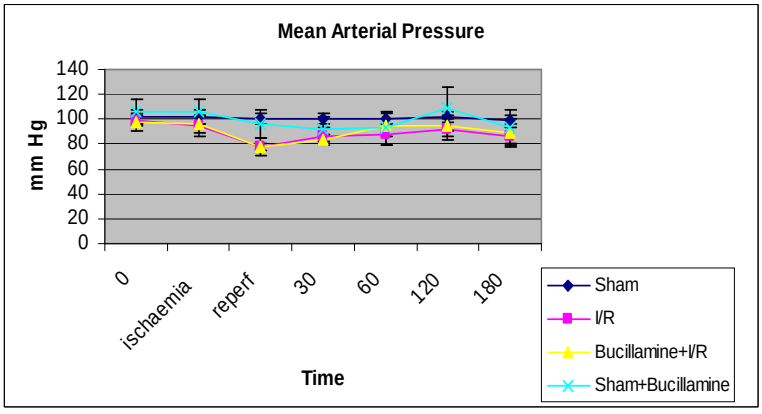
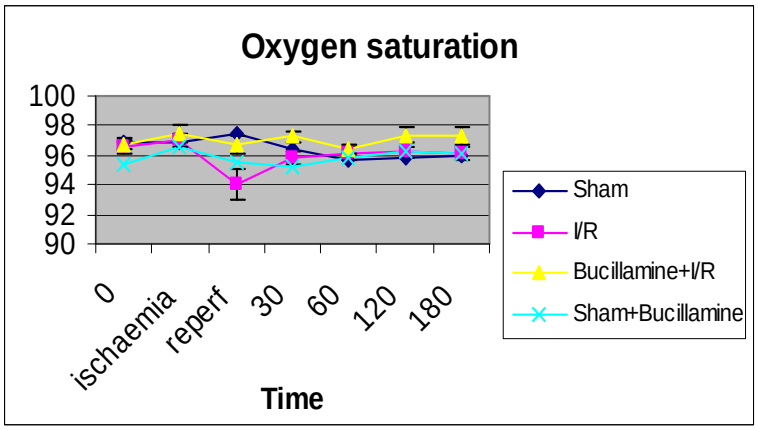


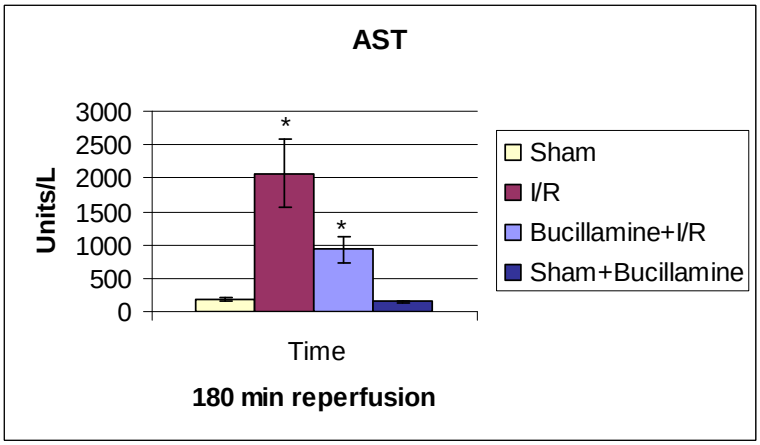
Figure 4.4.1c:



4.4.2 Biochemistry

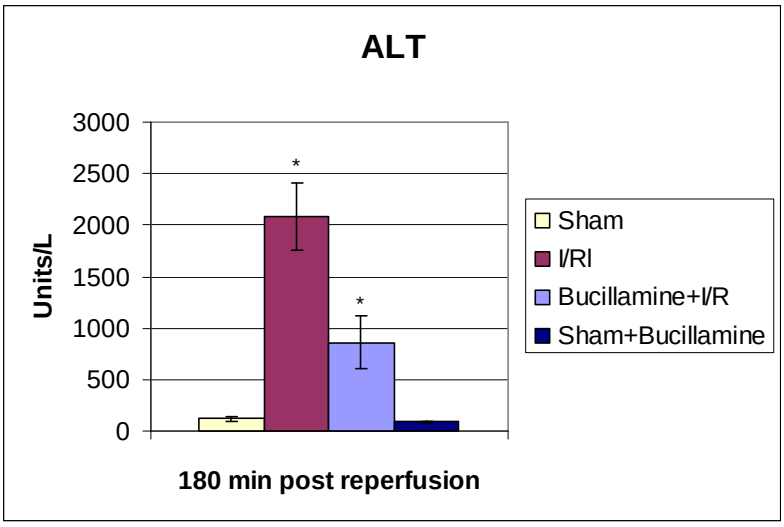
Transaminases were grossly elevated following I/R. The Bucillamine I/R group had lower AST and ALT than the IR group [(AST 932±201 vs. 2072.5±512, $p<0.05$), (ALT 862±263 vs. 2079±322, $p<0.05$)]. (Figure 4.4.2a, 2b).

Figure 4.4.2a:



*** p < 0.05**

Figure 4.4.2b:



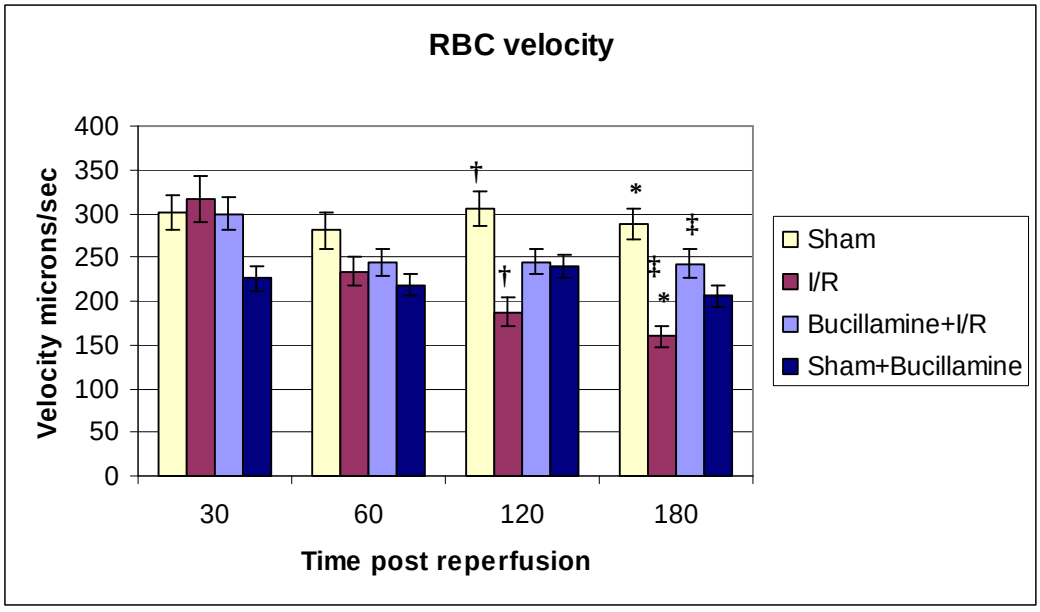
*** p<0.05**

4.4.3 Intravital microscopy

We did not find any difference in the perfusion, leukocyte adhesion, in the different sinusoidal regions (periportal, midzonal and pericentral sinusoids) within the same group of animals. RBC velocity was taken as average over the entire visible length of the sinusoid. Hepatocyte apoptosis/ necrosis were more severe in the pericentral region and we studied all the regions to calculate the number of nonviable nuclei.

4.4.3.1 RBC velocity

RBC velocity remained stable in the Sham group over the duration of the experiment. I/R produced a gradual fall in RBC velocity from baseline which was significant vs. sham operated animals at 120 and 180 minutes ($p<0.001$)). In the Bucillamine I/R group the initial fall in the RBC velocity at 60 minutes post reperfusion was similar to I/R(299.71±18.15 vs. 244±15.49, not significant) but after this the velocities remained steady for the subsequent 2 hours duration. The difference in velocity at 180 minutes reperfusion was statistically significant between the IR and Bucillamine I/R groups ($p<0.05$). Velocities were lower in the SB group as compared to sham group, but there was no change in the velocities over time (Figure 4.4.3.1).



† p<0.001, * p<0.001, ‡ p<0.05

Figure 4.4.3.1: Showing gradual fall in RBC velocity on reperfusion after I/R injury. Bucillamine+ IR group showing initial fall in RBC velocity with maintenance of RBC velocity.

4.4.3.2 Sinusoidal diameter

There was no statistically significant difference in the sinusoidal diameter in the I/R or Bucillamine I/R groups. (Figure 4.4.3.2).

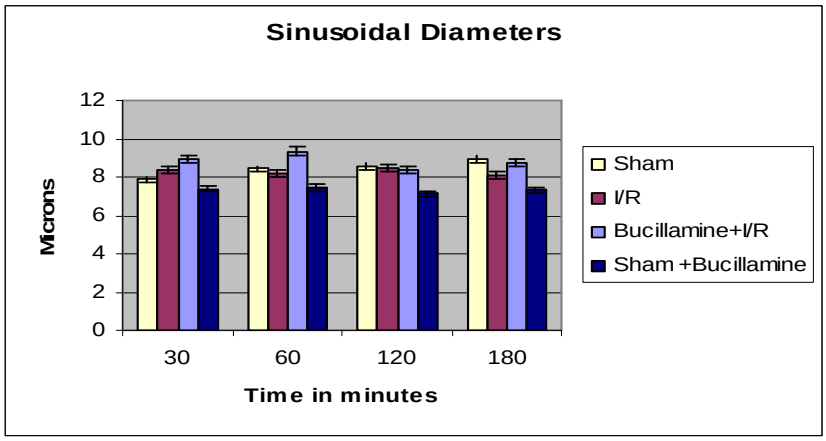


Figure 4.4.3.2: showing no change in sinusoidal diameter

4.4.3.3 Sinusoidal Perfusion Index

Sinusoidal perfusion index was lower in I/R group as compared to SB and sham groups at all time points. Bucillamine therapy with I/R increased the sinusoidal perfusion although values were not statistically significant. (Figure 4.4.3.3)

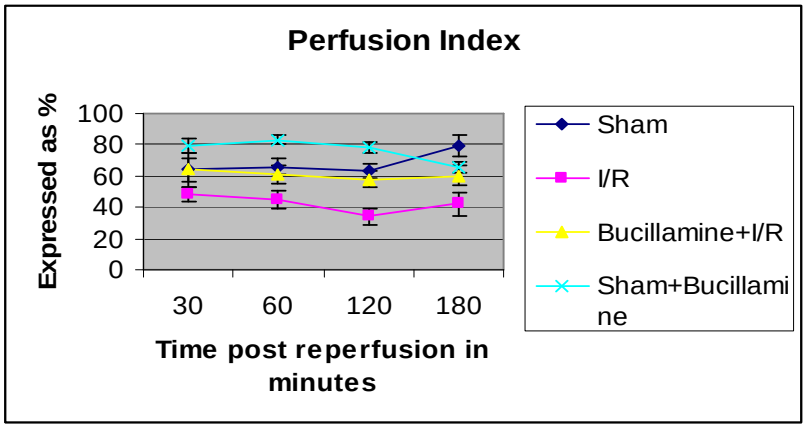


Figure 4.4.3.3: Showing no statistically significant change

4.4.3.4 Sinusoidal Blood Flow

I/R reduced sinusoidal blood flow over the reperfusion period. Bucillamine with I/R maintained the sinusoidal blood flow, after an initial fall, although, none of these changes, however, were statistically significant. (Figure 4.4.3.4)

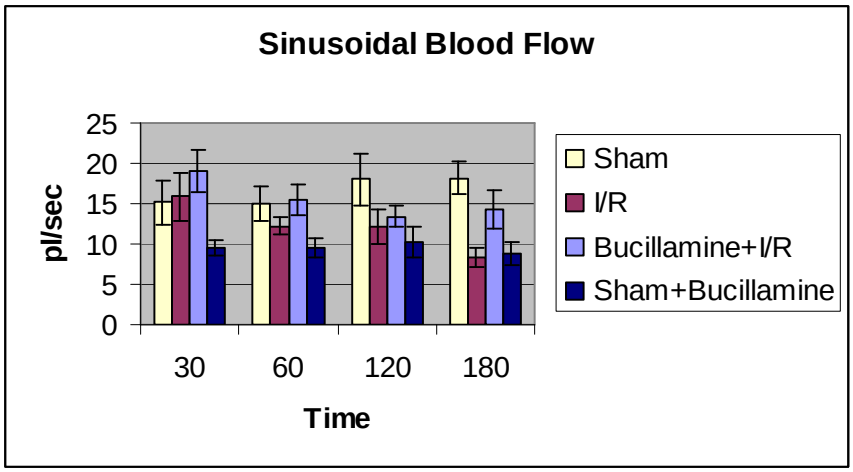


Figure 4.4.3.4: Showing a gradual fall in sinusoidal blood flow after IR. Bucillamine +IR group showed initial fall in sinusoidal blood flow which was maintained at same level after 60 minutes reperfusion.

4.4.3.5 Leukocyte adherence in venules and sinusoids

I/R injury were associated with adherence of leukocytes in the venules. Bucillamine with I/R reduced leukocyte adherence (385.66±142.69) in the venules, however this was not statistically reduced when compared with the I/R group (Figure 4.4.3.5.2). In the sinusoids I/R group (97.4±7.49) had a significantly higher number of adherent leukocytes as compared to sham(3.75±3.75), SB (22.48±11.46) and bucillamine groups(29.97±13.81) ($p<0.005$). (Figure 4.4.3.5.3)

Neutrophil adhesion

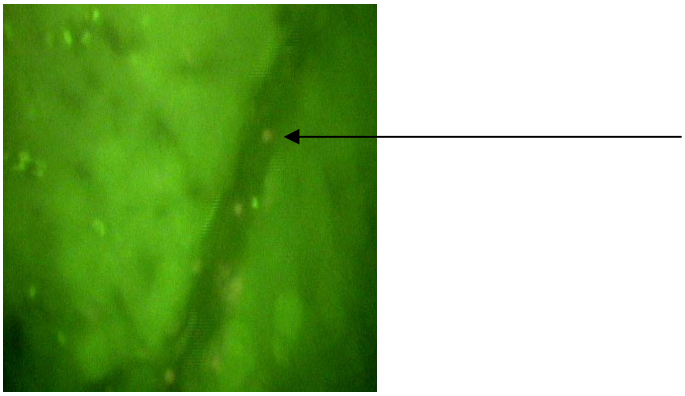


Figure 4.4.3.5.1 Neutrophils stained by rhodamine is seen adherent to post sinusoidal venular endothelium. The number of adherent neutrophils divided by the area of endothelial surface (л×D×L) gives the number of neutrophils/ mm2. D= sinusoidal diameter, L= length of segment along adherent neutrophils.

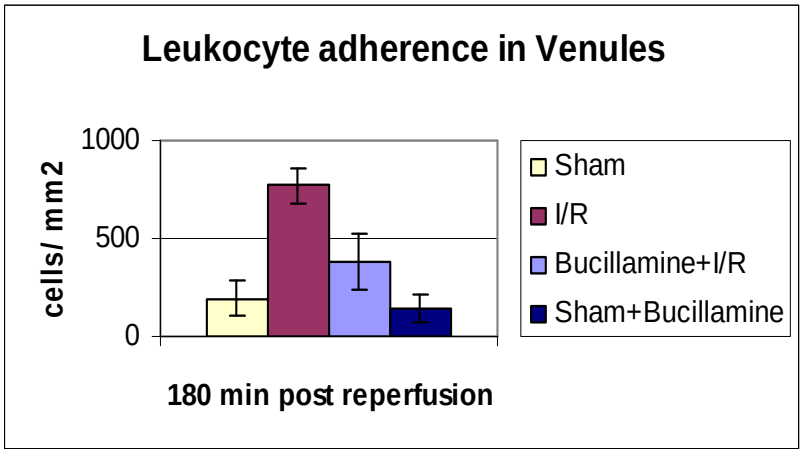
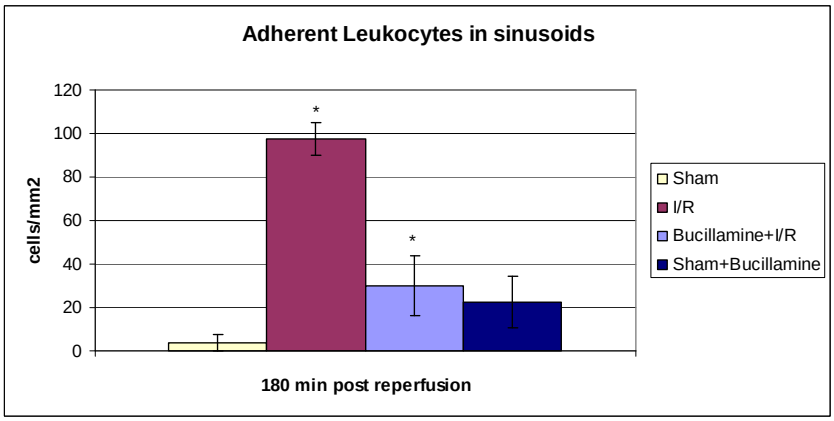


Figure 4.4.3.5.2: Showing no significant change



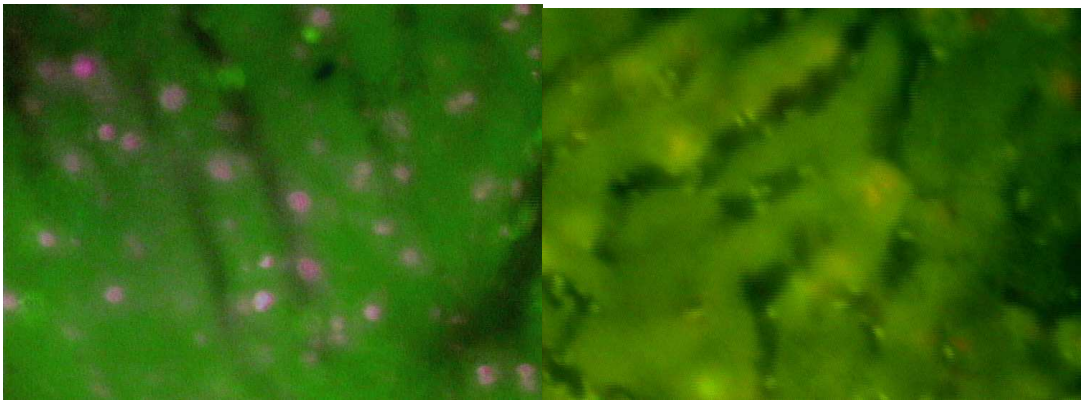
*** p<0.005**

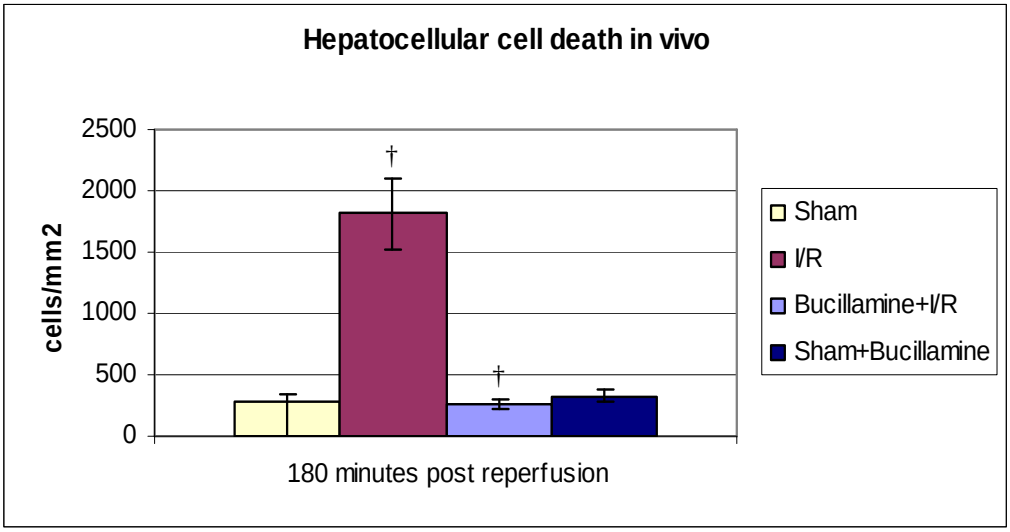
Figure 4.4.3.5.3: Statistically significant decreased adherent leukocytes in sinusoids in Bucillamine +IR group as compared to IR only group.

4.4.3.6 Hepatocellular cell death *in vivo*

The number of lethally damaged nuclei was assessed in each group after 3 hours of reperfusion. I/R produced significant hepatocellular death (1816±293.09 cells/ mm^2) which was significantly reduced with Bucillamine administration at 3 hours reperfusion (258.48±46.73 cells/ mm^2, $p<0.001$). (Figure 4.4.3.6)

IR **Bucillamine + IR**





† p < 0.001

Figure4.4.3.6: Significantly lesser nonviable nuclei at 3 hours reperfusion in Bucillamine+IR group as compared to IR only group ($p< 0.001$)

4.5 Discussion

This study has shown for the first time that Bucillamine can reduce the effects of liver warm ischaemia reperfusion injury. This is also the first study showing effect of Bucillamine on liver microcirculation in vivo. The model of partial liver ischaemia and reperfusion injury used in this study is stable, reproducible and avoids splanchnic congestion found with total liver inflow occlusion(Koti *et al.* 2005). Technical manoeuvres such as performing the laparotomy; mobilisation of the liver and performing Intravital microscopy did not affect the heart rate, oxygen saturation or mean arterial pressure as shown by stable parameters in the sham and the SB groups. There was a fall in mean arterial pressure following ischaemia reperfusion injury in both the I/R and Bucillamine I/R groups which was not statistically significant. Bucillamine administration without I/R has no effect on the vital parameters or haemodynamic stability of the animals suggesting that it has no direct effect on systemic or portal haemodynamics.

Intravital microscopy has allowed novel insights into hepatic haemodynamics following I/R and the effect of Bucillamine administration. Liver I/R is known to cause microcirculatory perfusion failure, activate polymorphonuclear leukocytes and increase leukocyte-endothelial cell interaction which in turn contribute to hepatocellular damage and liver dysfunction(Menger *et al.* 1999; Vollmar *et al.* 1994a; Vollmar *et al.* 1994c; Vollmar *et al.* 1996). Bucillamine administration with I/R was shown to maintain RBC velocity, sinusoidal blood flow and sinusoidal

perfusion in the reperfusion period. It has also shown to decrease leukocyte adhesions in sinusoids and decrease hepatocyte apoptosis/ necrosis.

RBC velocity

In our experiments there was a gradual drop in the RBC velocity in the I/R group, whereas the RBC velocity remained stable after an initial drop in the Bucillamine group. This would be consistent with the scavenging of oxygen free radicals by Bucillamine. RBC mechanical properties play a key role in tissue perfusion(Schmid- Schonbein H 1988; Shiga *et al.* 1990). RBC velocity can be affected by changes in both aggregability and deformability of the RBCs, and oxygen free radicals which form as a result of I/R affect RBC aggregation and deformability(Lowe GDO and Barbanel JC 1988). Decreased RBC deformability is associated with oxygen free radical damage during sepsis and is linked to multiorgan failure(Machiedo *et al.* 1989). Pre-treatment with an oxygen free radical scavenger prevents such adverse changes in deformability(Powell *et al.* 1989). RBC aggregation also has an impact on blood flow mainly in low shear regions(Cabel *et al.* 1997). Externally generated oxygen free radicals (i.e. outside RBCs) in experimental settings increases aggregation of RBCs whereas internally generated oxygen free radicals affects the deformability(Baskurt *et al.* 1998). By scavenging oxygen free radicals Bucillamine might be preventing RBC aggregation and deformability thus maintaining RBC velocity. After absorption Bucillamine enters the RBCs rapidly and is carried within the

erythrocytes(Horwitz 2003; Sugawara *et al.* 1985a; Sugawara *et al.* 1985b). This might be of importance in the beneficial effect of Bucillamine.

Sinusoidal perfusion

I/R injury decreased the sinusoidal perfusion which is a known consequence of severe liver ischaemia reperfusion injury(Vollmar *et al.* 1994a)and results in significant compromise of hepatic tissue oxygenation and damage and functional impairment of parenchymal and non parenchymal cells(Vollmar *et al.* 1994c; Vollmar *et al.* 1996). The severity of sinusoidal perfusion failure is proportional to the ischaemia time(Vollmar *et al.* 1994a). Bucillamine was shown to reduce the perfusion abnormality of I/R with an increased sinusoidal perfusion. This could be related to its effect on- 1. RBCs (decreased aggregability or better maintenance of deformability) and /or 2. WBC adhesions. Sinusoidal perfusion was also better maintained in the SB group, which although, as mentioned earlier had lower RBC velocity, had normal liver function, indicating that a combination of better perfusion and maintenance of RBC velocity would decrease abnormality in liver function.

Sinusoidal diameter

Changes in sinusoidal diameter by constriction of hepatic stellate cells mediated by endothelin-1 are known to influence the hepatic perfusion in endotoxaemia(Ring and Stremmel 2000; Croner *et al.* 2006; Horwitz 2003). Our results do not suggest such a role in liver I/R injury. The increase in sinusoidal

diameter in Sham group from 30 minutes to 180 minutes post reperfusion could be attributed to the effect of anaesthesia. This would suggest that there might be a relative decrease in the sinusoidal diameters in the I/R and Bucillamine groups. The sinusoidal dilatation in Bucillamine group at 30 minutes could explain the better perfusion and cytoprotection. Our results also do not suggest primary vasodilator function for Bucillamine as the sinusoidal diameter did not increase in the SB group.

Sinusoidal Blood flow

There was a progressive decrease in sinusoidal blood flow after I/R injury. Sinusoidal blood flow was maintained bucillamine administration. Sinusoidal blood flow is essentially related to the velocity of flow and diameter and our data on sinusoidal flow is similar to that for RBC velocity as there was no significant change in the diameter. The sinusoidal blood flow values were lower (although not significant as compared to sham) in the SB group demonstrating that the maintained blood flow in the group who had I/R with administration of Bucillamine was not related to a direct effect of Bucillamine but related to its effect on the inflammatory cascade of I/R.

Leukocyte endothelial interactions

The I/R group showed leukocyte adherence in the sinusoids and post sinusoidal venules. Jaeshcke et al first showed that leukocyte infiltration into liver parenchyma is associated with the development of liver I/R injury(Croner *et al.*

2006; Jaeschke *et al.* 1990). Hepatic I/R induces accumulation, adherence and extravasation of leukocytes in both sinusoids and post sinusoidal venules(Vollmar *et al.* 1994c). The increased number of adherent leukocytes in venules, but not in sinusoids, is known to correlate with the extent of liver dysfunction(Vollmar *et al.* 1994c). Leukocyte adherence in venules is mainly mediated by increased expression of ICAM-1(Croner *et al.* 2006; Vollmar *et al.* 1995b; Iwata *et al.* 1999). Our study showed a decreased leukocyte adherence in venules with Bucillamine following I/R. Bucillamine is known to scavenge ROS thus decreasing Kupffer cell and leukocyte activation. Leukocyte adhesion in sinusoids may decrease perfusion of sinusoids(Vollmar *et al.* 1994c). However, although we could show significantly increased adherent sinusoids in I/R group the number of adherent leukocytes were very few to account for no flow/ reduced flow in sinusoids by mechanical blockage, which would suggest that the adherent leukocytes are not the cause of reduced parenchymal perfusion. They might, however, be contributing to the hepatocellular injury and dysfunction by release of cytokines and generation of reactive oxygen species.

Hepatocellular injury/ apoptosis

The propidium iodide staining confirmed that there was significant hepatocellular necrosis associated with I/R injury in this model. Bucillamine administration reduced the number of nonviable nuclei by more than 80%. Liver warm I/R is associated with necrosis / apoptosis of hepatocytes(Jaeschke and Lemasters 2003). In liver I/R, oncotic necrosis and apoptosis share features and

mechanisms(Gujral *et al.* 2001; Jaeschke and Lemasters 2003). It is suggested that the ability of a necrotic process to be converted to an apoptotic one and vice versa illustrates that the pathways in the two processes could be shared, a phenomenon called necroapoptosis(Lemasters 1999; Formigli *et al.* 2000). Irreversible injury in anoxic hepatocytes is precipitated by an abrupt increase in plasma membrane permeability which results in uptake of propidium iodide, labelling the nonviable nuclei(Herman *et al.* 1988). We have used this previously used technique to demonstrate nonviable hepatocytes(Herman *et al.* 1988; Brock *et al.* 1999; Zhang *et al.* 1994). This study has shown that with better perfusion and decreased leukocyte adherence, Bucillamine reduces the hepatocyte damage, which could be related to its effect on scavenging of ROS.

4.6 <u>Conclusions:</u> These findings suggest that this agent may prove to be a useful target in liver protection against I/R injury and could be of clinical benefit in the field of liver transplantation or liver resection surgery.

In the subsequent chapter we investigate its effect on liver necrosis /apoptosis as assessed histologically and by genetic markers of apoptosis.

Chapter 5

The effect of Bucillamine infusion on Bax/ Bcl-2 and Histological changes following Liver Warm IR

5.1 Introduction:

During ischaemia the cell is deprived of the energy needed to maintain ionic gradients and homeostasis. Failure of enzyme systems inevitably leads to cell death(Mathews *et al.* 1994). Recovery from ischaemic injury is facilitated by reperfusion through the restoration of energy supply and removal of toxic metabolites(El-Wahsh *et al.* 2004). However, post-ischaemic reperfusion is a complex process with potentially damaging effects, giving rise to a phenomenon known as ischaemia-reperfusion injury (IRI).

Microscopically, IRI cell death occurs by uncontrolled and controlled mechanisms, known as necrosis and apoptosis respectively. The relative contributions of these processes are unclear but studies by Teoh et al.(Teoh and Farrell 2003), Selzner et al.(Selzner *et al.* 2003), and Jaeschke et al.(Jaeschke 2003b) suggest an overlap in necrotic and apoptotic pathway components. Additionally, such studies highlight discrepancies in methods used to measure apoptosis.

It has been shown that apoptosis of sinusoidal endothelial cells and hepatocytes is a cardinal feature of reperfusion injury in the cold(Gao *et al.* 1998) and warm ischaemic liver(Kohli *et al.* 1997; Kohli *et al.* 1999b; Natori *et al.* 1999). Several lines of evidence suggest that apoptosis critically contributes to organ viability in transplantation models as the number of apoptotic cells is positively correlated with ischaemia and animal survival. Moreover, inhibition of mediators of

apoptosis such as caspases(Natori *et al.* 1999) or calpain-like proteases(Kohli *et al.* 1997) ameliorates the effects of IRI(Piot *et al.* 1997; Vexler *et al.* 1997).

At a genetic level, protection against apoptosis ultimately involves decreased expression of pro-apoptotic genes such as Bax as well as up-regulation of anti-apoptotic mechanisms. Bcl-2 is a family of mitochondrial membrane protein which includes the proapoptotic Bax and anti apoptotic Bcl-2 gene. Rentsch et al found that induction of apoptosis in rat liver transplantation was associated with caspase 3 activation and Bax expression(Rentsch *et al.* 2001). Warm liver ischaemia reperfusion injury has also been shown to increase Bax expression(Ishigami *et al.* 2001). Increased apoptosis following liver warm ischaemia reperfusion injury has also been shown to occur in transgenic Bax inhibitor knock out mice as compared to normal mice suggesting that the proapoptotic Bax gene has a role in apoptosis following warm ischaemia reperfusion injury(Bailly-Maitre *et al.* 2006). Ablation of Bax has been shown to protect against warm ischaemia reperfusion injury in transgenic mice(Ben-Ari *et al.* 2007).

Bcl-2 is the most ubiquitous anti-apoptotic molecule protecting against a variety of apoptotic stimuli(Adams and Cory 2007; Reed 1994). Overexpression of Bcl-2 in transgenic mice is known to protect liver against I/R injury(Selzner *et al.* 2002). Bcl-2 overexpression suppresses cell apoptosis mainly by normalizing mitochondrial membrane permeability, so as to reduce cytochrome c release and thus preventing caspase 3 activation(Zamzami *et al.* 1996; Zhao *et al.* 2003).

Thus in simplistic terms Bax stimulates the release of mitochondrial cytochrome c(Rentsch *et al.* 2001), whereas Bcl-2 inhibits its release(Bossy-Wetzel *et al.* 1998). The release of mitochondrial cytochrome c results in activation of caspase 3 and fragmentation of DNA resulting in apoptosis(Rudiger *et al.* 2003).

Studies on Liver ischaemia reperfusion injury using antioxidants and effects on Bax/ Bcl-2

Various investigators have investigated effects of antioxidants on Bax and Bcl-2. These studies have been summarised in **Table 5.1**:

Table 5.1: Studies on Liver ischaemia reperfusion injury using antioxidants and effects on Bax/ Bcl-2

Author	Antioxidant	Model	Findings on using antioxidant
Kong, R (Kong *et al.* 2009)	Salvanoic acid B	Rat, 90 min ischaemia, 5 hr reperfusion	↑ Bcl-2 levels
Jha,S (Jha *et al.* 2008)	H_2S Donor (IK1001)	Mouse, 60 min ischaemia, 5hr reperfusion	↑ Bcl-2 levels
Duenschede, F.(Duenschede *et al.* 2007)	Lipoic Acid	Rat, 90 min ischaemia, 1 hr reperfusion	↓ Bax levels
Jia, CJ (Jia *et al.* 2006)	Alanyl-glutamine dipeptide	Rat, 30 min ischaemia, 1hr & 24 hr reperfusion	↑ Bcl-2 levels, ↓ Bax levels
Zhang, Y (Zhang *et al.* 2005)	Panax Notoginseng saponins	Rat OLTx model, 2 hr, 6hr and 24 hr reperfusion	↑ Bcl-2 levels
Wang, XH (Wang *et al.* 2004)	Hemin	Rat OLTx model, 12 hr reperfusion	↑ Bcl-2 levels
Jawan, B (Jawan *et al.* 2003)	Magnolol	Rat 2 hr ischaemia	↑ Bcl-xl levels
Altunkan,A (Altunkan *et al.* 2002)	Succinyl Gelatine	Rabbit 45 min ischaemia, 45 min reperfusion	↑ Bcl-2 levels, ↓ Bax levels
Ishigami,F (Ishigami *et al.* 2001)	Tauro Ursodeoxycholic acid	Rat 2 hr ischaemia, 6 hr reperfusion	↓ Bax levels
Singh,AK (Singh *et al.* 2000)	Picroliv	Rat 30 min ischaemia, 2 hr reperfusion	No change in Bax, Bcl-2 as compared to sham. However, caspase3 ↓

In the previous chapter we had shown an increase in non viable nuclei following ischaemia reperfusion as compared to sham group on Intravital microscopy, which was attenuated by bucillamine infusion. This correlated with better preservation of liver function as assessed by lesser derangement of liver enzymes. In this study we wanted to investigate the effect of Bucillamine on Bax and Bcl-2 and if there was any correlation with histological changes.

Studies have shown that the ratio of Bax to Bcl-2 expression is one factor that may determine the resistance of hepatocytes to apoptotic stimuli(Lacronique *et al.* 1996; Yamabe *et al.* 1998). We measured the levels of Bax and Bcl-2 and the ratio of Bax/Bcl-2. Histological assessment was done using modified Suzuki's criteria as described.

Aim- To study if protective effect –

1. correlated with histological changes.
2. associated with differences in expression of pro and anti apoptotic genes.

5.2 HYPOTHESIS

We hypothesised that Bucillamine infusion would have a protective effect against liver I/R by reducing Bax expression and increasing Bcl-2 expression.

Materials and Methods:

The drug was evaluated in an *in vivo* lobar liver ischaemia reperfusion model as previously described(Koo *et al.* 1992; Koti *et al.* 2005). This study was conducted under project license from the Home Office in accordance with the Animals (Scientific Procedures) Act 1986.

5.2.1 Animals and Surgical Procedure

Male Sprague-Dawley rats weighing 250-300g were used. The animals were kept in a temperature controlled environment with a 12 hour light-dark cycle. They were given tap water as well as standard rat chew pellets *ad libitum*.

Animals were anaesthetized with 4% Isoflurane and maintained with 2.0% Isoflurane. They were allowed to breathe spontaneously through a concentric mask connected to an oxygen regulator and monitored with a pulse oximeter (Ohmeda biox 3740 pulse oximeter, Ohmeda, Louisville, USA).

Polyethylene catheters (Portex 2 Fr, Portex, Kent, UK) were inserted into the carotid artery (right or left) for monitoring of mean arterial blood pressure and the

right jugular vein for administering normal saline (1ml /100gm body weight/hour) to compensate for intraoperative fluid loss.

Laparotomy was carried out through a midline incision. The ligamentous attachments of the liver were cut and the liver exposed. All animals were given heparin 20 units /kg and partial hepatic ischaemia of the left lateral and median lobes of (70% of liver) was induced by clamping the corresponding vascular pedicle with an atraumatic microvascular clamp for 45 minutes in the control and Bucillamine groups. This model prevents splanchnic congestion by allowing flow through the remaining liver(Koo *et al.* 1992; Koti *et al.* 2005). Animals in the Sham group did not have any vascular clamping. Animals were monitored for 3 hours in the reperfusion period. Animals were randomly allocated to the following groups:

5.2.2 Experimental groups (n=6 in each group)

Group1 - (Sham) – laparotomy and mobilisation of liver but no occlusion of the vascular pedicle.

Group2- IR- 45 minutes of partial hepatic ischemia followed by 3 hrs of reperfusion.

Group3- Bucillamine 15mg/ kg/hr intravenously + IR followed by 3 hrs of reperfusion.

Group4- (SB) - Sham + Bucillamine infusion 15mg/kg/hr (SB)

Subjects in the Bucillamine group were given Bucillamine infusion (15mg/kg/hr) over the operative period (preischaemia, ischaemia and 3 hour post reperfusion). Bucillamine was supplied by Santen pharmaceuticals, Osaka, Japan. Animals in the Sham and I/R groups were given an equivalent volume of saline up to 3 hours of reperfusion (or equivalent period in case of sham operated animals). Temperature of the animals was monitored and maintained at 36-38^{o}C by means of a heated platform (Harvard apparatus Ltd., Kent, UK). The experiments were terminated by exsanguination and serum and plasma samples were collected by spinning the blood at 3000rpm for 10 minutes and stored in aliquots of 0.2 mls at -80^{o} C. Liver tissue was stored in formalin for histopathology and immunohistochemistry. Liver tissue was also frozen immediately on termination of the experiment in liquid nitrogen and stored at -80^{o} C for further analysis.

5.2.3 RNA Extraction

RNA was extracted from the liver tissue samples that were frozen at -80℃ after the surgical procedure. Extraction followed the protocol as set out in the QIAGEN RNeasy® Mini Handbook (QIAGEN, Hilden, Germany). All centrifugations were carried out at room temperature (25℃) and maximum speed (1300 rpm).

A fraction of the tissue was excised from the main sample and placed in a suitably sized vessel. A volume of 550µl lysis buffer RLT was added to the

vessel and the tissue was homogenised using a rotor-start homogeniser. The tissue lysate was then centrifuged for 3 min. The supernatant was pipetted into a new microcentrifuge tube. 550µl of 70% ethanol was then added to the clear lysate and mixed immediately by pipetting. From this, 700µl was put in an RNeasy mini column placed in a 2ml collection tube and centrifuged for 30 s. The flow-through was discarded. 700µl of buffer RW1 was then added to the RNeasy column to wash it followed by centrifugation for another 30 s. The flow-through was ejected once more. 550µl of buffer RPE was subsequently pipetted into the RNeasy column and centrifuged for 30 s. The flow-through was discarded and 550µl of buffer RPE was added again. The tube was then centrifuged for 2 min and flow-through was thrown. The tube was then centrifuged for a further 1 min. The collection tube with any further flow-through was discarded. The RNeasy column was placed in a new 1.5ml collection tube. To elute the RNA, 40µl of RNase-free water was directly added onto the silica-gel membrane of the mini column. The tube was centrifuged for 1 min. The RNeasy mini column was taken out as the collection tube contained the RNA. The RNA was then quantified as absorbance at 260nm using a Gene Spec spectrophotometer (Hitachi Gene systems, Alameda, CA, USA).

5.2.4 Kinetics Study

The graph shape produced by a polymerase chain reaction is sigmoidal (y axis = quantification of electrophoresis gel bands, x-axis = number of PCR cycles). It is therefore important to ensure that the number of PCR cycles chosen for any

particular reaction restricts the amount of product produced to the exponential phase of the graph. More cycles do not guarantee a greater yield as items run out and start to hinder the reaction (for example, reduction in the number of deoxynucleotide triphosphates [dNTPs] that are available). Thus, to determine the optimum number of cycles a RNA sample was chosen at random and run for 20, 30 and 40 cycles with each pair of primers for GAPDH, Bcl-2 and Bax. The amplification products were separated by 2% agarose gel electrophoresis and the band patterns quantified (see below).

5.2.5 Reverse-Transcriptase Polymerase Chain Reaction

Message levels of the genes being investigated in this study were determined using reverse-transcriptase polymerase chain reaction (RT-PCR). The commercially available QIAGEN OneStep RT-PCR Kit (QIAGEN, Hilden, Germany) was used and the protocol followed was of that set out in the handbook. This method combined the reverse transcription from messenger RNA (mRNA) to DNA followed by the PCR proper.

Each PCR tube contained 10µl of the 5x QIAGEN OneStep RT-PCR Buffer, 10µl of the Q-Solution, 2µl dNTP Mix, 2µl Enzyme Mix, and 0.5µl each of forward and reverse primer solutions. However, these concentrations were all prepared in a master mix whose volume was 10% greater than the total number of reactions to be performed and 25µl was subsequently added to each individual PCR reaction. Having previously quantified the RNA samples the volume needed to make a

concentration of 0.5µg/µl was calculated and added to the PCR tube. A variable volume of RNase-free water was then added to make up the PCR tube solution to a total of 50µl. The PCR reactions also included negative controls, i.e. no RNA was included and 25µl of RNase-free water was added.

Below is an outline of the PCR conditions and primer sequences:

Reverse Transcription
Step 1: T = 50℃ for 30 min
Step 2: T = 95℃ for 15 min

PCR Proper
Step 1: T = 94℃ for 1 min (denaturation)
Step 2: T = 58, 58 and 56℃ for 1 min - GAPDH, Bcl-2 and Bax respectively (annealing)
Step 3: T = 72℃ for 1 min (extension)
Step 4: Repeat steps 1-3 with number of cycles -1 (GAPDH = 25, Bcl-2 = 30, Bax = 30)
Step 5: T = 72℃ for 10 min (final extension)
Step 6: Hold at 4℃ (if kept overnight)

Primer Sequences
GAPDH
CCATCACCATCTTCCAGGAG (forward)
CCTGCTCACCACCTTCTTG (reverse)

Bcl-2
GCTACGAGTGGGATACTGGAGA (forward)
AGTCGTCCACAGAGCGATGTT (reverse)

Bax
AGGATGATTGCTGATGTGGATAC (forward)
CACAAAGATGGTCACTGTCTGC (reverse)

Product size

GAPDH	576 base pairs
Bcl2	446 base pairs
Bax	300 base pairs

5.2.6 Agarose Electrophoresis Gel

Amplification products were resolved by 2% agarose gel electrophoresis, stained with ethidium bromide (EtBr) (Sigma chemical Co., St Louis, MO,USA) and photographed under ultraviolet light. Each gel was run at 80 volts for 1 hour.

To make the 2% agarose gel, 2g of agarose were added to 100ml 1.0x EDTA (TAE) buffer. In a microwave oven the solution was brought to the boil to dissolve the agarose. Once the solution had cooled down to about 60℃ at room temperature, 1µl of EtBr was added. The gel was poured into the gel rack, a "comb" was placed in it to create the wells, and the gel was left to set. After some time the gel tank was filled with remaining buffer solution and the ''comb'' removed for the loading of the amplification products.

20µl was removed from each PCR tube and put into a new vessel. To each tube 4µl of DNA loading dye was added. One tube containing 20µl of DNA ladder and 4µl of DNA loading dye was also created. The gels included DNA markers to help resolve the molecular weights of the bands but quantification of individual bands was done using the computer software Syngene (Syngene, Cambridge, UK).

5.2.7 Histological assessment

Samples of liver were taken, at the end of the experiment from the left lobe. These were fixed in 10 per cent neutral buffered formalin and embedded in paraffin; paraffin section 4µm thick were cut using a microtome and mounted on

slides for haematoxylin and eosin staining. Assessment of liver injury was performed with light microscopy by Consultant pathologist who was blinded from the study groups, by a scoring system devised by Suzuki *et al*(Suzuki and Toledo-Pereyra 1993). In this system, sinusoidal congestion, hepatocyte ballooning or vacuolation, and necrosis are graded from 0 to 4. No congestion, vacuolation or necrosis is given a score of 0, while severe congestion, vacuolation and necrosis is given a score of 4, as shown in table 5.2.1.

Table 5.2.1

Suzuki's Criteria for Liver I/R

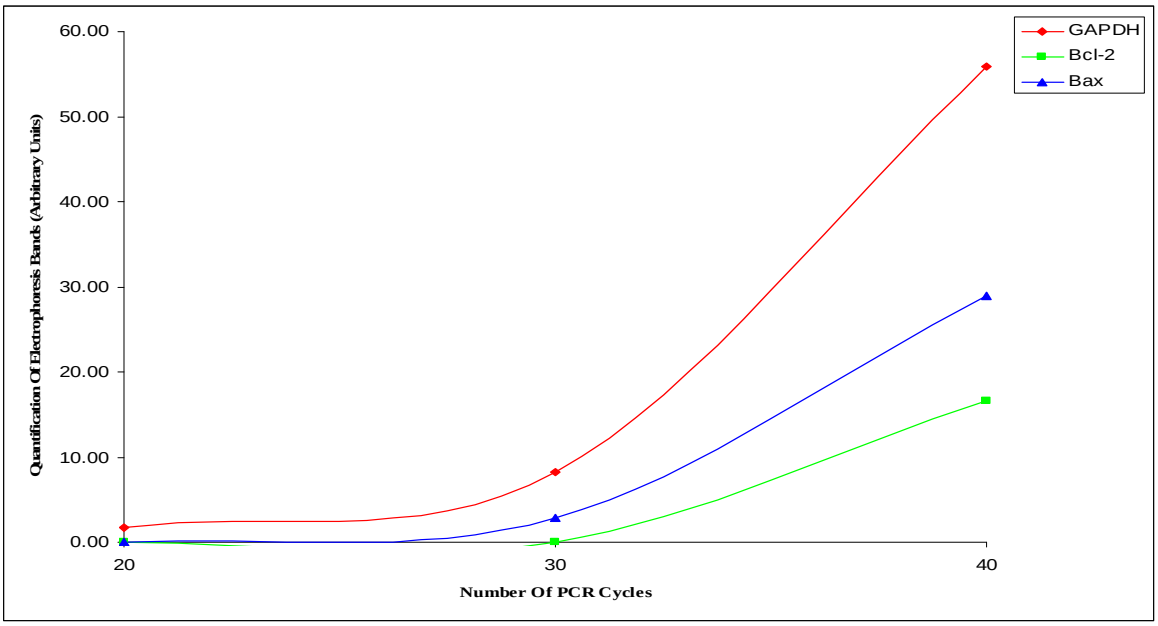
Numerical assessment	**Congestion**	**Vacuolation**	**Necrosis**
0	None	None	None
1	Minimal	Minimal	Single cell
2	Mild	Mild	< 30%
3	Moderate	Moderate	30- 60%
4	Severe	Severe	>60%

5.3 Data Collection and Statistical Analysis

As the data was not distributed normally a non-parametric test was used to determine whether the means of the groups were significantly different. The Student's *t* test was used to compare the IRI alone and Bucillamine plus IRI groups as these were the two groups of greatest relevance. All data are expressed as mean values ± standard deviations. Statistical significance was set at $p < 0.05$.

5.4 Results:

GAPDH, Bcl-2 and Bax expression was measured by agarose gel electrophoresis (Figures 5.4.2, 5.4.3A and 5.4.4A respectively) and the intensity of the expression bands compared in the following experimental groups: sham operated *versus* IR alone *versus* Bucillamine + IR. Gels were also run to separate the amplification products for Bax expression in the groups sham operated *versus* IR alone *versus* Bucillamine + IR. The optimum number of PCR cycles for GAPDH, Bcl-2 and Bax amplification were determined to be 25, 30 and 30 respectively (Figure 5.4.1).



Number Of PCR Cycles	Quantification (Arbitrary Units)		
	GAPDH	Bcl-2	Bax
20	1.71	0.00	0.00
30	8.17	0.00	2.84
40	55.87	16.60	28.97

Figure 5.4.1. Graphical and tabular representations of kinetic studies to determine the optimum number of PCR cycles for GAPDH, Bcl-2 and Bax expression.

5.4.2Effect of Bucillamine on Bcl-2 Expression in Rat Liver

There was decrease in Bcl-2 expression following IR as compared to sham group. An increase in mean Bcl-2 expression was demonstrated in the rat livers of the Bucillamine + IR group compared to the IR alone group (10720± 7320 vs 1517± 929, $p<0.05$) (Table 5.4.2). The increase in mean Bcl-2 expression is demonstrated graphically in Figure 5.4.3B.

5.4.3 Effect of Bucillamine on Bax Expression in Rat Liver

An increase in the Bax expression in the IR alone group was seen as compared to the sham group (12312.8±2553.2 vs. 1829.8± 721.51, p, 0.001) (Table 5.4.2). In the Bucillamine + IR group the Bax expression was significantly attenuated (2036±759, p=0.000468). The increase in mean Bax expression is demonstrated graphically in Figure 5.4.4B.

5.4.4 Bax/Bcl-2 ratio

A mean Bax/Bcl-2 expression ratio was calculated for the IR, Bucillamine and sham groups (Table 5.4.2). The ratio was higher in the IR alone group as compared to the Bucillamine + IR group (10.13±5.12 vs. 0.36±0.33, p=0.013). The difference in ratio sizes is demonstrated graphically in Figure 5.4.5.

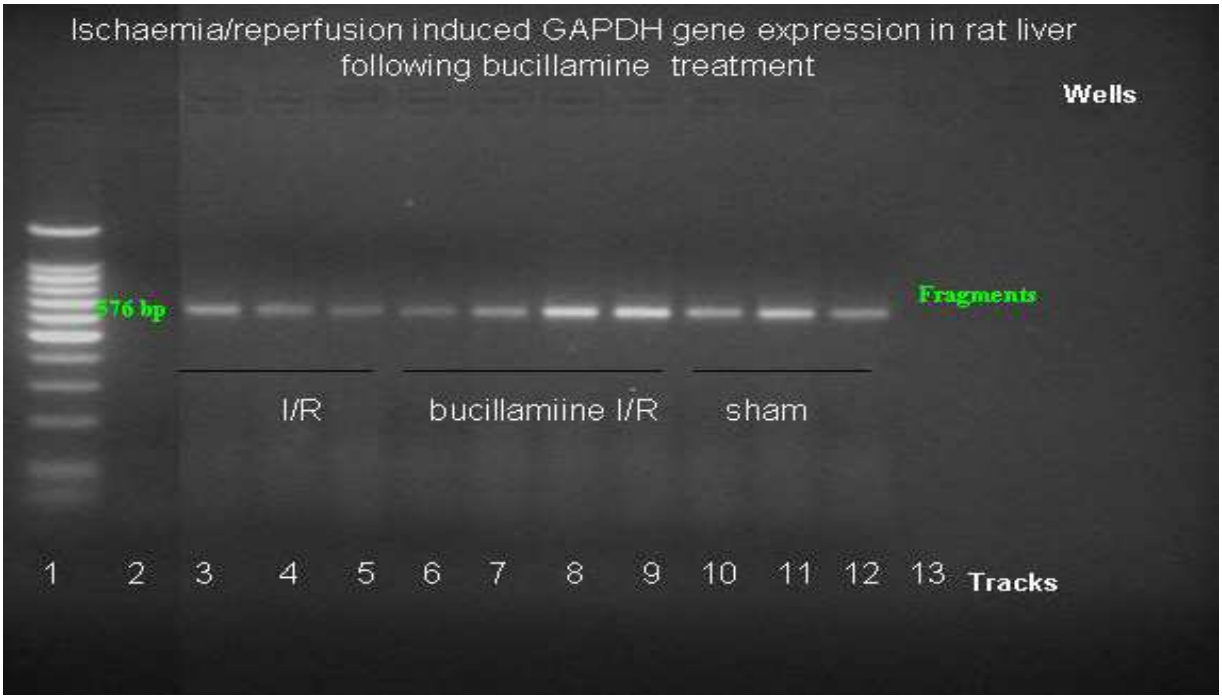
Finally, track 2 in each agarose electrophoresis gel (Figures 5.4.2, 3 and 4) was blank due to lack of any fragment. This was to be expected as track 2 acted as a negative control with no RNA added to the PCR reaction tube prior to loading.

Table 5.4.2 :

		Quantification Of Bands (Arbitrary Units)		
	Track Number	**Bax**	**Bcl-2**	**Bax/Bcl-2 Ratio**
IR alone	3	12512	750	16.68
	4	10351	1500	6.90
	5	16585	3050	5.44
	6	10431	1485	7.02
	7	11685	800	14.61
MEAN		12312.80	1517	10.13
±S.D.		2553.20	929.22	5.13
Bucillamine + IR	8	3060	12340	0.25
	9	2615	3040	0.86
	10	1385	17680	0.08
	11	1412	17430	0.08
	12	1708	3020	0.57
MEAN		2036	10702	0.36
±S.D.		759.04	7320.53	0.33
Sham	13	3080	800	3.85
	14	1412	570	2.48
	15	1308	10444	0.13
	16	1563	11251	0.14
	17	1786	13520	0.13
MEAN		1829.8	7317	1.74
±S.D.		721.61	6158.84	1.34

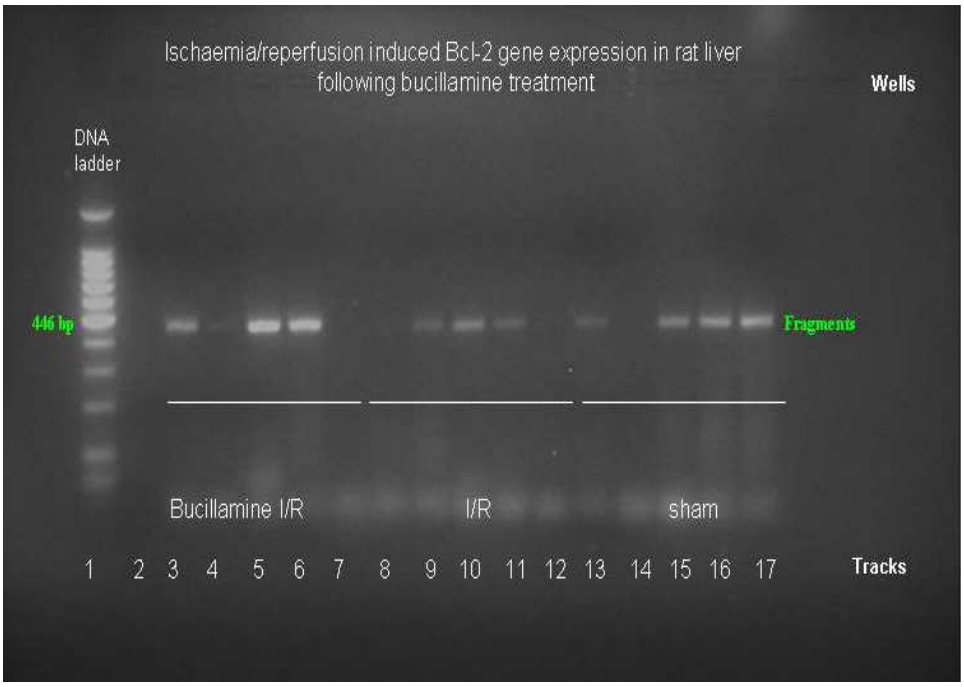
Table 5.4.2. Quantification of Bcl-2 and Bax agarose gel electrophoresis band patterns in the following experimental groups: ischaemia-reperfusion injury (IR) alone and Bucillamine + IR. Statistical analysis of the two groups (Student's *t*-test) was carried out. Significant increase in Bax expression seen in IR group which was attenuated by Bucillamine infusion ($p<0.0005$). Bucillamine infusion also significantly up regulated Bcl-2 expression as compared to IR only group ($p<0.005$)**.**

Figure 5.4.2:



Track Key: Track1- DNA molecular marker, Track2- Negative control

Figure 5.4.3A:



Track Key: Track1- DNA molecular marker, Track2- Negative control

Figure5.4.3B:

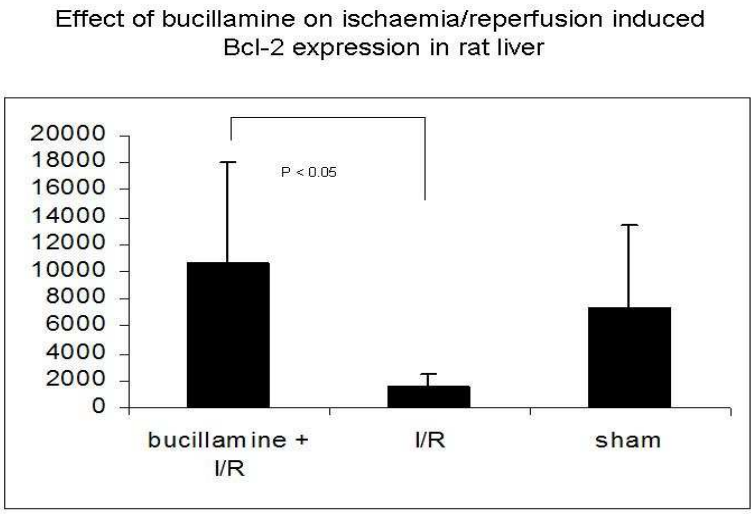
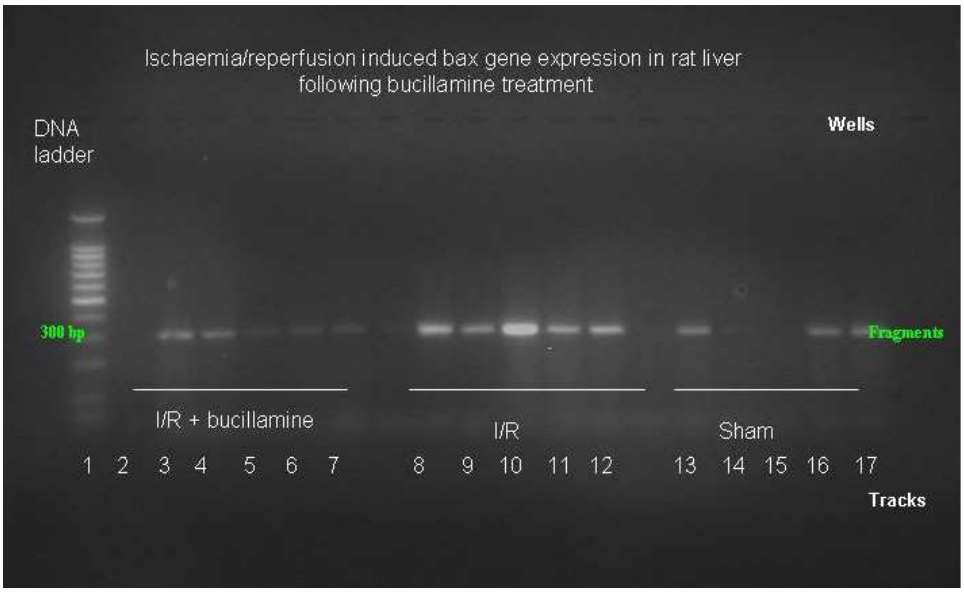


Figure 5.4.4 A:



Track Key: Track1- DNA molecular marker, Track2- Negative control

Figure 5.4.4B

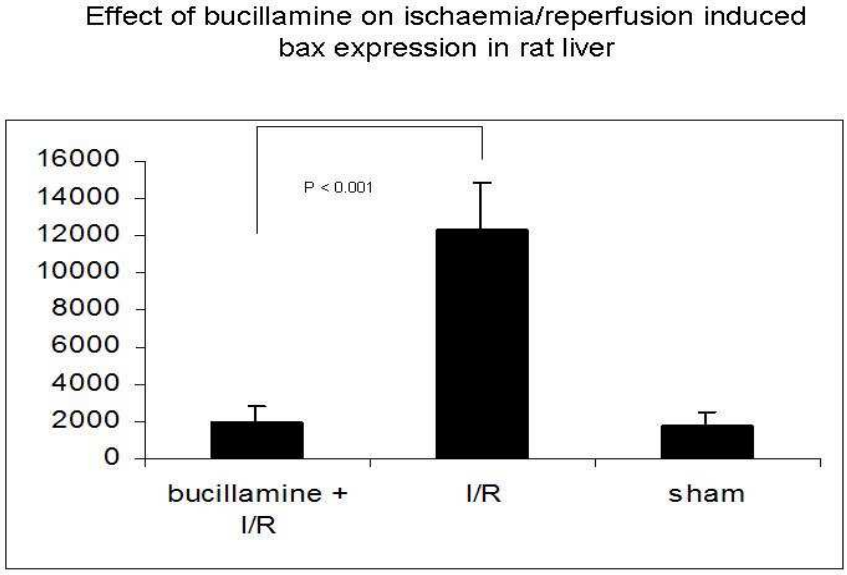
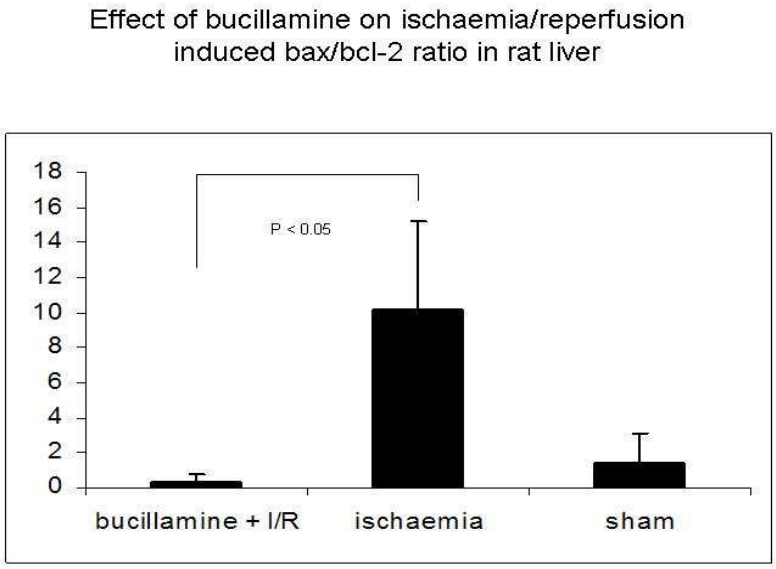


Figure 5.4.5:



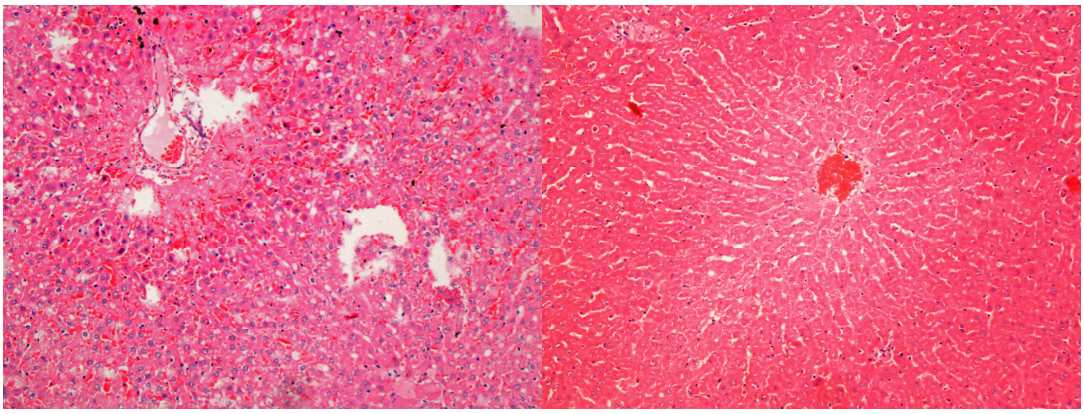
5.4.6 Histology

I/R injury caused significant periportal congestion with severe necrosis in zones 2 and 3 (Figure 5.4.6a), with a statistically significant rise in the Suzuki score as compared to sham group ($p < 0.001$). The Bucillamine I/R group showed less damage with a statistically significantly lower Suzuki score as compared to I/R only group ($p < 0.05$). Portal as well as central venous congestion was seen but there was no significant spill out into the surrounding parenchyma. The only change appeared to be some degenerative changes in the perivenular hepatocytes (Figure 5.4.6b). The SB group had well preserved architecture and the sham group revealed minimal changes (Figure 5.4.6c and d). No apoptotic changes were seen on histology at 3 hours reperfusion.

Table 5.4.6:

	Sham	IR	Bucillamine + I/R	Sham+ Bucillamine
Suzuki score	**4.6 ± 0.68**	**9 ± 0.68**	**6.57 ± 0.65**	**1.8 ± 0.2**

a.IR group **b.Bucillamine+I/R group**



c.Sham **d.Sham+ Bucillamine**

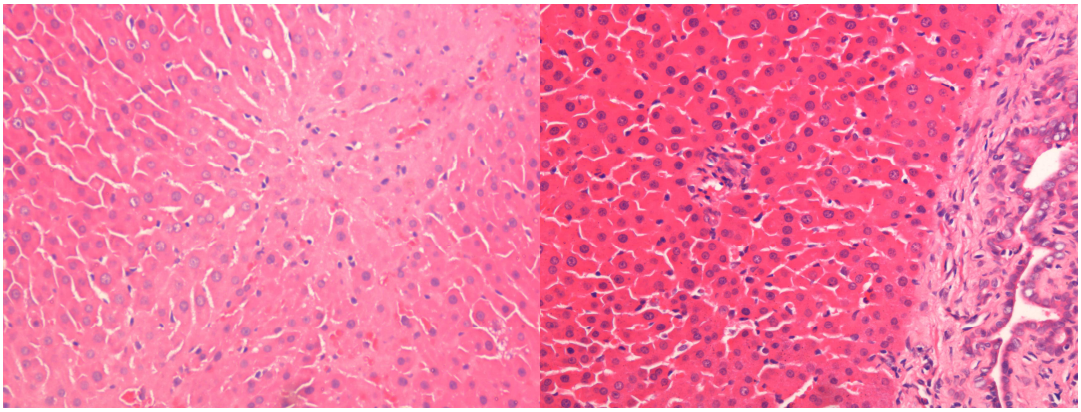


Figure 5.4.6 Histology a).IR- The HE section shows large areas of necrosis and sinusoidal congestion, normal residual hepatocytes noted at bottom of the frame
b).Bucillamine+IR-The HE section shows sinusoidal congestion, some hepatocyte vacuolation but no significant necrosis
c).Sham- The HE section reveals no significant damage.
d).Sham+Bucillamine- The HE section reveals no significant change.

5.5 DISCUSSION

Findings in this study

In this study apoptotic gene expression was measured in a rat hepatic IR model, which has shown for the first time a decreased expression of the proapoptotic Bax gene and increased expression of Bcl-2 gene after Bucillamine infusion in Liver IR. Bucillamine infusion during ischaemia reperfusion also showed a protective effect as seen on histology.

Methodology

RT- PCR for Bax and Bcl-2 is a very well described and validated technique(Oshiro *et al.* 2002; Tannuri *et al.* 2008). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is an enzyme that catalyses the sixth step in the glycolytic pathway. As the gene is required to maintain basic cellular functions, an inherent assumption is that the expression of this gene remains constant in all cells. For this reason GAPDH was utilised as an internal standard in this study to normalise the gene expression analysis of Bcl-2 and Bax, as a 'housekeeping' gene.

We used Suzuki criteria for assessing the histological changes as this allows the changes occurring to be allocated a score which could be compared between the groups(Suzuki and Toledo-Pereyra 1993). All samples being assessed by a Consultant Pathologist who was blinded to the sample allocation. Other investigators have used degree of necrosis as the only histological

parameter(Noh *et al.* 2006) however, the severity of necrosis seen at 3 hours reperfusion is not as marked with more marked changes seen at 24 hours following reperfusion. Initial changes in liver IR seen on histology are congestion, cytoplasmic vacuolation, nuclear pyknosis, cytoplasmic eosinophilia and neutrophil infiltration. Other investigators have used gradations in these changes to assess severity of liver injury in early phase of liver IR(Cavalieri *et al.* 2002; Kong *et al.* 2009). This is the first study on liver warm ischaemia reperfusion injury using Bucillamine infusion. In the ex vivo cold ischaemia model Amersi et al(Amersi *et al.* 2002) used Banff score(Demetris *et al.* 1996) for grading histological changes and showed better protection of cytoarchitecture with Bucillamine.

An increase in Bax and decrease in Bcl-2 expression leads to increase in mitochondrial permeability resulting in increased release of cytochrome c which in turn upregulates caspase 3 activation leading to apoptosis. An increase in Bax and decrease in Bcl-2 was seen in the IR group. Bucillamine by its anti oxidant effect seems to decrease the expression of Bax and upregulates the anti apoptotic gene Bcl-2.

Liver warm I/R is associated with necrosis / apoptosis of hepatocytes(Jaeschke and Lemasters 2003). In liver I/R, oncotic necrosis and apoptosis share features and mechanisms(Gujral *et al.* 2001; Jaeschke and Lemasters 2003). It is suggested that the ability of a necrotic process to be converted to an apoptotic

one and vice versa illustrates that the pathways in the two processes could be shared, a phenomenon called necroapoptosis(Lemasters 1999; Formigli *et al.* 2000). Our results agree with this theory of common phenomenon of necroapoptosis. Liver I/R showed cellular congestion, vacuolation and necrosis along with inflammatory changes which are a feature of oncotic necrosis(Majno and Joris 1995). However, Liver I/R also showed an increase in Bax/ Bcl-2 ratio. Bucillamine ameliorated both the severity of morphological changes as well as the rise in Bax/ Bcl-2 ratio. This co-related with our earlier observation of increased non viable nuclei with propidium iodide on intra vital microscopy and its attenuation by Bucillamine infusion.

Conclusion:

This study shows a protective role of Bucillamine infusion in the early phase in the subsequent chapters we concentrate its role in late phase of IR and the possible mechanisms.

Chapter 6

New insights into the late phase of hepatic IR and the effect of Bucillamine on hepatocellular and microvascular changes after 24 hrs of reperfusion

6.1 Introduction

The previous chapters have analysed the early phase of liver warm ischaemia reperfusion injury and demonstrated the protective hepatocellular and microvascular changes associated with Bucillamine in hepatic IR injury. In this chapter we have studied the late phase of hepatic IR and the effect of Bucillamine on the late phase of IR. In the clinical setting ischaemia reperfusion injury in the late phase has a major bearing on patient survival, graft function and morbidity. Therefore it is imperative that experimental studies investigate the pathophysiology of the late phase, the mechanism of hepatocellular injury caused in the late phase of hepatic IR and the effect of protective strategies in the late phase of hepatic IR.

Pathophysiological changes in IR (early and late phase)

The key event in the initial phase of reperfusion injury is activation of macrophages which are the primary source of extracellular ROS (free radicals). Endothelial cells and parenchymal cells are activated leading to release of ROS originating from intracellular mitochondria, xanthine oxidase system, NADH oxidase system and iron redox system. There is an imbalance between endothelial NO and ROS leading to impairment of protective effects of NO on the microvasculature. Free radicals (ROS) are the key initiators of reperfusion injury which lead to endothelial injury and further release of pro inflammatory cytokines. Simultaneous activation of complement pathway in the early phase serves to prime macrophages and neutrophils for release of ROS(Jaeschke *et al.* 1993).

The complement cascade along with cytokines and chemokines leads to recruitment of neutrophils, increased expression of adhesion molecules and adhesion of neutrophils to the endothelium in venules. Neutrophils damage endothelium, extravasate, adhere to parenchymal cells and plug the local microvasculature which along with vasoconstriction due to increased endothelin release exacerbates tissue ischaemia. In the late phase of IRI neutrophils produce an oxidative burst causing parenchymal injury due to release of free radicals and proteases.

Microvascular, biochemical and histological changes (comparison of early and late phase of hepatic IR)

In the early phase of hepatic IR there is significant impairment of RBC velocity, sinusoidal flow, sinusoidal perfusion and significant increase in sinusoidal and postsinusoidal venular neutrophil adhesion and hepatocellular death by intravital microscopy as demonstrated by the findings in this study and previously by Menger and Vollmar(Menger *et al.* 1999; Vollmar *et al.* 1994a). In the late phase of hepatic IR there is significant impairment of sinusoidal perfusion and significantly increased neutrophil adhesion in sinusoids and postsinusoidal venules. Neutrophil adhesion is markedly increased as compared to the early phase. In both phases there is increased parenchymal cell death and evidence of raised hepatic transaminases suggestive of hepatocellular injury. In both phases there is histological evidence of apoptosis, necrosis and neutrophilic infiltration with these changes being significantly more in the late phase of hepatic IR.

Studies involving Bucillamine and other thiol antioxidants in late phase of liver I/R

In the in vivo study of syngeneic OLT in rats Amersi et al have shown a improved survival and better preservation of hepatic cytoarchitecture in livers given Bucillamine intraportally(Amersi *et al.* 2002). Our group has previously shown a protective role of continuous infusion of N acetylcysteine (NAC) in late phase of warm liver I/R(Fusai *et al.* 2005; Glantzounis *et al.* 2004). Chavez-Cartaya et al however, did not show any beneficial effect with NAC given preoperatively intramuscularly at 24 hour reperfusion(Chavez-Cartaya *et al.* 1999). Data from clinical trials in the use of NAC in OLT is unclear(Bromley *et al.* 1995; Thies *et al.* 1997; Steib *et al.* 1998; Bucuvalas *et al.* 2001; Weigand *et al.* 2001).

Effect of Bucillamine on late phase of warm liver I/R has not been studied. Its effect on liver microcirculation in the late phase has also not been studied. We hence carried out this study to see if the protective effect of Bucillamine infusion could be seen in the late phase of liver I/R.

6.2 Materials and Methods

Animal care and experimental protocols were performed in accordance with the Home Office Guidance in the Operation of the Animals (Scientific Procedures) Act 1986. Male Sprague-Dawley rats weighing 300- 350g were be used in this study. Rats were kept in temperature controlled environment with 12 hours light-dark cycle and allowed tap water and standard rat chow pellets ad libitum.

6.2.1 Operative Procedures

Animals were anesthetized using isoflurane (Baxter, Norfolk, UK) and allowed to breathe spontaneously via concentric mask connected to an oxygen regulator. The animal's body temperature was maintained at 36-38 ^{o}C using a heating pad (Harvard apparatus Ltd., Kent, UK) and monitored with a rectal temperature probe. The arterial oxygen saturation (SaO_2) and heart rate (HR) were continuously monitored with a pulse oximeter (Ohmeda Biox 3740 pulse oximeter, Ohmeda Louisville, Colorado, USA). Polyethylene catheters (Portex 2 Fr) were inserted into the right or left jugular vein for administering normal saline (1ml /100gm body weight/hour) [or Bucillamine reconstituted to same volume depending on the experimental group] to compensate for intraoperative fluid loss.

Laparotomy was carried out through a midline incision. The ligamentous attachments of the liver were cut and the liver exposed. All animals were given heparin 20 units /kg and partial hepatic ischaemia of the left lateral and median lobes of (70% of liver) induced by clamping the corresponding vascular pedicle

with an atraumatic microvascular clamp for 45 minutes in the control and Bucillamine groups. This model prevents splanchnic congestion by allowing flow through the remaining liver(Koti *et al.* 2005). Reperfusion was started by releasing the atraumatic clamp. Rats were randomly allocated to 2 study groups (n=6/group).

6.2.2 Experimental Protocol

Group 1. I/R- 45 minutes ischaemia followed by 24 hour reperfusion

Group 2. Bucillamine + I/R

In the Bucillamine group Bucillamine was given as an infusion through the jugular line at a rate of 15mg/kg/hr through the period of ischaemia and for 3 hours after reperfusion. Animals in the I/R group received same volume of normal saline. Abdomen was closed with 4-0 Vicryl. The neck line was removed after the period of infusion and the neck wound closed using 4-0 Vicryl continuous suture.

Rat recovery was closely monitored. Animal behaviour was assessed every hour for 4 hours and then at 22 and 24 hours. Signs of poor clinical condition were lethargy , ruffled fur and guarding upon abdominal palpation, lack of grooming and decreased food intake.

After 24 hours of reperfusion right carotid artery was cannulated with a polyethylene catheter (0.40-mm inner diameter, Portex, Kent, UK) for administering fluorochromes. The abdomen was re-opened through the previous

incision. The left lobe of liver was mobilised gently after dividing filmy adhesions that had formed following previous laparotomy and put under the intravital microscopy for visualisation. In each group, Intravital microscopy was performed to assess RBC dynamics and interactions of leukocyte to the endothelium, prior to giving propidium iodide to assess nonviable nuclei.

6.2.3 Intravital Microscopy

As described previously in Chapter 3 (page 65)

6.2.3.1 Leukocyte parameters

As described previously in Chapter 3 (page 68)

6.2.3.2 Mean RBC velocity

As described previously in Chapter 3 (page 65)

6.2.3.3 Sinusoidal diameter

As described previously in Chapter 3 (page 67)

6.2.3.4Sinusoidal perfusion

As described previously in Chapter 3 (page 67)

6.2.3.5 Sinusoidal Blood Flow

Sinusoidal blood flow was calculated using formula for capillary blood flow as previously described(Rucker *et al.* 1999).

Sinusoidal blood Flow= Velocity x π x $(d/2)^2$ (expressed as pl/sec)

6.2.3.6 Detection of hepatocellular death in vivo.

As described previously in Chapter 3 (page 69)

At the end of the experiment animals were killed by exsanguinations. Serum and Plasma samples were collected by spinning the blood at 3000rpm for 10 minutes and stored in aliquots of 0.2 mls at -80^{o} C. Liver tissue was stored in formalin for histopathology and immunohistochemistry. Liver tissue was also frozen immediately on termination of experiment in liquid nitrogen and stored at -80^{o} C for further analysis.

6.2.4 Biochemical Assay

Blood samples were collected from the Carotid artery and were centrifuged at 3000 rpm for 10 minutes and serum was removed and analysed on an autoanalyzer (Hitachi 747, Hitachi, Tokyo, Japan) using commercially available kits (Boehringer Mannheim, Lewes, East Sussex, UK) for serum aspartate transaminase and serum alanine transaminase.

6.2.5 Histological assessment

As described previously in Chapter 5 (page 109)

6.3 Data collection and statistics

All the data is expressed as mean± standard error of mean (sem). Analysis of data was done using SPSS 14.0.Differences in data between groups was assessed by using one way ANOVA with Bonferroni's post hoc test. Data was considered statistically significant if $p< 0.05$.

6.4 Results:

There were no procedure related deaths in either group.

It was found to be difficult to mobilise the liver on account of adhesions and tissue oedema as compared to 3 hour reperfusion group (Figure 6.1). Intravital microscopy was not possible due to oedema and necrosis in one animal in the I/R group and was only possible to get partial data in one animal in the Bucillamine group. Complete Intravital microscopy data could be obtained in 5 animals in both groups.

Biochemistry

The AST as well as ALT were less raised in the Bucillamine group, though this was statistically non significant (AST, 3053±1322.06 vs. 3379±1501.51) (ALT, 1611±588.12 vs. 1740.67±723.11). (Figure 6.2 and 6.3)

Intravital microscopy

RBC velocity

RBC velocity was higher in the Bucillamine group as compared to the I/R group (481.38± 33 vs. 400.47±25.4, p=0.062) (Figure 6.4).

Sinusoidal diameter

There was no statistically significant difference between the two groups (7.37±0.23 vs. 7.27±0.21). (Figure6.5).

Sinusoidal Perfusion Index

There was no statistically significant difference between the two groups (76.61±5.78 vs. 67.03±7.73, p=0.35). (Figure 6.6)

Sinusoidal Blood Flow

There was no statistically significant difference between the two groups (19.13±2.63 vs. 16.28±1.83, p=0.401). (Figure 6.7)

Leukocyte adherence in sinusoids

I/R group (176.92±48.68) showed a significant higher number of adherent leukocytes in the sinusoids as compared to bucillamine group (43.09±8.42) ($p<0.05$). (Figure 6.9)

Leukocyte adherence in venules

There were significantly lesser number of adherent leukocytes in the venules in the Bucillamine group as compared to I/R group (217.33±50.15 vs. 737.62±132.52, $p < 0.01$) (Figure 6.10)

Hepatocellular cell death *in vivo*

There was statistically significant difference in the nonviable nuclei between the Bucillamine and I/R groups (385.37±49.37 vs. 923.98±116.68, $p<0.005$). (Figure 6.11)

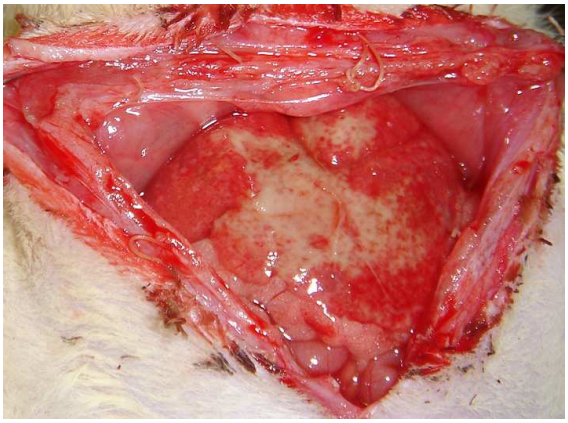


Figure 6.1: Photograph showing severe necrosis and oedema seen 24 hours post reperfusion

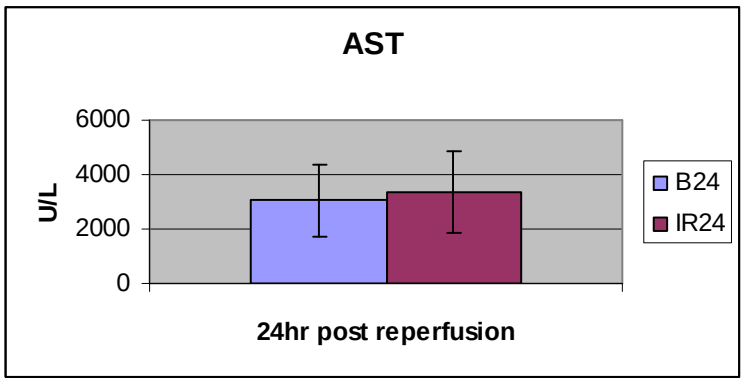


Figure 6.2: Showing serum AST levels 24 hours post reperfusion values expressed as Mean ± SEM

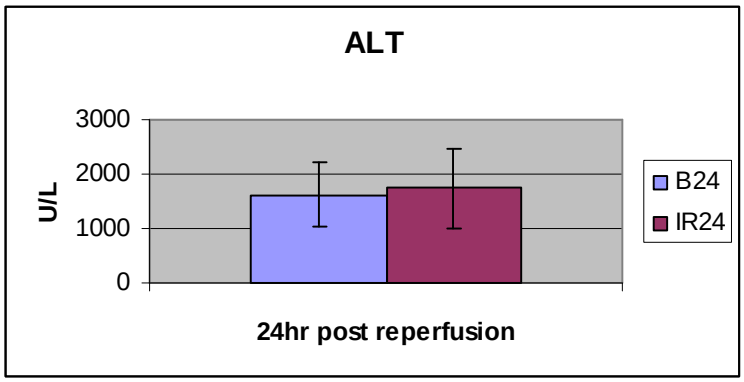


Figure 6.3: Showing serum ALT levels 24 hours post reperfusion values expressed as Mean ± SEM

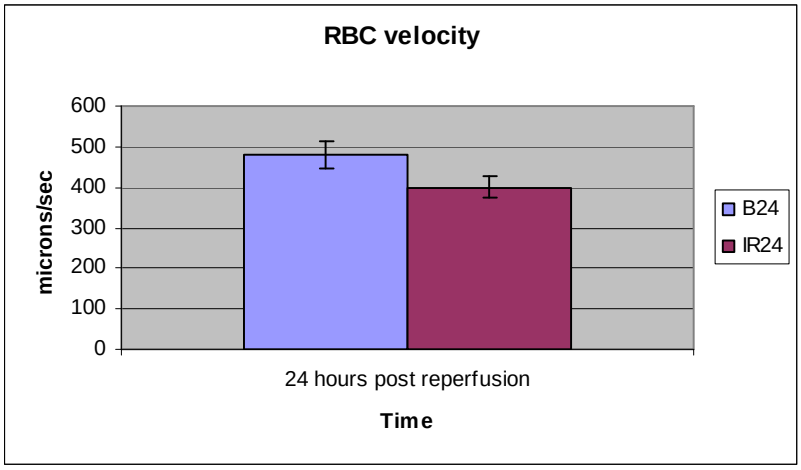


Figure 6.4: Velocity of RBC flow in Bucillamine+IR-24 as compared to IR-24. Values expressed as mean ± sem. (No significant difference seen between IR-24 and Bucillamine +IR-24)

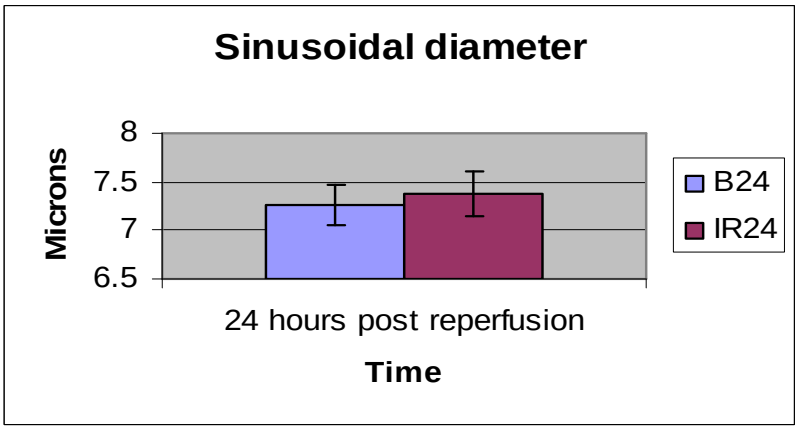


Figure 6.5: Figure - Sinusoidal diameter- No Significant Difference seen. Values expressed as mean ± sem.

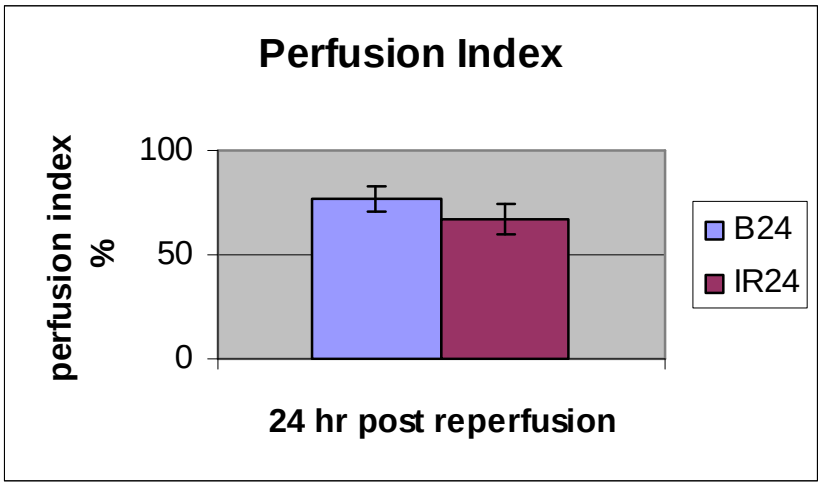


Figure 6.6: Sinusoidal perfusion index – No significant difference seen. Values expressed as mean ± sem.

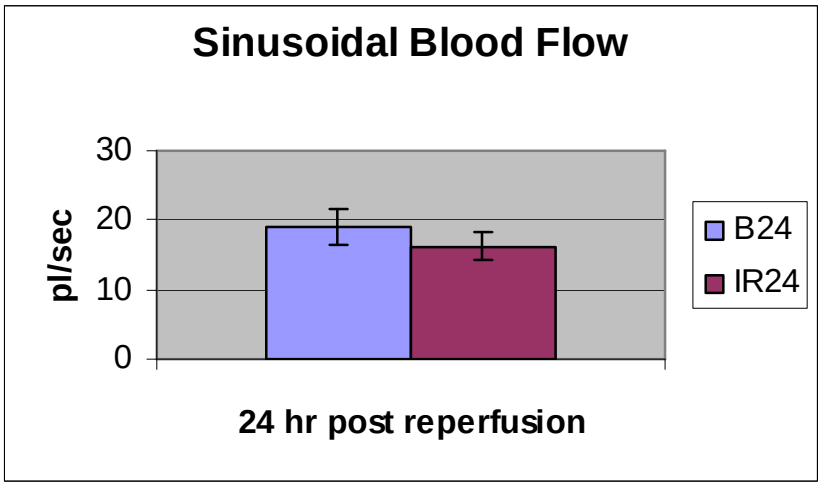
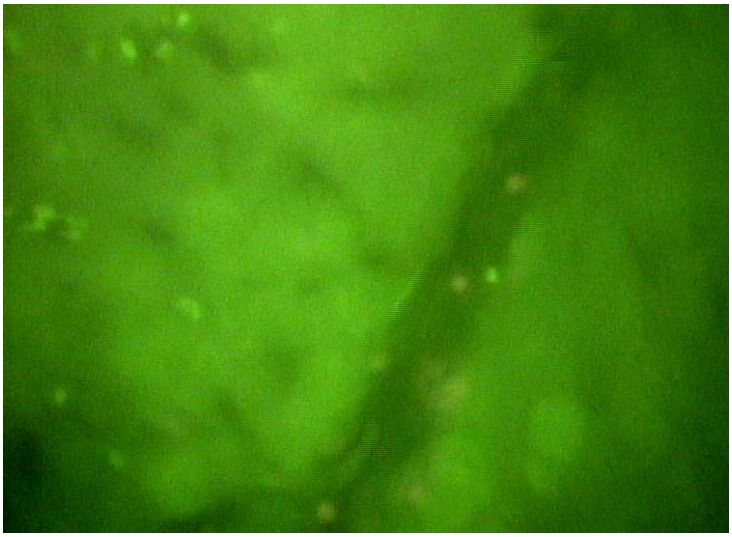


Figure 6.7: Sinusoidal flow = V× (D/2)2×л. V is velocity of RBC, D is sinusoidal diameter. No significant difference seen. Values expressed as mean ± sem.



Figures 6.8: Neutrophils stained by rhodamine are seen adherent to post sinusoidal venular endothelium and to hepatic sinusoids. The number of adherent neutrophils divided by the area of endothelial surface (л×D×L) gives the number of neutrophils/ mm2. D= sinusoidal diameter, L= length of segment along adherent neutrophils.

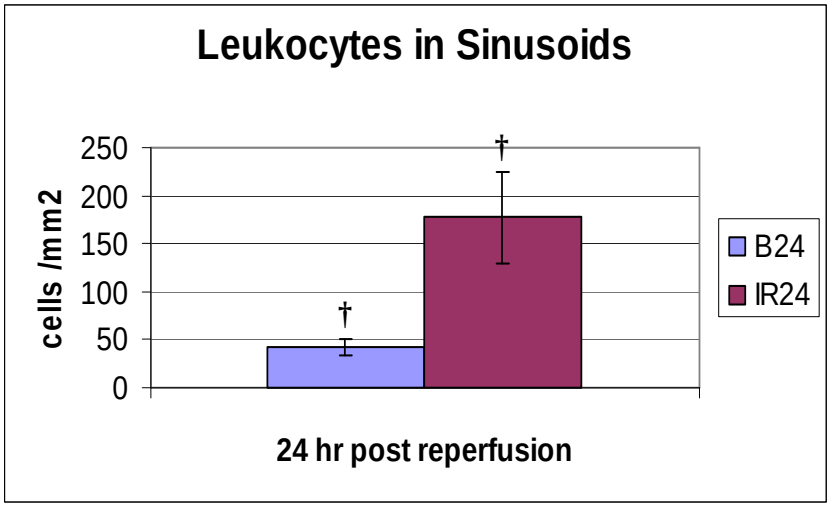


Fig 6.9: Significantly reduced venular neutrophil adhesion in Bucillamine (B-24) group compared to IR only group (IR-24). Values expressed as mean ± sem. † $p<0.01$

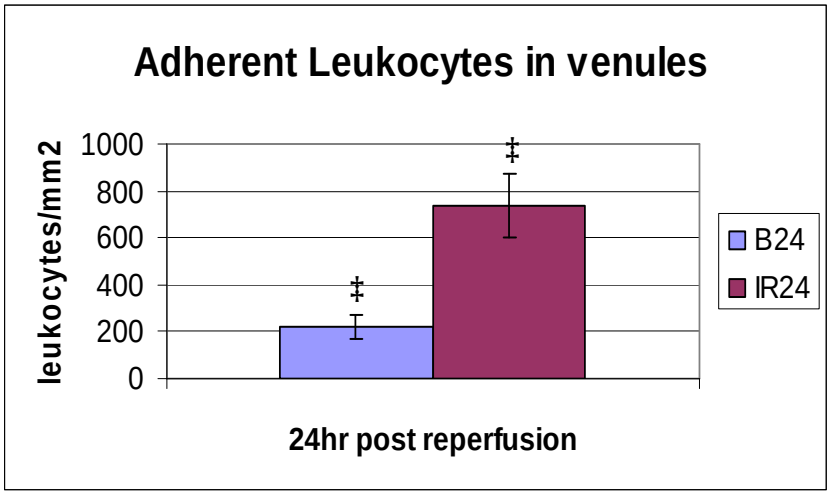


Figure 6.10- Significantly reduced sinusoidal neutrophil adhesion in Bucillamine group (B24) compared to IR only group (IR24). Values expressed as mean ± sem. ‡ $p<0.05$

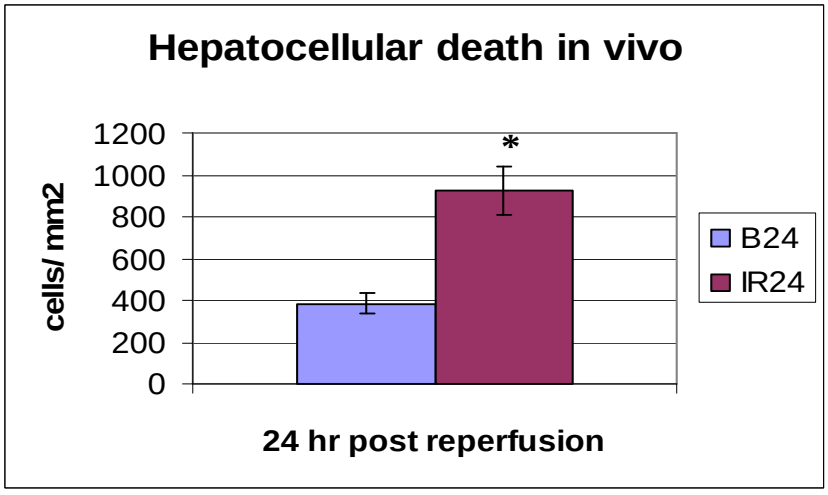
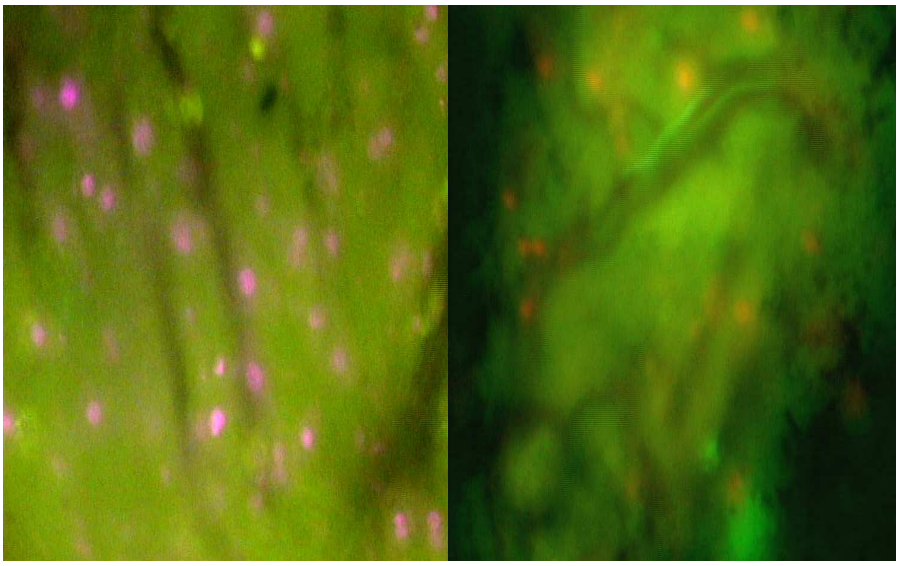


Figure 6.11- Hepatocellular cell death in Bucillamine group (B-24) is significantly less compared to IR only (IR-24) group. Values expressed as mean± sem. * **$p<0.005$**

Hepatocellular death.



IR-24 Bucilamine+IR-24

Figure 6.12: Hepatocellular l death in IR by propidium iodide staining (IVM). The dead cells appear pink stained by propidium iodide. The number of cells divided by the surface area of the field above gives the number of cells/mm2.

Fig 6.13 - Hepatocellular cell death seen in Bucillamine+IR-24 by propidium iodide staining (IVM).

Histology

In the IR24 group severe damage with abundant ballooning degeneration and necrosis was seen which was reduced by Bucillamine administration (Figure 6.14 and 6.15). Apoptotic changes were seen in the IR only group.

Figure 6.14: IR-24

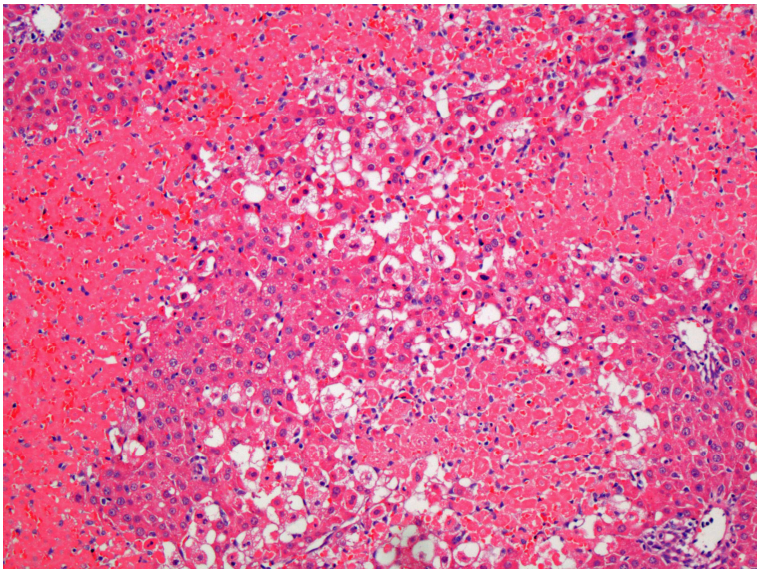


Figure 6.15: B-24

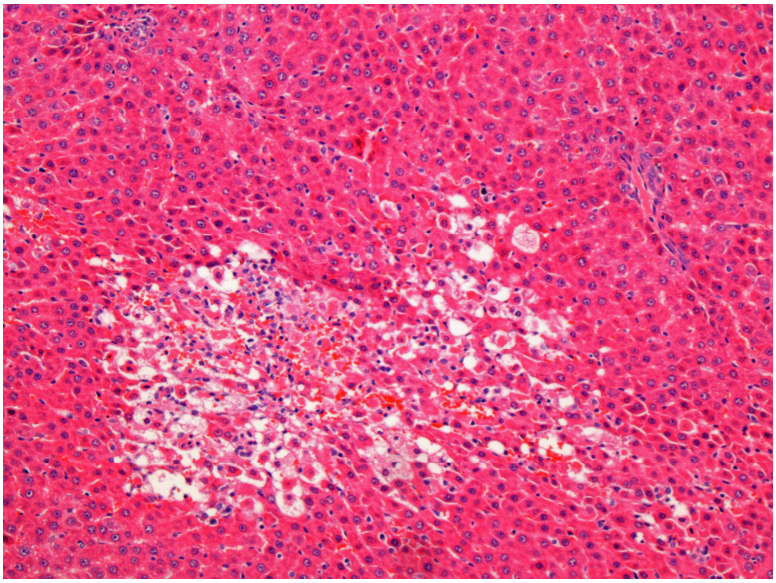


Figure 6.14 &6.15: Very severe injury with abundant ballooning degeneration and necrosis is seen in the IR injury group. Very diffuse and significant neutrophil adhesion is seen in the IR group. Apoptosis is evident in the IR group. Bucillamine group shows less injury with some ballooning and degeneration as well as neutrophilic infiltration.

6.5 Discussion

Model stability, selection

The animal model was haemodynamically stable during the period of ischaemia and recovery. The animals were resuscitated with intravenous fluids prior to closure of laparotomy wound and recovery from anaesthesia. All animals in our study received either Bucillamine or normal saline for 3 hours post reperfusion only. The effect of this was assessed at 24 hours post reperfusion. This was to keep uniformity in the protocol for early phase (Chapter 4) and late phase experiments. Furthermore, we wanted to see if the beneficial effect of Bucillamine seen in the early phase of liver I/R was maintained in the late phase of I/R. Equivocal results with the use of NAC have suggested that the beneficial effect of NAC might be lost after the continuous infusion of NAC is stopped(Glantzounis *et al.* 2004). Our results show a beneficial effect with Bucillamine in the late phase even after stopping of continuous infusion which agrees with the findings of Amersi et al(Amersi *et al.* 2002).

Microcirculatory changes in hepatic IR injury at 24 hours and comparison with early phase (3hrs)

The velocity of flow and sinusoidal flow in hepatic IR after 24 hours of reperfusion are not significantly less in comparison to Bucillamine + IR group in the late phase of hepatic IR which is in contrast to the early phase where we found the velocity of flow and sinusoidal flow to be significantly lower in the IR group. The sinusoidal perfusion in both the early (Chapter 4) and late phase of hepatic IR

were found to be lower in the IR group, although this was not statistically significant. There was no change in the sinusoidal diameter in early or late phase of IR injury in either group.

Neutrophil adhesion in the early and late phase of hepatic IR

The sinusoidal neutrophil adhesion and postsinusoidal venular neutrophil adhesion in both the early and late phase of hepatic IR was significantly more in comparison to the sham group. However the number of adherent neutrophils was significantly more in the late phase of hepatic IR in comparison to the early phase of hepatic IR suggesting that there is increased neutrophil activation and adhesion as IR injury progresses over a period of time. Previous intravital studies by Menger et al have shown that increased neutrophil adhesion to endothelium is responsible for increased endothelial injury and poor sinusoidal perfusion(Menger *et al.* 1999).

Neutrophil adhesion in Bucillamine + IR (3 hrs and 24 hrs)

The data here clearly demonstrate that Bucillamine markedly attenuates neutrophil adhesion to endothelium in the IR injury group in both sinusoids and venules and the reduced neutrophil adhesion may account for the improved sinusoidal perfusion seen in the Bucillamine + IR group. In the early phase of hepatic IR significantly reduces neutrophil adhesion in both sinusoids and venules. Previous studies on direct ischaemic preconditioning have demonstrated modulation of sinusoidal perfusion by reduced neutrophil

activation and adhesion at 24 hours(Vollmar *et al.* 1994c). The number of adherent neutrophils seen in the sinusoids in our study, however, does not suggest a mechanistic role for adherent leukocytes in reducing the sinusoidal perfusion by mechanically blocking the sinusoidal flow. Neutrophil activation by IR and its amelioration by Bucillamine seem to be an important mechanism of action of Bucillamine.

Liver injury and Hepatocellular death in IR and the effect of Bucillamine. (3hrs and 24 hrs)

Hepatic transaminases were used as surrogate markers of liver injury and are significantly higher in the IR injury group at 3 hours as compared to the Bucillamine group suggesting reduced hepatocellular injury in the Bucillamine group. Transaminase levels were also slightly reduced in the late phase of hepatic IR although the difference was not statistically significant. However, the direct marker of liver injury as assessed by necrotic/apoptotic nuclei stained with propidium iodide showed a significant decrease with Bucillamine infusion.

Histological changes in IR -24 and the effect of Bucillamine. (Early and late phase)

Histological findings in this study clearly demonstrate increased parenchymal necrosis and neutrophilic infiltration in the IR injury group at 24 hours as compared to the Bucillamine group. In the early phase the modified Suzuki score was significantly more in IR injury in comparison to Bucillamine+ IR group.

However the Suzuki score is an objective score described only for assessing the early phase of hepatic IR and cannot be used for describing the late phase changes.

6.6 Conclusions

This is the first study to demonstrate the protective effects of Bucillamine on hepatic microcirculation in a recovery model of hepatic IR in rats. The study showed that although there was no significant difference in the level of liver enzymes which are an surrogate marker of liver injury the protective effect of Bucillamine seemed to be preserved as assessed by histology and assessment of non viable nuclei on Intravital microscopy which are more direct methods of assessing liver injury.

In the next chapter we investigated possible mechanisms of action and role of Bucillamine in cytokine activation.

Chapter 7

Effect of Bucillamine on Oxidant stress: is mediated by the replenishment of intracellular glutathione levels?

7.1Introduction:

Reperfusion of ischaemic tissue generates reactive oxygen species (ROS) which have deleterious effects on various cellular functions(Werns and Lucchesi 1990). ROS activate cytokines, macrophages and other components of the inflammatory pathway(Entman *et al.* 1991; Jaeschke and Farhood 1991a; Le *et al.* 1997). When generated in large numbers they can also cause direct oxidative damage to the cells through iron mediated reactions(Horwitz *et al.* 1998). Kupffer cells become activated during ischaemia(Rymsa *et al.* 1991)and this is exacerbated during reperfusion by complement activation(Jaeschke *et al.* 1990). Activated Kupffer cells generate ROS(Jaeschke and Farhood 1991a). The Kupffer cell induced oxidant stress in early reperfusion injury leads to neutrophil activation and these activated neutrophils along with Kupffer cells contribute to further oxidant stress during the late phase of reperfusion injury(Jaeschke and Farhood 1991a). In this chapter we investigated if the beneficial effect of Bucillamine could be through suppression of oxidant stress.

7.1.1 Lipid Peroxidation assays

Oxidant stress in tissue and plasma is measured by lipid peroxidation assays. Several *in vitro* markers of oxidative stress are available, but most are of limited value in vivo because they lack sensitivity and specificity or require invasive methods(Halliwell 2000). The most widely used test for measurement of oxidative stress is measurement of malondialdehyde (MDA), a product of lipid peroxidation by a thiobarbituric acid reacting substances assay (TBARS assay)(Del *et al.*

2005). However, the use of this assay has flaws as MDA is not a specific product of lipid peroxidation(Gutteridge and Halliwell 1990), and TBARS assay is not specific for MDA(Halliwell 2000). Thiobarbituric acid (TBA) reacts with several other compounds derived by oxidation(Knight *et al.* 1988). Moreover, the treatment of biological samples to obtain condensation product is carried out at high temperatures (around 100^{o} C) and may result in generation of further products of oxidation with obvious overestimation of results(Del *et al.* 2005). Another method of assessing lipid peroxidation in vivo is measurement of exhaled volatile alkanes, such as ethane and pentane(Knutson *et al.* 1999). However, these hydrocarbons are minor end-products of peroxidation and their concentrations are influenced by the breakdown rates of peroxides(Halliwell 2000). The accuracy of exhaled pentane as a marker of lipid peroxidation has also been questioned(Cailleux and Allain 1993). Lipid hydroperoxides have been measured by various methods(Gay and Gebicki 2003), however, this has been found to be inconsistent(Halliwell 2000).

7.1.2 The ideal Lipid Peroxidation assay

The ideal assay of lipid peroxidation should satisfy the following conditions(Halliwell 2000):

1) It should quantitate a major product of lipid peroxidation

2) The coefficient of variance between multiple runs of the same sample should be very small in comparison with the differences between subjects.

3) It must not be subject to interference by other biomolecules.

4) It must employ chemically robust measurement methods (e.g. mass spectrometry or HPLC with good identification methods such as diode array or coulometric detectors) or be validated by such methods.

5) It must not be confounded by uptake of oxidised lipids present within foods.

6) It should be able to assess both steady state levels of products of lipid peroxidation (i.e. the balance between rate of peroxidation and rates of metabolism/ clearance of peroxidation products) and the total rates of ongoing lipid peroxidation.

7) The parameter measured should be stable on storage and should not be formed artefactually in stored samples.

7.2. F_2 isoprostanes

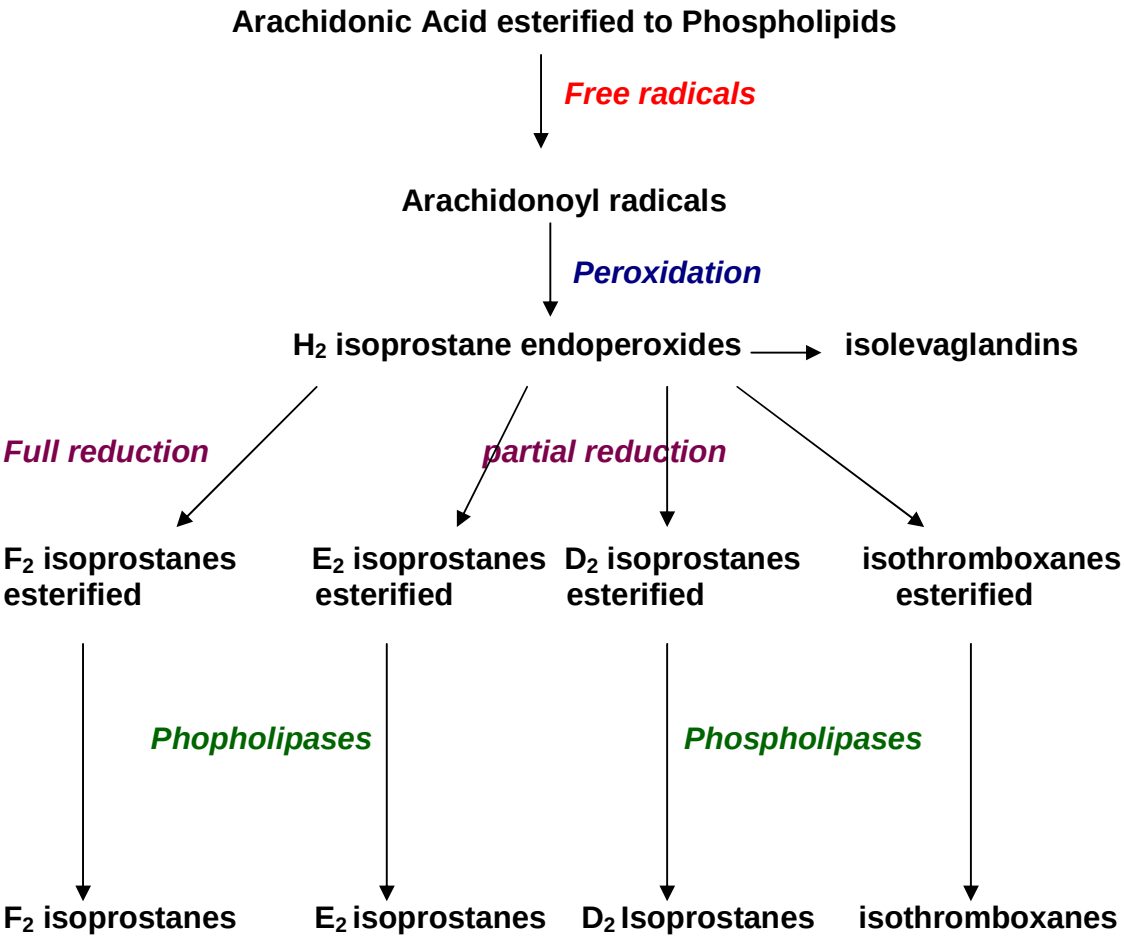
Isoprostanes are prostaglandin like substances that are produced in vivo by free radical induced peroxidation of arachidonic acid(Morrow *et al.* 1990) esterified to tissue lipids. They have been detected in a variety of normal animal tissues including liver, kidney, testes, heart, brain, skeletal muscle, aorta, ocular lens and lungs(Morrow and Roberts 1994). The formation of PG like compound during auto oxidation of poly unsaturated fatty acids was reported first in mid 1970s(Pryor *et al.* 1976), but isoprostanes were not discovered in vivo until 1990(Morrow *et al.* 1990). F_2 isoprostanes are a group of 64 compounds isomeric in structure to cyclooxygenase derived $PGF_2\alpha$. Other products of the isoprostane pathway are also formed in vivo by the rearrangement of labile PGH_2 like

isoprostane intermediates. These include E_2 and D_2 isoprostanes(Morrow and Roberts 1997), cyclopentenone A_2 and J_2 isoprostanes(Chen *et al.* 1999) and highly reactive acyclic ketoaldehydes (isoketals)(Brame *et al.* 1999). Oxidation of docosahexaenoic acid (an abundant unsaturated fatty acid in the central nervous system) leads to formation of isoprostane like compounds called neuroprostanes(Roberts *et al.* 1998).

Isoprostanes are formed in a free radical dependent manner and are chemically stable. They are initially generated in cell membranes at the site of free radical attack from which they are cleaved presumably by phospholipases, circulate and are excreted in the urine(Montuschi *et al.* 2004). (Figure 7.2.1)

Figure 7.2.1

The Isoprostane Pathway



F_2 isoprostanes:

1. are chemically stable(Roberts and Morrow 2000)
2. are specific products of peroxidation(Roberts and Morrow 2000)
3. are formed in vivo(Roberts and Morrow 2000)
4. are present in detectable amounts in all normal tissues and biological fluids, thus allowing definition of a normal range(Roberts and Morrow 2000)
5. are unaffected by lipid content in the diet(Richelle *et al.* 1999; Gopaul *et al.* 2000)
6. levels are found to increase in animal models of oxidant injury(Morrow *et al.* 1992; Awad and Morrow 1995)
7. might provide a sensitive biochemical basis in dose finding studies with antioxidants(Roberts and Morrow 2000).

Roberts et al compared and reviewed various methods of measuring lipid peroxidation and found F_2 isoprostanes to be the most accurate method(Roberts and Morrow 2000).

7.2.1 Biological properties of F_2 isoprostanes

F_2 isoprostanes are potent vasoconstrictors and induce their effects through the induction of Thromboxane A_2 receptors and also through release of products of the cyclooxygenase pathway(Cracowski *et al.* 2001). They have been shown to have a vasoconstrictor effect in rat glomerular arteries(Takahashi *et al.* 1992) and cerebral arterioles(Hoffman *et al.* 1997). F_2 isoprostanes also are known to mediate rapid neutrophil adhesion through either Thromboxane A_2 receptors or

specific isoprostane receptors present on neutrophils(Fontana *et al.* 2001). They are also known to cause platelet activation and platelet adhesion(Pratico *et al.* 1996; Minuz *et al.* 1998). All of these effects are known to play a part in ischaemia reperfusion injury.

7.2.2 Studies involving ischaemia reperfusion injury:

Mathews et al studied plasma F_2 isoprostanes in Sprague Dawley rats after 45 minute liver ischaemia and 1, 5 and 24 hours of reperfusion, and showed a significant increase in plasma F_2 isoprostanes in the reperfusion period with a peak rise at 1 hour reperfusion(Mathews *et al.* 1994). This was accompanied by a rise in plasma and liver tissue reduced glutathione (GSH) levels and a rise in hydroxyeicosatetraenoic acids. F_2 isoprostanes have been found to be increased in brain and heart muscle in ischaemia reperfusion models involving cardioplegia and reperfusion, in animals(Idris *et al.* 2005; Xia *et al.* 2003; Fischer *et al.* 2003) and human subjects(Mehlhorn *et al.* 2003). They have also been shown to increase in lung tissue ischaemia reperfusion model(Becker *et al.* 1998a). In kidneys F_2 isoprostanes have been shown to increase during ischaemia and reduce in the reperfusion period(Favreau *et al.* 2004). Increase in urinary F_2 isoprostanes has also been shown in human subjects undergoing percutaneous transluminal angioplasty following myocardial infarction(Reilly *et al.* 1997; Guan *et al.* 2003). All of these studies suggest that F_2 isoprostanes could be used as a marker of oxidative stress in ischaemia reperfusion injury.

Studies on Ischaemia reperfusion injury and F2 isoprostanes Table 7.2.3

First author and year	Organ	F2 isoprostane measurement (serum/tissue/urine)	Observation	Notes
Matthews(1994)(Matthews *et al.* 1994)	Rat Liver	Plasma	Significant increase post reperfusion with peak rise 1 hr post reperfusion	↑ Liver GSSG, ↑ Liver hydroxyeicosatetranoic acid
Kelly (2008)(Kelly *et al.* 2008)	Human Stroke	Plasma	Increased F2 isoprostane at 6hrs post stroke	
Basu (2007)(Basu *et al.* 2007)	Human renal transplant	Plasma	Increased F2 isoprostane on reperfusion	
Zhang (2007)(Zhang *et al.* 2007)	Rat Brain	Brain	Increased F2 isoprostane	
Lin (2006)(Lin *et al.* 2006)	Human Sub arachnoid haemorrhage	CSF, Plasma	Increased F2 isoprostane	
Idris (2005)(Idris *et al.* 2005)	Pig Heart	Arterial, Venous, and Brain tissue	Significant increase post reperfusion	
Xia (2003)(Xia *et al.* 2003)	Rat Heart	Heart tissue	Significant increase during ischaemia	Protection by propofol, high dose better than lower dose
Fischer (2003)(Fischer *et al.* 2003)	Pig Heart	Heart tissue immunocytochemistry	Increased expression of F2 isoprostanes	
Mehlhorn (2003)(Mehlhorn *et al.* 2003)	Human heart	Left ventricular biopsy immunocytochemistry	Increased expression of F2 isoprostanes	
Guan W (2003)(Guan *et al.* 2003)	Human undergoing Percutaneous transluminal angioplasty(PTCA)	Urine	Increased F2 isoprostane	No increase in Allopurionol infusion group
Basu (2001)(Basu *et al.* 2001)	Pig Spine	Urine, Plasma	Increased F2 isoprostane on reperfusion	
Heindl (2001)(Heindl and Becker 2001)	Guinea pig heart	Heart tissue	No significant increase	
Fischer (2000)(Fischer *et al.* 2000)	Rat Lung transplantation	Lung tissue, Plasma	Increased F2 isoprostane	Decreased F2 isoprostanes with Captopril
Marin (2000)(Marin *et al.* 2000)	Rat Cerebral ischaemia	Brain tissue	Increased F2 isoprostane	
Basu (2000)(Basu *et al.* 2000)	Pig Heart	Plasma	Increased F2 isoprostane	
Becker (1998)(Becker *et al.* 1998b)	Ferret lung	Lung tissue	Increased F2 isoprostane	
Reilly (1997)(Reilly *et al.* 1997)	Human PTCA	Urine	Increased F2 isoprostane	

7.3 Glutathione and Liver I/R:

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

The antioxidant function of GSH is implicated through two general mechanisms of reaction with ROS: direct or spontaneous and glutathione peroxidase (GPX) catalysed. As a major by-product of such reactions oxidised glutathione (GSSG) is produced (reaction 1). GSSG, can then be again reduced to GSH with the participation of glutathione reductase and NADPH (reaction 2):

$$2GSH + H_2O_2 \longrightarrow GSSG + 2H_2O \qquad (1)$$

$$GSSG + NADPH + H + \longrightarrow 2GSH + NADP + \qquad (2)$$

In the only other study on Bucillamine in Liver I/R Amersi et al showed increased levels of GSH in the liver and decreased levels of oxidized glutathione in both, the liver and blood after Bucillamine use and suggested that the beneficial effect of Bucillamine could be due to replenishment of endogenous glutathione levels(Amersi *et al.* 2002).

Aim of this Study:

To determine whether the beneficial effect of bucillamine in warm IRI is mediated by preservation of intracellular glutathione levels

Hypothesis:

We hypothesize that:

1. ischaemia reperfusion injury in rat livers there is increased oxidative stress and this would result in raised F2 isoprostane levels
2. Bucillamine by its anti oxidant property would reduce the rise in the levels of F2 isoprostanes.
3. Bucillamine would increase the level of tissue glutathione.

7.4 Materials and Methods:

Animals and surgical preparation

As described in Chapter 3 page 48

7.4.1 Experimental groups (n=6 in each group)

Group1 - (Sham) – laparotomy and mobilisation of liver but no occlusion of the vascular pedicle.

Group2- IR- 45 minutes of partial hepatic ischemia followed by 3 hrs of reperfusion.

Group3- Bucillamine 15mg/ kg/hr intravenously + IR (as above) followed by 3 hrs of reperfusion.

Group4- (SB)- Sham + Bucillamine infusion 15mg/kg/hr (SB)

Group5- IR24- 45 minutes ischaemia (as above) followed by 24 hour reperfusion

Group6- B24- 45 minutes ischaemia + Bucillamine 15mg/ kg/hr intravenously for 3hours followed by 24 hour reperfusion

7.4.2 Liver Injury Assay

As described in Chapter 3 page 71.

7.4.3 Measurement of Hepatic F_2-Isoprostanes

As described in Chapter 3 page 71.

7.4.4 Measurement of Plasma F_2-Isoprostanes

As described in Chapter 3 page 72.

7.4.5 Liver Tissue Glutathione Assay

As described in Chapter 3 page 72.

7.5 Statistical analysis

All the data is expressed as mean± standard error of mean (sem). Analysis of data was done using SPSS 14.0 (SPSS Inc., Chicago, Illinois, USA). Differences in data between groups were assessed by using one way ANOVA with Bonferroni's post hoc test. Data was considered statistically significant if $p< 0.05$.

7.6 Results

There were no procedure related deaths. The model was haemodynamically stable. There was a transient fall in oxygen saturation immediately after reperfusion which was reduced by Bucillamine therapy. There was a transient fall in blood pressure following reperfusion in both the I/R only and Bucillamine + I/R groups, which were not statistically significant.

7.6.1 Biochemistry

Transaminases were grossly elevated following I/R. The Bucillamine I/R group had lower AST and ALT than the I/R group [(AST 932±200.81 vs. 2072.5±511.79, $p<0.05$), (ALT 861.4±262.63 vs. 2079.3±322.33, $p<0.05$)]. (Figure 4.2.2a & b). The AST as well as ALT were lower in the Bucillamine group at 24 hours (B24) as compared to the IR only group at 24 hours (IR24), though this was statistically non significant (AST, 3053±1322.06 vs. 3379±1501.51) (ALT, 1611±588.12 vs. 1740.67±723.11). (Figure 6.2 & 6.3)

7.6.2 Hepatic F_2-Isoprostanes

The I/R group and Bucillamine groups had lower levels of liver tissue F2 isoprostanes than the Sham groups although this difference was statistically not significant. (Figure 7.6.1 & 2)

7.6.3 Plasma F_2-Isoprostanes

There was an increase in the plasma F_2 isoprostane levels in the I/R group at 3 hours reperfusion, although this rise was statistically non significant. In the Bucillamine + I/R group no rise in the plasma F_2 isoprostane levels was seen. There was no difference in the levels at 24 hour post reperfusion. (Figure 7.6.3 & 4)

7.6.4 Liver Tissue Glutathione

There was no statistically significant difference in the total Glutathione or GSH/GSSG ratio in any of the groups. Although the GSH/GSSG ratio seemed to better maintained in the Bucillamine group as compared to the I/R group both at 3 hour and 24 hour reperfusion this was not statistically significant. (Figure 7.6.5-8)

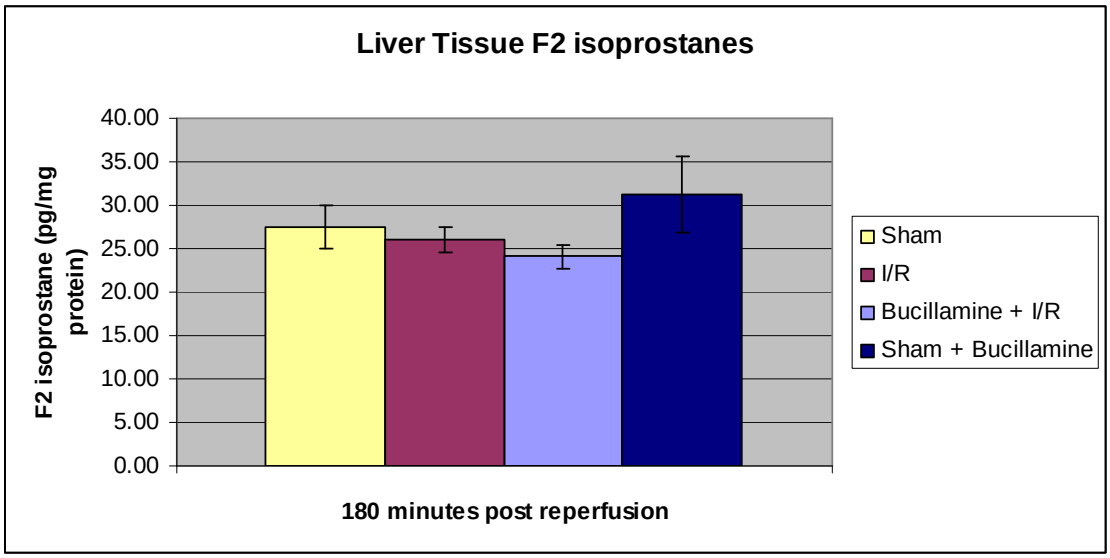


Figure 7.6.1

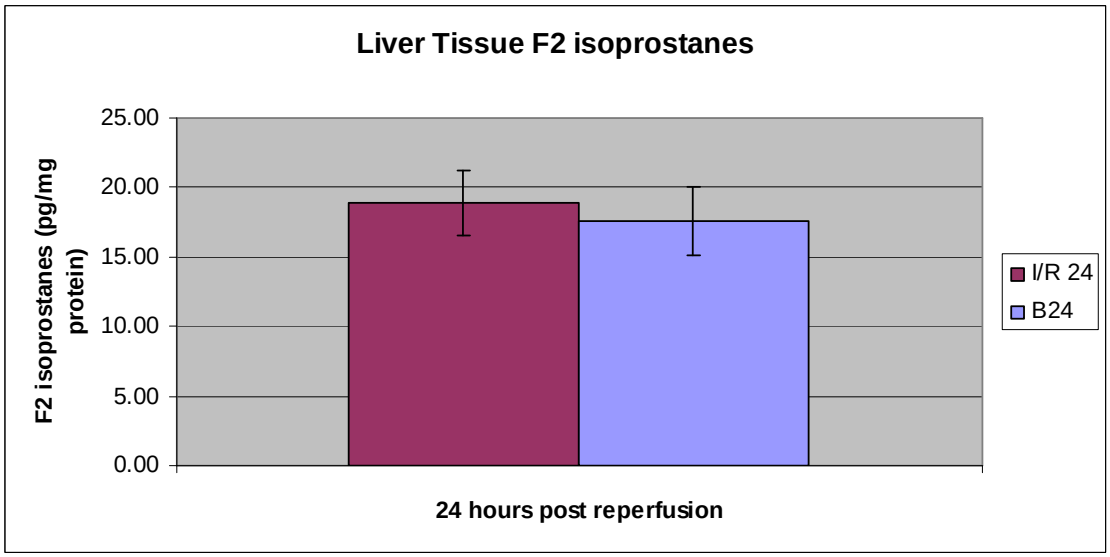


Figure 7.6.2

Figure 7.6.1&2: Liver Tissue F_2 isoprostane levels showing no significant difference in the groups.

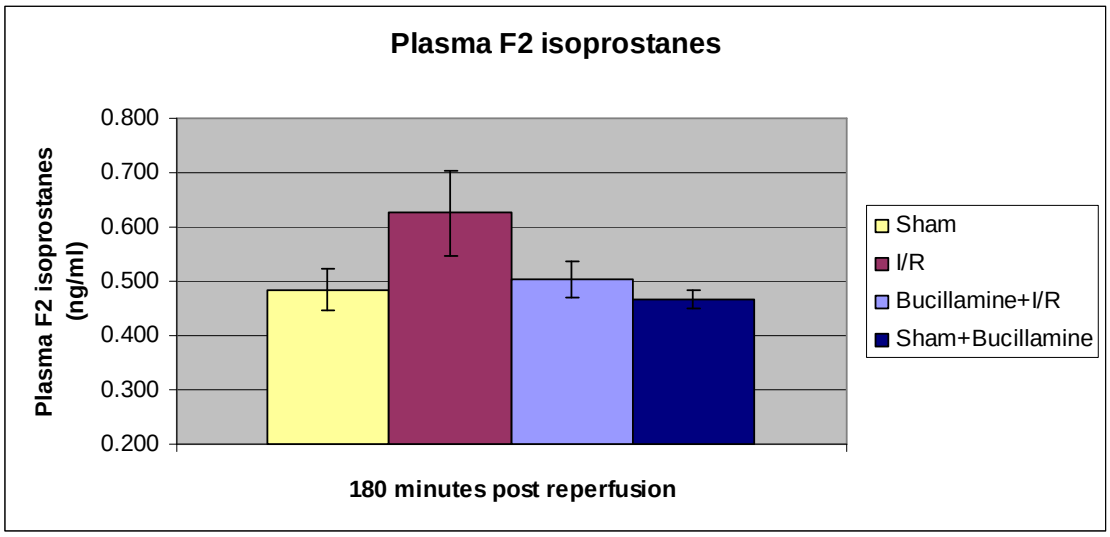


Figure 7.6.3

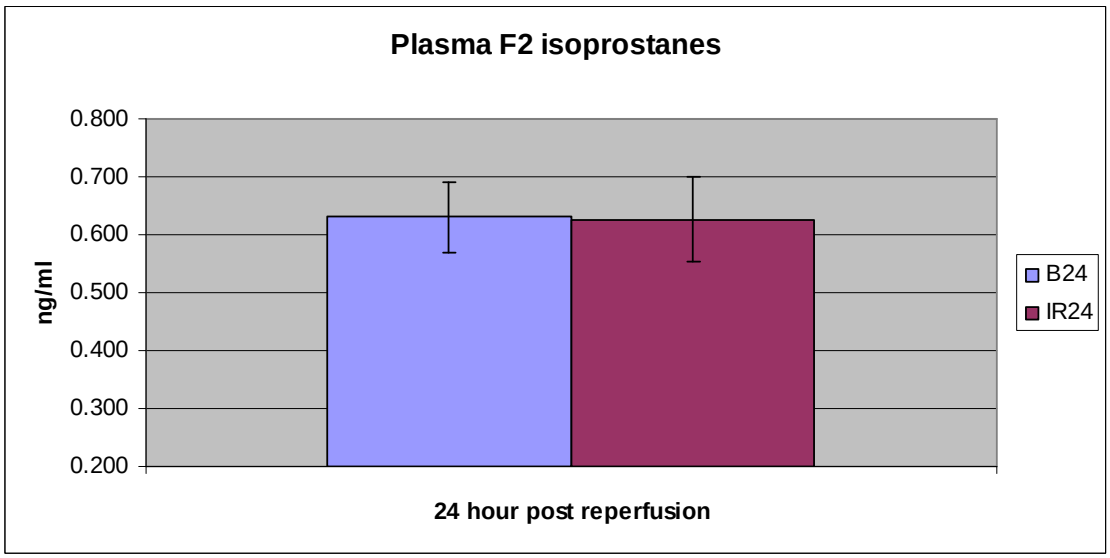


Figure: 7.6.4

Figure 7.6.3&4: Plasma F_2 isoprostane levels showing no significant difference between groups

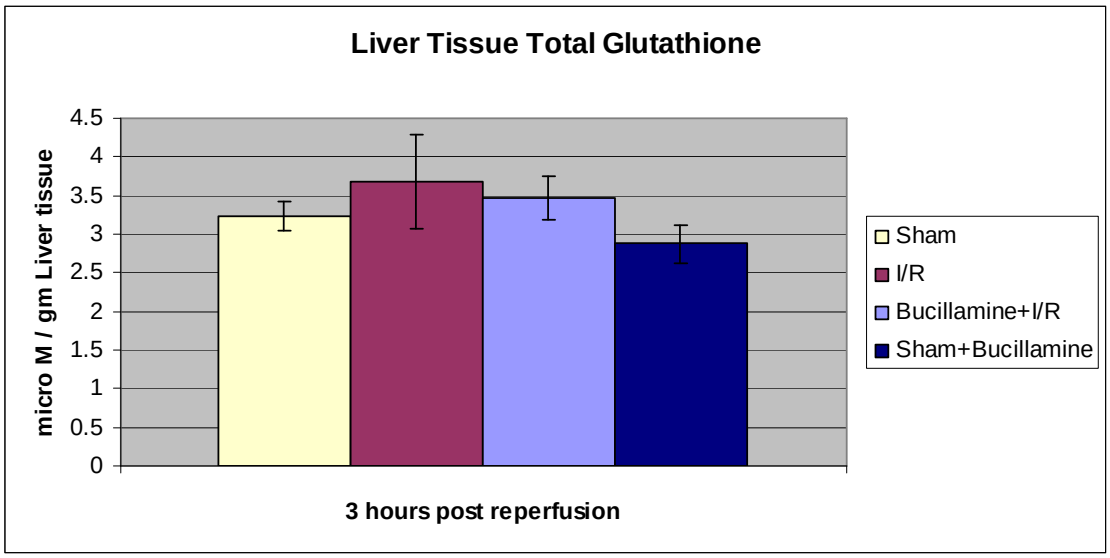


Figure 7.6.5

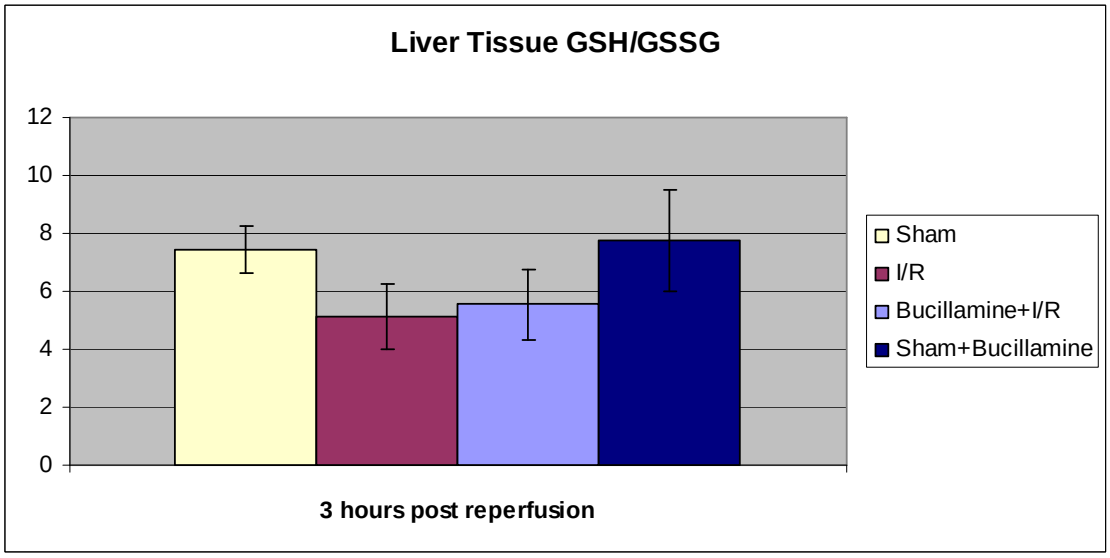


Figure 7.6.6

Figure 7.6.5&6: Liver Total Glutathione and GSH/GSSG levels following 45 minutes ischemia and 3 hours reperfusion showing no significant difference between groups.

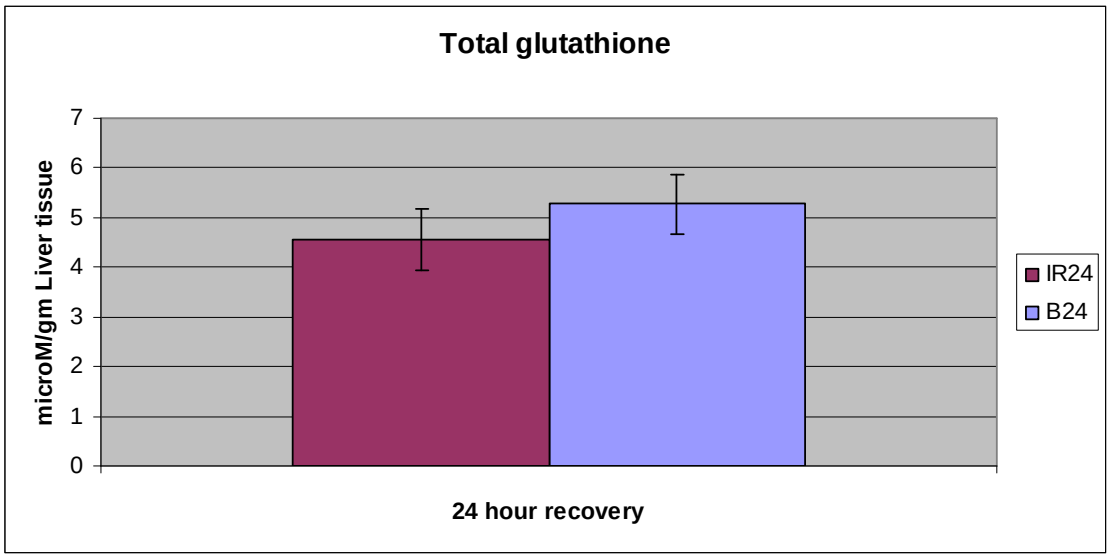


Figure 7.6.7

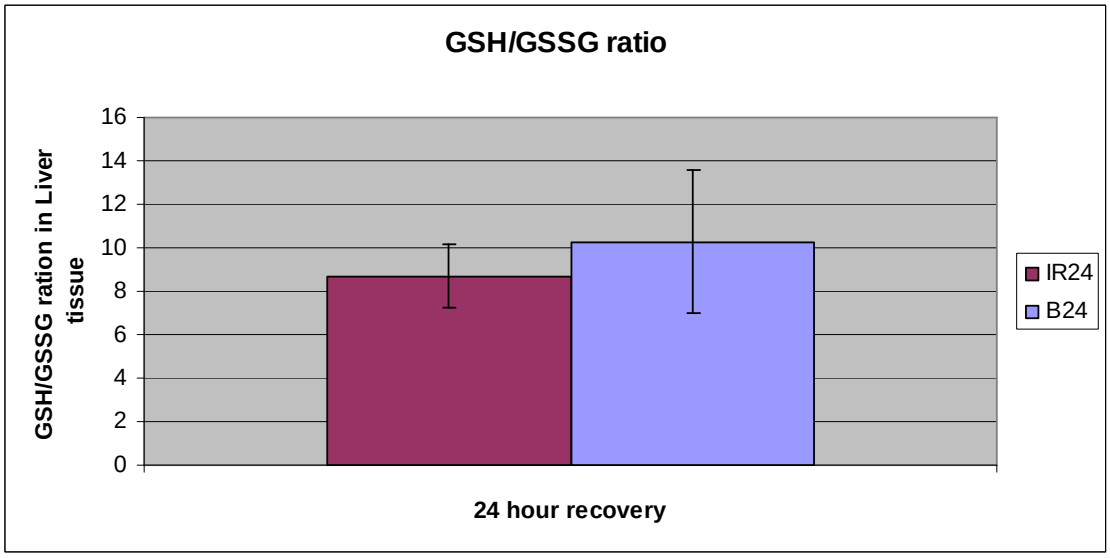


Figure 7.6.8

Figure 7.6.7& 8: Liver Total Glutathione and GSH/GSSG levels following 45 minutes ischemia and 24 hours reperfusion showing no significant difference between groups.

7.7 Discussion

7.7.1 F_2 Isoprostanes methodology:

.The previously published literature would suggest that F_2 isoprostanes could be used as a marker of oxidative stress in ischaemia reperfusion injury(Becker *et al.* 1998a; Gopaul *et al.* 2000; Idris *et al.* 2005; Mathews *et al.* 1994; Mehlhorn *et al.* 2003; Morrow and Roberts 1997; Richelle *et al.* 1999; Roberts and Morrow 2000; Xia *et al.* 2003). We have used a previously well described method to measure plasma and liver tissue F_2 isoprostane levels(Morrow and Roberts 1997). Errors could arise in measurement of F_2 isoprostanes due to generation of these in tissue by auto oxidation(Morrow and Roberts 1997). We took care to ensure that samples were snap frozen to -80^0 C and used immediately on thawing. Errors could arise during homogenisation of tissue, pipetting which were minimised by these being carried out by one individual.

7.7.2 Plasma F_2 isoprostanes, liver I/R and effect of Bucillamine infusion:

F_2 isoprostanes are present in very small amounts in free form plasma (in the range of 0.02 ng/ml)(Morrow *et al.* 1995) under normal circumstances. Over 90% of F_2 isoprostanes present in plasma are carried as lipid esters(Moore 2004). We measured esterified concentration of F_2 isoprostanes in the plasma. Our results showed no significant difference in plasma F_2 isoprostane levels in any of the groups. In the only previous study of liver I/R and F_2 isoprostane Mathews et al(Mathews *et al.* 1994) showed a significant rise in plasma F_2 isoprostane levels with a peak rise at I hour post reperfusion. In our experiments although in the IR

group there appears to be an increase in the levels as compared to the sham group, this was not significant. The half life of plasma F_2 isoprostanes in rats is also very short (about 20 min)(Moore 2004). Our results could be hence different due to different time of sampling.

7.7.3 Liver tissue F_2 isoprostanes, liver I/R and effect of Bucillamine infusion:

The levels of F2 isoprostanes in normal liver tissue are 1-10 pg/mg of wet tissue(Fernando and Mendis 2001; Harry *et al.* 1999) . This is the first study on liver I/R using liver tissue F_2 isoprostane levels. We did not find any increase in liver tissue F_2 isoprostane levels following I/R. Jaeschke et al had suggested that the molecular mechanism of ischaemia reperfusion injury does not seem to be due to lipid peroxidation(Jaeschke 2003b) and our results agree with this. There was no rise in hepatic F_2 isoprostanes which would confirm that lipid peroxidation does not play a primary role in ischaemia reperfusion injury. However, it is known that ischaemia reperfusion injury causes an increase in phospholipase levels(Caro and Cederbaum 2006). It is suggested that this up regulation of phospholipases might be resulting in an increased hydrolysis of F_2 isoprostanes which could keep the levels of F_2 isoprostane within normal range(Moore 2004). Hence, Moore et al have suggested that the timing of sample collection might be critical(Moore 2004). This would need to be proved in further studies using different time points. Alternatively, Phospholipase levels could be measured in future studies.

7.7.4 Liver tissue Glutathione methodology:

Liver tissue Glutathione was measured by modified Tietze method(Baker *et al.* 1990; Eyer and Podhradsky 1986; Tietze 1969) which utilizes a carefully optimized enzymatic recycling method, using glutathione reductase, for the quantification of GSH. GSH concentrations of the sample, was measured by the kinetic method as described in the protocol to avoid interference by other thiol groups. Variability in the assay can arise due to errors in homogenisation of tissue, pipetting and, dilution. These were minimised by using standard techniques of dilution, assays being done by the same person. Inter assay coefficient of variance is 3.6% and intra assay 1.6%. The dynamic range of the assay is 0-16 µM for GSH and 0-8 µM for GSSG.

7.7.5 Liver Tissue Glutathione, effect of I/R and effect of Bucillamine infusion:

In our study we did not find any statistically significant difference in any groups. In the only other stody on Bucillamine and Liver I/R in an *ex vivo* liver transplant Amersi et al found a significantly increased level of reduced glutathione (GSH) in the liver with a significantly decreased level of oxidized Glutathione (GSSG) in the liver and blood. Lipid peroxidation was also found to be significantly less in this model of cold ischaemia with 2 hour reperfusion(Amersi *et al.* 2002).These differences in results could be due to the different experimental models used. In the *ex vivo* model the effect of cold ischaemia was studied by storing the livers in

24 hour in UW solution(Amersi *et al.* 2002). Cold storage is known to decrease liver tissue glutathione levels(Sumimoto *et al.* 1996). The effect on liver tissue glutathione levels in warm ischaemia using *in vivo* models of liver partial ischaemia (*similar to ours*) is variable.

The difference in the result seems to be related to the duration of ischaemia and reperfusion used in these models. Some investigators – Stein et al (partial ischaemia 45 min and 45 min reperfusion)(Stein *et al.* 1991), Nagai et al (partial ischaemia 15 min and 60 min reperfusion)(Nagai *et al.* 1991), Ajamieh et al (partial ischaemia 90 min and 90 min reperfusion)(Ajamieh *et al.* 2004) , Caraceni et al (partial ischaemia 1 hr and 2 hr reperfusion)(Caraceni *et al.* 2005), Demir et al (partial ischaemia 30 min and 20 min reperfusion)(Demir and Inal-Erden 1998), Sener et al (partial ischaemia 45 min and 6 hr reperfusion)(Sener *et al.* 2003), Domenicali et al (partial ischaemia 1 hr and 2 hr reperfusion in fatty liver)(Domenicali *et al.* 2005), Duenschede et al (partial ischaemia and 1 hr reperfusion)(Duenschede *et al.* 2007), Dulundu et al (partial ischaemia 60 min and 60 min reperfusion)(Dulundu *et al.* 2007), Saavedra-Lopes et al (60 min ischaemia and 6hr reperfusion)(Saavedra-Lopes *et al.* 2008), have shown a decrease in liver tissue glutathione levels as compared to sham animals.

Whereas other investigators – Kobayashi et al (partial ischaemia 60 min and 60 min reperfusion)(Kobayashi *et al.* 1992), Schaeur et al (partial ischaemia, 60, 90 and reperfusion 120 min)(Schauer *et al.* 2004), Zumbado et al (75 min ischaemia and 1 hr reperfusion)(Zumbado *et al.* 2003), Accatino (30 min ischaemia and 1 hr, 6 hrs, 1 day, 3 days and 7 days reperfusion)(Accatino *et al.* 2003), have shown no difference in liver tissue glutathione levels.

It has been shown by Jaeschke et al that plasma levels of GSSG increased significantly during the reperfusion period(Jaeschke 1991). At the same time biliary efflux of glutathione was not enhanced and the increase in plasma GSSG was not affected by inhibition of glutathione reductase in the liver. Hepatocytes are known to release glutathione into sinusoids in the reduced form with an extracellular oxidation in the vasculature(Jaeschke 1991). They also showed that stimulation of Kupffer cells by complement pathway and other inflammatory mediators not only cased increased generation of ROS but also simultaneously increased the sinusoidal GSH efflux(Jaeschke 1992). Increase in plasma GSH and GSSG was seen as early as 15 minutes post reperfusion and although both plasma GSH and GSSG increased, GSH values increased by 3 to 6 fold whereas plasma GSSG increased by 9 to 27 fold as compared to pre ischaemic values(Jaeschke and Farhood 1991a).

Oxidation of GSH in vivo was also studied by giving a bolus dose of GSH after ligating blood vessels of both kidneys(Jaeschke and Farhood 1991a). The injection of GSH in sham animals showed a decrease in plasma GSSG, which recovered to a basal level of 24-27% of Total plasma Glutathione in 10-15 minutes. In contrast animals in the liver IR group showed significantly higher plasma GSSG levels which suggested that the high post ischaemic plasma levels of GSSG is not due to spontaneous oxidation of GSH in the plasma but reflects a higher extracellular oxidant stress. In another study Saito et al showed that the tissue ratio of GSH/GSSG decreased significantly in the ischaemic lobe at 60

minutes post ischaemia there was a fall in GSH/GSSG levels at 60 min post reperfusion which reached pre ischaemia levels by 180 min post reperfusion(Saito *et al.* 1999).

These studies might suggest that the fall in glutathione could be related to the length of ischaemic period (i.e. longer the ischaemic period more the decrease in glutathione levels) and that glutathione levels in the liver tissue might be getting replenished after a certain period of reperfusion. In a study using total liver ischaemia, Kirimlioglu et al (total ischaemia 20 min and 22 hr reperfusion) showed a significant fall in glutathione levels as compared to sham animals(Kirimlioglu *et al.* 2006). Our results do not support the hypothesis of replenishment of liver glutathione as the main mechanism of action of Bucillamine, however, further research could be carried out using a different model of warm ischaemia reperfusion injury before we could rule this out. Future studies could look at plasma GSSG and GSH levels in addition to the liver tissue GSH and GSSG levels. Furthermore, newer more accurate methods of GSSG and GSH levels in vivo could be used to avoid interference by Bucilamine and other thiol groups(Jones and Liang 2009; Shaik and Mehvar 2006).

Bucillamine has also been shown to stimulate glutathione synthesis(Wielandt *et al.* 2006). Our results however, do not suggest this to be the mechanism of action either. In a model of partial warm ischaemia reperfusion injury, Kurokawa et al showed a fall in total liver tissue glutathione at 60 min post reperfusion after 120

minutes of ischaemia. This was accompanied by a significant fall in mitochondrial GSH in the ischaemia period and a significant rise in mitochondrial GSH levels at 60 minutes reperfusion suggesting that endogenous GSH might be transported into mitochondrial matrix from the cytosol in the reperfusion period to compensate for the fall in mitochondrial GSH in the ischaemic period(Kurokawa *et al.* 1996). Further research into the mechanism of action of Bucillamine could also look at mitochondrial GSH levels.

7.8 Conclusion

This study shows that the hepato protective effect of Bucillamine in warm Liver ischaemia reperfusion injury is not by direct replenishment of Glutathione level. We have shown in the previous chapters the beneficial effect of Bucillamine and its effect on reducing leukocyte endothelial interaction. In the next chapter we investigate if this is related to any changes in cytokine activation which could explain its mechanism of action.

Chapter 8

Modulation of Neutrophil activation by Bucillamine induced inhibition of Cytokine-induced neutrophil chemoattractant in the early and late phase of hepatic IR.

Introduction:

IR injury is associated with increased venular and sinusoidal neutrophil adhesion seen by intravital microscopy and histologically in both the phases of hepatic IR. Bucillamine reduced neutrophil adhesion in both phases of hepatic IR and this was associated with preservation of liver cytoarchitecture and improved liver function and decreased apoptosis/ necrosis.

What is CINC-1?

Cytokine induced neutrophil chemoattractant (CINC-1) is an 8kd polypeptide that was originally identified in the conditioned media of IL-1β- stimulated rat glomerular epithelial cells(Watanabe *et al.* 1989a). It consists of 72 amino acids having similar amino acid sequence to human peptides with gro/melanoma growth stimulatory activities, indicating that rat CINC belongs to the IL-8 family of cytokines(Watanabe *et al.* 1989b; Watanabe *et al.* 1992). Cells known to express CINC-1 include hepatocytes(Planaguma *et al.* 2002; Spitzer and Zhang 1996), kupffer cells(Hisama *et al.* 1996; Spitzer and Zhang 1996), macrophages(al-Mokdad *et al.* 1998; Hisama *et al.* 1996; Mitsui *et al.* 2002; Shibata *et al.* 1998), mast cells(Ramos *et al.* 2003), neurons(Sakamoto *et al.* 1996), fibroblasts(Huang *et al.* 1992), type II greater alveolar cells(Crippen *et al.* 1995) and cardiac muscles(Seino *et al.* 1995).

CINC-1 is a major neutrophil chemoattractant and activator(al-Mokdad *et al.* 1998; Nakagawa *et al.* 1994; Ramos *et al.* 2003; Shibata *et al.* 2000; Suzuki *et*

al. 1994). CINC-1 is induced by IL-1β, TNF-α and bacterial products and promotes both neutrophil rolling and adhesion, most probably through upregulation of surface integrins(Aoki *et al.* 1997; Davenpeck *et al.* 1998; Dolecki and Delarco 1994; Mitsui *et al.* 2002; Nakagawa *et al.* 1994; Suzuki *et al.* 1994). It is also reported to stimulate neutrophil activity by promoting cathepsin G release from azurophilic granules(Shibata *et al.* 2000). Relative to CINC-2 and CINC-3, CINC-1 seems to be equal in chemotactic activity but less efficient in inducing calcium mobilization. It is also induced earlier in macrophages than CINC-2 and -3 and declines more quickly in expression(Shibata *et al.* 2000). The significance of this is unclear.

Hisama et al have shown in an ischaemia reperfusion model of the liver an increase in serum CINC-1 levels which peaked at 6 hours post reperfusion and gradually came down to normal levels after 24 hours(Hisama *et al.* 1996). They also showed that CINC production has at least two factors involved: 1. activation of coagulation system and 2. Kupffer cell activation(Hisama *et al.* 1996). Thrombin and Factor Xa have been shown to enhance production of CINC in liver ischaemia reperfusion injury(Yamaguchi *et al.* 2000). Production of CINC by Kupffer cells has been shown to be attenuated by calcium channel blockers(Liang *et al.* 2000). It has also been shown to be attenuated by antithrombin presumably by increased production of PGI_2 by endothelial cells(Harada *et al.* 1999). CINC production has also been shown to be attenuated by xanthine oxidase inhibition(Matsumura *et al.* 1998).

Aim of this study

The aim of this study was to investigate the effects of Bucillamine on CINC levels in hepatic IR and correlate with microcirculatory data from the previous chapters. In this study the effect of Bucillamine on the inflammatory response of I/R has been evaluated. Measurement of CINC-1 has been used to determine cytokine activation. We measured neutrophil adhesion with sinusoids by intravital microscopy as a measure of leukocyte-endothelial interaction and inflammatory cell infiltration.

8.2 Materials and Methods:

Animals and surgical procedures

As detailed in the chapter on methodology, page 50.

Experimental groups (n=6 in each group)

Group1 - (Sham) – laparotomy and mobilization of liver but no occlusion of the vascular pedicle.

Group2- IR- 45 minutes of partial hepatic ischemia followed by 3 hrs of reperfusion.

Group3- Bucillamine 15mg/ kg/hr intravenously + IR followed by 3 hrs of reperfusion.

Group4- (SB) - Sham + Bucillamine infusion 15mg/kg/hr (SB)

Group5- IR24- 45 minutes ischemia followed by 24 hour reperfusion

Group6- B24- 45 minutes ischaemia + Bucillamine 15mg/ kg/hr intravenously followed by 24 hour reperfusion

CINC Elisa

Elisa for quantification of CINC was done as described in chapter 3, page 73.

Intravital microscopy

As described in chapter 3, page 54.

8.3 Data collection and statistics

All the data is expressed as mean± standard error of mean (SEM). Analysis of data was done using SPSS 14.0.Differences in data between groups was assessed by using one way ANOVA with Bonferroni's post hoc test. Data was considered statistically significant if $p< 0.05$.

8.4 Results

There were no procedure related deaths in either group. The model was haemodynamically stable.

Effect of IR on CINC-1 in the early and late phase of hepatic IR

Hepatic IR injury produced a high serum CINC level in comparison to sham animals in the early phase of hepatic IR (2247.67 ± 237.86 vs. 5936.3 ± 727.06). The serum CINC levels were significantly high in the late phase of hepatic IR (15306± 1222.04). (Fig 8.1, 8.2)

Effect of Bucillamine on CINC-1 in the early and late phase of hepatic IR

Bucillamine reduced CINC -1 level in both the early (3409.73 ± 755,2, ns) and late phase (183.95 ± 28.44, $p< 0.001$) of hepatic IR in comparison to IR only.

Correlation of CINC-1 with intravital findings on neutrophil adhesion

I/R produced a significant increase in the number of adherent leukocytes in the venules at the end of 180 minutes of reperfusion between the I/R and sham groups (769.05±87.481 vs. 195.79±90.79 leukocytes per mm^2, $p<0.05$). Bucillamine group showed less number of adherent leukocytes (385.66±142.69) however, there was no statistically significant difference with either the IR or sham groups (Figure 4.4.3.5.1). I/R group (97.4±7.49) showed a significant higher number of adherent leukocytes in the sinusoids as compared to

sham(3.75±3.75), SB (22.48±11.46) and bucillamine I/R groups(29.97±13.81) ($p<0.005$). (Figure 4.4.3.5.2)

In the recovery group there were significantly lesser number of adherent leukocytes in the venules in the Bucillamine group (B-24) as compared to I/R (IR-24)group (217.33±50.15 vs. 737.62±132.52, $p < 0.01$) (Figure 6.9) I/R (IR-24) group (176.92±48.68) showed a significant higher number of adherent leukocytes in the sinusoids as compared to bucillamine (B-24)group(43.09±8.42) ($p<0.05$). (Figure 6.10)

Correlation of Hepatocellular death with CINC-1

Increased hepatocellular death on propidium iodide staining was observed in IR injury in both phases of hepatic IR in comparison to animals in the Bucillamine infusion group (Chapter 4 and 5).

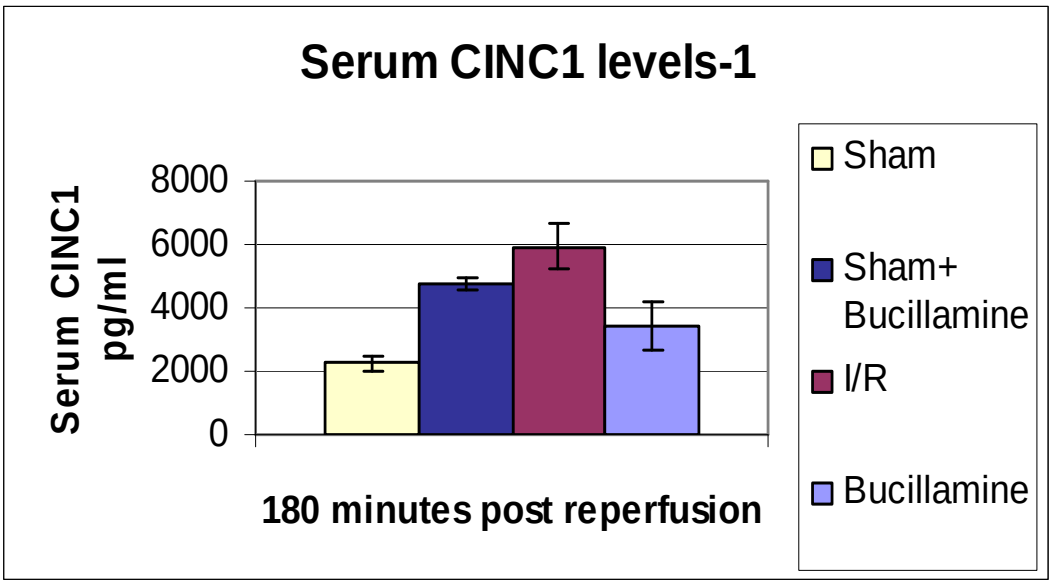


Figure 8.1 Modulation of CINC in early phase of hepatic IR

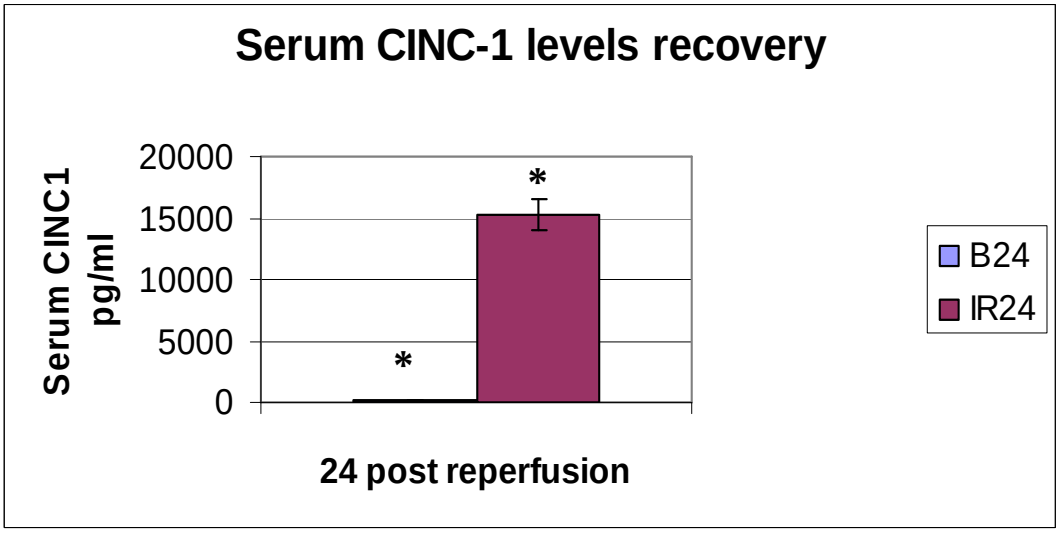


Figure 8.2 Modulation of CINC in late phase of hepatic IR*p<0.001

8.5 Discussion

Assay, controls and variability

If samples generate values higher than the highest standard, samples were further diluted with the Calibrator Diluent and the assay was repeated. Any variation in operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding. This assay is designed to eliminate interference by soluble receptors, binding proteins and other factors present in biological samples.

Reliability of technique and validity cross reactions, sensitivity and specificity.

Twelve assays were evaluated and the minimum detectable dose (MDD) of rat CINC-1 ranged from 0.7 - 1.3 pg/mL. The mean MDD was 1.1 pg/mL.
The MDD was determined by adding two standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration. This assay recognizes both recombinant and natural rat CINC-1. Several factors were prepared at 50 ng/mL in Calibrator Diluent RD5-4 and assayed for cross-reactivity. No significant cross-reactivity or interference was observed

Findings in this study

Serum CINC-1 levels in hepatic IRI and correlation with intravital findings of neutrophil adhesion and hepatocellular death.

The serum CINC-1 levels in the early phase of hepatic IR were higher compared to sham animals. Liang et al have shown that CINC transcript expression in the liver peaks at 3 hours following hepatic IR and serum CINC levels peak at about 6 hours(Liang *et al.* 2000). Oxidative stress due to ROS results in increased NF-κB production and NF-κB stimulates CINC production by kupffer cells. CINC-1 is known to increase neutrophil infiltration which can be reduced by giving anti CINC-1 antibody as shown by Hisama et al. In this study increased CINC levels correlate with increased neutrophil adhesion and hepatocellular death in the early and late phase of hepatic IR.

Effect of Bucillamine on CINC levels in hepatic IR and correlation with intravital findings of neutrophil adhesion, hepatocellular death (early and late phase).

Bucillamine reduced CINC-1 in the early phase of hepatic IR and in the late phase of hepatic IR (24hrs). The serum CINC-1 levels correlate with reduced neutrophil adhesion and hepatocellular death. This suggests that the beneficial effect of Bucillamine could be through modulation of CINC-1 and neutrophil activation in hepatic IR. This needs to be clarified in future studies.

Conclusion:

This study shows that the hepato protective effect of Bucillamine in warm Liver ischemia reperfusion injury could be through the modulation of CINC-1 levels and the resultant decreased neutrophil activation and recruitment.

Chapter 9

Discussion of the Thesis

9.1 Chapter 1 Introduction

This chapter was a review of the published literature on the mechanism of Liver ischaemia reperfusion injury highlighting the changes occurring in the early and late phase of Liver ischaemia reperfusion injury. The central role of reactive oxygen species in IR, the role of nitric oxide and the interplay between the antioxidant protective mechanisms and oxidative stress was discussed. Both intra and extracellular defence mechanisms are important in protection against IR. Reactive oxygen species (ROS) have a central role to play in Ischaemia – Reperfusion injury (IR)(Waxman 1996).

ROS activate cytokines, macrophages and other components of the inflammatory pathway(Entman *et al.* 1991; Jaeschke and Farhood 1991a; Le *et al.* 1997). When generated in large amounts they can also cause direct oxidative damage to the cells through iron mediated reactions(Horwitz *et al.* 1998). Thiol donors are antioxidants which can interrupt redox signalling pathway and thereby reduce cytokine and macrophage activation(Sano *et al.* 2001). In addition, thiol donors can protect against oxidative injury by replenishing intracellular glutathione and other endogenous thiol compounds(Ceconi *et al.* 1988a).

9.2 Chapter 2 Bucillamine

In this chapter we have given an introduction of Bucillamine, its pharmacological properties, its uses and adverse effects and the current available literature on the effect of Bucillamine in IR injury.

Bucillamine is a low molecular weight thiol donor that is capable of rapidly entering cells. As an oral formulation it is marketed in Japan and Korea for the treatment of rheumatoid arthritis(Matsuno *et al.* 1998).

Similar to other cysteine derivatives such as N- acetylcysteine (NAC), bucillamine has the ability to replenish intracellular GSH(Amersi *et al.* 2002). These compounds can directly scavenge peroxides, but less efficiently than the glutathione/ glutathione peroxidase system. Bucillamine preserves a high concentration of oxidized glutathione, which may be its primary action.

Bucillamine has two donatable thiol groups and is fourfold more potent than NAC in preventing ischaemia reperfusion injury in *in vitro* studies(Horwitz and Sherman 2001) and 16-fold more potent *in vivo* study(Whitekus *et al.* 2002). Bucillamine is metabolised to SA 969 which has one donatable thiol group and SA 981 which has a structural similarity to D- Penicillamine and ha a immunomodulating effect.

Bucillamine has undergone some preliminary investigations in experimental studies of IR. In cardiac I/R injury Horwitz and Sherman(Horwitz and Sherman 2001) demonstrated in isolated rat cardiac myocytes that bucillamine is a potent antioxidant. Bucillamine (125-500 microM) prevented lactate dehydrogenase (LDH) release in cardiac myocytes exposed to hydrogen peroxide or a

xanthine/xanthine oxidase system. Further, in dogs subjected to 90 min of coronary artery occlusion and 48 h of reperfusion, bucillamine, administered during reperfusion decreased myocardial infarct size by 41%(Amersi *et al.* 2002).

In the only other study of effect of Bucillamine in Liver IR injury, Amersi and colleagues(Amersi *et al.* 2002) studied Bucillamine and I/R in a rat *ex vivo* liver transplantation model. Bucillamine decreased liver IR injury with increased levels of GSH in the liver and decreased levels of oxidized glutathione in both the liver and blood. Thus on the basis of these ex vivo experiments replenishment of glutathione was thought to be the mechanism of action. Bucillamine was also shown to improve survival in a separate group of rat liver transplantations.

The study by Amersi et al suffers from few criticisms. No studies trying to confirm mechanism of action were performed in the animals that underwent the transplants. Furthermore, cold storage (as in their *ex vivo* model) is known to decrease liver tissue glutathione levels(Sumimoto *et al.* 1996). The dose of Bucillamine used was also much higher than the dose used in our study (10 mg intraportally at the time of liver retrieval and 90 mg in 90 ml of blood perfusate). Furthermore, added thiols cause interference in estimation of GSH levels and no mention is made in the study about what precautions were taken to prevent this and what method (end point/ kinetic method) was used for estimation of GSH levels. The effect on liver tissue glutathione levels in warm ischaemia using *in*

vivo models of liver partial ischaemia (similar *to ours*) is variable (as discussed in Chapter 7).

From previous studies bucillamine seems to be an agent which could be used to reduce liver IR injury. The effects of bucillamine in the treatment of liver warm I/R injury has not been investigated before, furthermore its effect on liver microcirculation was not known. The mechanism of action has also not been fully studied. This formed the basis of studying the effect of Bucillamine in warm liver IR.

9.3 Chapter 3 Methodology

Adequacy of model

The model of partial liver ischaemia and reperfusion injury used in this study is stable, reproducible and avoids splanchnic congestion found with total liver inflow occlusion(Koti *et al.* 2005). Technical manoeuvres such as performing the laparotomy; mobilisation of the liver and performing intravital microscopy did not affect the heart rate, oxygen saturation or mean arterial pressure as shown by stable parameters in the sham and the SB groups. There was a fall in mean arterial pressure following ischaemia reperfusion injury in both the I/R and Bucillamine I/R groups which was not statistically significant. Bucillamine administration without IR has no effect on the vital parameters or haemodynamic stability of the animals suggesting that it has no direct effect on systemic or portal

haemodynamics. Liver I/R is known to cause microcirculatory perfusion failure, activate polymorphonuclear leukocytes and increase leukocyte-endothelial cell interaction which in turn contribute to hepatocellular damage and liver dysfunction(Vollmar *et al.* 1994a; Vollmar *et al.* 1994c; Vollmar *et al.* 1995b; Vollmar *et al.* 1996; Menger *et al.* 1999). We hence used intravital microscopy to study the microcirculatory changes in IR and the effect of Bucillamine.

Transplant vs. partial hepatic IR model, global IR vs. lobar IR.

Since this study was limited to investigating the effects of warm IR and the effects of Bucillamine in warm IR a partial hepatic IR model was chosen as against a transplant model. Global hepatic IR would result in congestion of intestine and release of cytokines resulting in confounding results. To avoid this, a portosystemic shunt is needed in a global IR model. This was another reason to use a partial ischaemia reperfusion model in our study. Partial liver ischaemia avoids the intestinal congestion and resulting release of cytokines(Koti *et al.* 2005). It is a stable model and previously well described(Koti *et al.* 2005).

Correlation of the model with other animal models and human IR

The rat was selected as the experimental animal due to its resistance to surgical trauma and infection, the advantage of size and the liver microvascular anatomy which makes the technical procedure possible, compared with other laboratory animals; the availability and the relative economy of maintenance. Sprague

Dawley is a very docile out bred albino rat (Rattus norvergicus), originated in Madison, Wisconsin in 1925, by R. Dawley. The original colony was closed shortly after its development and no new stock has been introduced since then, producing stable colonies which are descended directly from the original stock. The rat model of hepatic ischaemia and reperfusion is a useful procedure for the study of the local and systemic effects of ischaemia and reperfusion injury. This model offers a well defined volume of tissue that can be easily rendered ischaemic with no significant alteration of systemic haemodynamics.

In vivo fluorescent microscopy (Intra vital Microscopy)

In vivo microscopy of the liver allows assessment of the hepatic microvascular perfusion, the analysis of dynamic processes such as changes in diameters of blood vessels, interactions between leukocytes and endothelium.

For the purposes of in vivo microscopic assessments, a plane organ surface is necessary to provide clear and sharp images without inducing trauma to the tissue under the objective. The anaesthetized animals were placed on the stage of a Nikon custom built microscope (Nikon, Japan) with an integrated heating system where the temperature was maintained at 37˚C. The whole set up was placed on a pneumatic vibration isolation workstation (Newport, USA) to minimise vibration. The liver was exteriorised by adequate mobilisation and division of the hepatic ligaments and mounted on a plane glass surface. The liver was continuously irrigated with saline to wet the surface of the liver and prevent drying as dryness of the liver would aggravate hepatic ischaemia. This procedure

was easily tolerated by the animals and allowed the tissue to be exteriorised with minimum trauma. This method of exteriorisation of the liver also eliminated the respiratory movements in the tissue. Liver microcirculation could be affected by exteriorisation and handling of the liver and great care and precaution was taken in performing the manoeuvre. During the experiments, the animal's abdomen was covered with a plastic wrap (Saran wrap®, Dow Chemical, Michigan, USA) to prevent fluid evaporation. The liver was carefully handled, as mechanical trauma induces disturbances in microcirculation. In order to obtain clear images, it was necessary to match the concentration of fluorescent dyes with the filter systems, the light intensity and the magnification. Off line microcirculatory analysis was performed from recorded images to measure RBC velocity and sinusoidal diameter using Lucia G software (Laboratory Universal Computer Image Analysis, Nikon, Tokyo, Japan). This software allows measurement of length and area seen in the image, which allows the parameters studied to be measured. Intravital microscopy has the advantage of allowing changes to be visualised in real time *in vivo*. It however, gives only a 2 dimensional view of what is essentially 3 dimensional liver anatomy, this however, is the limitation of the equipment currently available. Only peripheral tissue upto a limited depth can be visualised. Furthermore, liver mobilisation and handling is required which in itself could contribute to liver injury.

Serum liver enzymes

Blood levels of intracellular enzymes are a way of estimating tissue damage: tissue or organ specific enzyme levels provide valuable information about related tissues. Transaminases released from the damaged liver during ischaemia enter the circulation during reperfusion and their blood levels increase. ALT and AST are indicators of major alterations of liver integrity, and were utilized in this study as a marker of hepatocellular injury. Measurement of liver enzymes as surrogate markers of liver injury has its limitations. They do not give any indication of sub-lethal cell injury. They also do not provide any information about the mechanism of injury and there is poor correlation with ability for liver to recover.

9.4 Chapter 4- The effect of Bucillamine infusion on microvascular changes in liver warm IR

Intravital microscopy has allowed novel insights into hepatic haemodynamics following I/R and the effect of Bucillamine administration. Liver I/R is known to cause microcirculatory perfusion failure, activate polymorphonuclear leukocytes and increase leukocyte-endothelial cell interaction which in turn contribute to hepatocellular damage and liver dysfunction(Vollmar *et al.* 1994a; Vollmar *et al.* 1994c; Vollmar *et al.* 1995b; Vollmar *et al.* 1996; Menger *et al.* 1999). Bucillamine administration with I/R was shown to maintain RBC velocity, sinusoidal blood flow and sinusoidal perfusion in the reperfusion period. It has

also shown to decrease leukocyte adhesions in venules and decrease hepatocyte apoptosis/ necrosis. After absorption Bucillamine enters the RBCs rapidly and is carried within the erythrocytes(Horwitz 2003; Sugawara *et al.* 1985a; Sugawara *et al.* 1985b). The effect of bucillamine on RBC velocity may be secondary to its rapid uptake by RBCs which may reduce oxygen free radical mediated deformability and aggregation(Baskurt *et al.* 1998; Cabel *et al.* 1997; Lowe GDO and Barbanel JC 1988; Machiedo *et al.* 1989; Powell *et al.* 1989).

This might be of importance in the beneficial effect of Bucillamine. Bucillamine was shown to reduce the perfusion abnormality of I/R with a significantly increased sinusoidal perfusion. This could be related to its effect on- 1. RBCs (decreased aggregability or better maintenance of deformability) and /or 2. WBC adhesions; 3. Decreased platelet aggregability/ fibrin deposition. Sinusoidal perfusion was also better maintained in the SB group, which although, as mentioned earlier had lower RBC velocity, had normal liver function, indicating that a combination of better perfusion and maintenance of RBC velocity would decrease abnormality in liver function. Furthermore, Bucillamine was also shown to reduce apoptosis/necrosis following Liver IR. Apoptosis of sinusoidal endothelial cells following liver IR could give rise to decreased sinusoidal perfusion due to endothelial cellular congestion(Vollmar *et al.* 1996). Bucillamine seems to attenuate these changes.

We were unable to measure the portal venous flow simultaneously as this would interfere with the Intravital microscopy equipment set up. Further manipulation of the liver for setting up venous Doppler or laser Doppler also would have affected the liver microcirculation. Hence we could not correlate our findings with changes in portal venous and hepatic arterial haemodynamics. Bucillamine enters erythrocytes rapidly and hence precise measurement of bioavailability requires whole blood measurements. This can be done using high performance- liquid chromatography- mass spectrometry technique(Beaudry *et al.* 2004). Unfortunately we could not perform whole blood Bucillamine measurement and this could be undertaken in future studies. Further studies could also concentrate on changes in blood viscosity and RBC deformability by Bucillamine.

9.5 Chapter 5- Effect of Bucillamine infusion on Bax/ Bcl-2 and histological changes seen after liver IR.

In Chapter 4 it was shown that Bucillamine administration can reduce the microcirculatory failure associated with severe IR and that this results in a reduced hepatocellular injury as indicated by lowered serum transaminases. However the mechanism was not investigated. In this chapter the histology of the resected livers was examined in detail with particular regard to the degree of hepatocyte necrosis or apoptosis.

Histology

Light microscopy examination allows excellent appraisal of degree of tissue injury. In the liver the end points of necrosis, vacuolation, sinusoidal congestion and neutrophil infiltration were chosen as histological markers, realizing that more detailed ultrastructural studies may be useful either to confirm or to explain histological findings in some cases. In this study, clear differences were found between groups, and histology was of great help to understand the nature of ischaemic injury. An objective scoring system using modified Suzuki's criteria was used to assess histological changes in the early phase of hepatic IR. Bucillamine infusion during ischaemia reperfusion also showed a protective effect as seen on histology.

In the early phase of liver IR changes seen on gross haematoxylin and eosin staining are subtle and difficult to quantify in descriptive terms only. Furthermore there could be observer variation. All samples being assessed by a Consultant Pathologist who was blinded to the sample allocation. Other investigators have used degree of necrosis as the only histological parameter(Noh *et al.* 2006) however, the severity of necrosis seen at 3 hours reperfusion is not as marked with more marked changes seen at 24 hours following reperfusion. All scoring systems that are used however, suffer from the fact that they are semi quantitative in nature and there could be interobserver variations. Furthermore there could be sampling errors in spite of best precautions.

In view of our histological findings we measured the level of the Bcl-2 gene family which are known to have a vital role in programmed cell death. Liver IR is known to increase the expression of the proapoptotic Bax gene(Bailly-Maitre *et al.* 2006; Ben-Ari *et al.* 2007; Ishigami *et al.* 2001) and decrease the expression of anti apoptotic Bcl-2 gene. In this study apoptotic gene expression was measured in a rat hepatic IR model, which has shown for the first time a decreased expression of the proapoptotic Bax gene and increased expression of Bcl-2 gene after Bucillamine infusion in Liver IR. These findings co-related with our earlier observation of increased non viable nuclei with propidium iodide on intra vital microscopy and its attenuation by Bucillamine infusion.

9.6 Chapter 6- The effect of Bucillamine infusion on late phase of liver IR

The introductory chapter has highlighted the previous studies which have demonstrated a two phase response to IR injury with an early phase characterised by Kupffer cell activation, activation of complement cascade and production of C5a by proteolytic cleavage (Jaeschke *et al.* 1994) and generation of reactive oxygen species (ROS) (Jaeschke and Farhood 1991a; Bailey and Reinke 2000; Liu *et al.* 1995; Shiratori *et al.* 1994), and a late phase characterised by influx of polymorpho nuclear cells and organ injury. Drugs influencing the early phase of IR may not necessarily have a prolonged effect into the late phase or indeed influence the long term viability of the liver.
A late phase IR model was therefore established.

Our results show a beneficial effect with Bucillamine in the late phase even after stopping of continuous infusion after 3 hours. The sinusoidal neutrophil adhesion and postsinusoidal venular neutrophil adhesion in the late phase of hepatic IR was significantly more in comparison to the Bucillamine + IR group. The direct marker of liver injury as assessed by necrotic/apoptotic nuclei stained with propidium iodide showed a significant decrease with Bucillamine infusion. Histological findings in this study also demonstrated increased parenchymal necrosis and neutrophilic infiltration in the IR injury group at 24 hours as compared to the Bucillamine group.

Our model unfortunately necessitates a second general anaesthetic and relaparotomy which could contribute to the liver injury. Intravital microscopy was not possible in one animal in the Bucillamine +IR group at 24 hr reperfusion on account of oedema and adhesions making it impossible to mobilise the left lobe of liver onto the slide. It was also impossible to perform intravital microscopy in one animal in the IR only group on account of severe necrosis. Histological changes at 24 hours reperfusion are also more marked and Suzuki's score does not give objective score for comparison.

9.7 Chapter 7- The effect of Bucillamine on oxidant stress: is mediated by glutathione replenishment?

In the only other study of Bucillamine in liver IR in an *ex vivo* liver transplant model Amersi et al found a significantly increased level of reduced glutathione (GSH) in the liver with a significantly decreased level of oxidised Glutathione (GSSG) in the liver and blood(Amersi *et al.* 2002). In view of these findings they concluded that Bucillamine could be acting by replenishing the tissue reduced glutathione levels. The effect on liver tissue glutathione levels in warm ischaemia using *in vivo* models of partial liver ischaemia (*similar to ours*) is variable. This variability could be due to different duration of ischaemia and the different time after reperfusion studied in the various studies as discussed in Chapter 7. Our study shows no significant decrease in glutathione levels at 45 minutes ischaemia followed by 3 hour and 24 hour reperfusion. Our results hence do not support the hypothesis of replenishment of liver glutathione as the main mechanism of action; however, further research could be carried out using a different model of warm ischaemia reperfusion injury before we could rule this out.

Bucillamine has also been shown to stimulate glutathione synthesis(Wielandt *et al.* 2006). Our results however, do not suggest this to be the mechanism of action either. In a model of partial warm ischaemia reperfusion injury, further research could also look at the mitochondrial GSH levels to investigate the mechanism of action.

In this study oxidant stress in tissues and plasma was measured by measuring F_2 isoprostane levels. F_2 isoprostanes measurement is the most accurate method of measuring lipid peroxidation(Roberts and Morrow 2000). This is the first study on liver I/R using liver tissue F_2 isoprostane levels. We did not find any increase in liver tissue F_2 isoprostane levels following I/R. Jaeschke et al had suggested that the molecular mechanism of ischaemia reperfusion injury does not seem to be due to lipid peroxidation(Jaeschke 2003b) and our results agree with this. There was no rise in hepatic F_2 isoprostanes which would confirm that lipid peroxidation does not play a primary role in ischaemia reperfusion injury. However, it is known that ischaemia reperfusion injury causes an increase in phospholipase levels(Caro and Cederbaum 2006). It is suggested that this up regulation of phospholipases might resulting in an increased hydrolysis of F_2 isoprostanes which could keep the levels of F_2 isoprostane within normal range(Moore 2004). Hence, Moore et al have suggested that the timing of sample collection might be critical(Moore 2004). This would need to be proved in further studies using different time points.

We also studied plasma F_2 isoprostane levels. Our results showed no significant difference in plasma F_2 isoprostane levels in any of the groups. In the only previous study of liver I/R and F_2 isoprostane Mathews et al(Mathews *et al.* 1994) showed a significant rise in plasma F_2 isoprostane levels with a peak rise at I hour post reperfusion. In our experiments although in the IR group there appears

to be an increase in the levels as compared to the sham group, this was not significant. The half life of plasma F_2 isoprostanes in rats is also very short (about 20 min)(Moore 2004). Our results could be hence different due to different time of sampling.

9.8 Chapter 8- Cytokine studies (CINC-1 levels)

In Chapter 7 we showed that the hepato protective effect of Bucillamine in warm Liver ischaemia reperfusion injury is not by direct replenishment of Glutathione level. We have shown in the study previous chapters the beneficial effect of Bucillamine and its effect on reducing leukocyte endothelial interaction. In this study we investigated if this is related to any changes in cytokine activation which could explain its mechanism of action.

Cytokine induced neutrophil chemoattractant (CINC-1) which is secreted by kupffer cells induces activation of neutrophils in IR injury. CINC belongs to the IL-8 cytokine family which mediates the recruitment of neutrophils into sites of inflammation. Quantification of CINC-1 by Elisa is a well described technique (Nakagawa et al). A sensitive enzyme–linked immunosorbent assay for rat CINC using biotin-conjugated anti-CINC rabbit immunoglobulin has been established. The biotin-streptavidin sandwich enzyme-linked immunosorbent assay detects CINC at concentrations of 3 pg/ml to 30ng/ml. Bucillamine reduced CINC-1 levels in the early phase of hepatic IR and in the late phase of hepatic IR (24hrs). The

serum CINC-1 levels correlate with reduced neutrophil adhesion and hepatocellular death. This suggests that the beneficial effect of Bucillamine could be through modulation of CINC-1 and neutrophil activation in hepatic IR.

9.9 Overall conclusion and Future studies

This study has shown for the first time that Bucillamine can reduce the effects of liver warm ischaemia reperfusion injury. This is also the first study showing effect of Bucillamine on liver microcirculation in vivo. The protective effect of Bucillamine was also seen at late phase of reperfusion injury. This study also showed that Bucillamine up regulates Bcl-2, and down regulates the expression of Bax gene, this is accompanied by decreased leukocyte endothelial interaction seen in liver sinusoids and post sinusoidal venules, decreased levels of CINC-1 levels and better preservation of the liver cytoarchitecture. We also showed that the mechanism of action might not be related to replenishment of glutathione levels; however, further studies could be carried out using a different model and different time points to assess this further.

Our study however, is open to criticism. We used a model of partial ischaemia and reperfusion for the reasons given (page 185). A model of total ischaemia reperfusion however, mimics the conditions encountered in clinical practice and further studies could be carried out using this model. As this was an experimental study we had not performed a sample size calculation and hence there is a

likelihood of Type II errors and using more animals in the experiments might have given us statistically significant results which should be borne in mind when performing further studies. We also did not have a sham group and a sham + Bucillamine group in our recovery experiments.

We were also unable to perform Laser Doppler flow measurements or direct portal flow measurements as this would have interfered with our set up of Intravital microscopy. Our data of nonviable nuclei, etc could have been measured more objectively by measuring the fluorescence, which unfortunately could not be done due to the limitations of the available software (LUCIA G). We would have liked to study ultrastructurally changes with electron microscopy to correlate with the Intravital microscopy findings.

Although we showed that the protective effect of Bucillamine was preserved in the late phase by Intravital microscopy and histology there was no difference in the liver enzymes which suggests the possibility that Bucillamine might be delaying the inflammatory response rather than reducing it and studies at later time points could be undertaken to investigate this further.

Further studies looking at the mechanism of action of Bucillamine could look at its effect on mitochondrial redox potential. Using Intravital microscopy Sun et al measured the fluorescence intensity of Rhodamine(Rh) 123 as a marker of mitochondrial redox potential and showed that a change in mitochondrial

membrane potential is the key mechanism in early warm liver IRI(Sun *et al.* 2005). A statistically significant fall in fluorescence in Rh123 was seen as early as 15 minutes post reperfusion which correlated with liver injury markers. This model could be used for assessing the effect of Bucillamine. NADH autofluorescence also has be used as a measure of mitochondrial redox state(Chance *et al.* 1962). Using oxygen sensitive fluorescent dye tris(1,10-phenanthroline)ruthenium(II) chloride hydrate [$Ru(phen)_3^{2+}$], the activity of which is directly dependent on the tissue PO_2, is another approach to study the metabolic state of liver using Intravital microscopy(Vollmar and Menger 2009). Changes in the levels of tissue and serum glutathione and F_2 isoprostanes could also be looked at in the study at earlier time points (for e.g 15 min., 30 min and 60 min. post reperfusion). Alternatively, mitochondrial GSH and tissue phospholipases levels as marker of lipid peroxidation could be looked at. Inflammatory process and its modulation also affects liver regeneration(Devey *et al.* 2009; Jaeschke 2006; Silva *et al.* 2008) and a liver resection model studying the effect of Bucillamine on liver regeneration could also be undertaken.

The findings of our study suggest that this agent may prove to be a useful target in liver protection against IR injury and could be of clinical benefit in the field of liver transplantation or liver resection surgery. In phase I human studies in normal volunteers, bucillamine at doses up to 25 mg/kg/h i.v. for 3 h elicited no serious drug related adverse effects(Horwitz 2003). Bucillamine is however, not

available for use in intravenous form at present. We had to freshly prepare Bucillamine solution prior to each experiment.

In a recent review post resectional liver failure was found to have an incidence of 0.7-9.1% in all patients undergoing liver resections(van den Broek *et al.* 2008) with an incidence of 9.1 % in patients undergoing major liver resections (>3 segments). Patients with post resectional liver failure have a mortality risk of 59%(van den Broek *et al.* 2008). Liver IRI directly contributes to development of post resectional liver failure(van den Broek *et al.* 2008). Hence, post resectional liver failure would be clinically the most relevant end point for studying any effect on modulation of liver IR. We calculate that a clinical trial involving patients undergoing major liver resections (> 3 segments) using incidence of post resectional liver failure as an clinical end point would require 383 patients in each arm to achieve a 50% reduction in incidence with a p value of <0.05 and a power of 80%. This would require a multicentre study with care being taken to match other risk factors evenly between the study groups (remnant liver volume, blood loss, pre existent liver disease, age of patient, etc). A pilot study using surrogate markers (ICG clearance, serum LFT levels, etc) could however, be undertaken with smaller numbers.

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Appendix – abstracts and publications arising from the thesis

Abstracts

1. Bucillamine ameliorates liver warm ischaemia reperfusion injury in a rat model

SP Junnarkar, N Tapuria, W- X Yang, B Fuller, AM Seifalian, BR Davidson

Introduction- Liver transplantation and resection surgery involve a period of ischaemia and reperfusion to the liver which initiates an inflammatory cascade resulting in liver and remote organ injury. Bucillamine is a low molecular weight thiol antioxidant that is capable of rapidly entering cells. The effect in liver warm ischaemia reperfusion injury has not been studied.

Hypothesis- Bucillamine would protect against warm ischaemia reperfusion injury through its antioxidant and anti-inflammatory effects.

Aim- To use a well described model of liver ischaemia reperfusion to determine the effect of Bucillamine administration on liver function, liver microcirculation and hepatocyte apoptosis.

Materials and Methods- Effect of bucillamine was studied in a rat model of liver ischaemia-reperfusion injury with 45 minutes partial(70%) ischaemia and 3 hours reperfusion. Liver injury was assessed by serum Transaminases (AST and ALT) and by assessing sinusoidal blood flow and staining of apoptotic hepatocytes by propidium iodide on intravital microscopy.

Results- Liver injury was significant lesser in Bucillamine group as compared to Control group as assessed by serum AST (932±200.8 vs 2072.5±511.79, $p<0.05$) and serum ALT (861.4±262.63 vs 2079.25±322.33, $p<0.05$). The number of apoptotic cells at the end of 3 hours of reperfusion was also significantly lesser in the Bucillamine group ($p >0.001$). There also was better perfusion of sinusoids in the Bucillamine group at the end of 3hours of reperfusion (RBC velocity 242.66±16.86 vs 181.11±17.59).

Conclusions- Bucillamine protects hepatocytes from ischaemia- reperfusion injury through its anti inflammatory and anti oxidant effect.

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2. Attenuation of Liver ischaemia reperfusion injury by the thiol antioxidant Bucillamine

SP Junnarkar, N Tapuria, B Fuller, AM Seifalian, BR Davidson

Introduction- Liver transplantation and resection surgery involve a period of ischaemia and reperfusion to the liver which initiates an inflammatory cascade resulting in liver and remote organ injury. Bucillamine is a low molecular weight thiol antioxidant that is capable of rapidly entering cells. Its effect in liver warm ischaemia reperfusion injury has not been studied.

Hypothesis- Bucillamine would protect against warm ischaemia reperfusion injury through its antioxidant and anti-inflammatory effects.

Aim- To use a well described model of liver ischaemia reperfusion to determine the effect of Bucillamine administration on liver function, microcirculation and cytokine production.

Materials and Methods- Effect of bucillamine was studied in a rat model of liver ischaemia-reperfusion injury with 45 minutes partial(70%) ischaemia and 3 hours reperfusion. Liver injury was assessed by serum transaminases (AST and ALT) and propidium iodide staining of apoptotic hepatocytes intravital microscopy. Liver microcirculation sinusoidal perfusion and leukocyte adhesions. Cytokine response was assessed by measuring serum CINC-1 levels.

Results- The model produced a significant liver injury with elevated Transaminases and an acute inflammatory response. Bucillamine reduced the liver injury as indicated by a reduced AST(932±200.8 vs 2072.5±511.79, $p<0.05$) and ALT (861.4±262.63 vs 2079.25±322.33, $p<0.05$). The number of apoptotic cells at the end of 3 hours of reperfusion was also significantly lesser in the Bucillamine group ($p >0.001$). Serum CINC-1 levels were found to be lesser in animals given Bucillamine with a very significant difference at 24 hours post reperfusion ($p<0.001$). There also was better perfusion of sinusoids in the Bucillamine group at the end of 3hours of reperfusion.

Conclusions- Bucillamine therapy reduces the deranging effects of warm ischaemia reperfusion injury

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3. Bucillamine inhibits neutrophil activation and decreases liver warm ischaemia reperfusion injury

SP Junnarkar, N Tapuria, AR Mani, B Fuller, AM Seifalian, BR Davidson

Introduction- Liver transplantation and resection surgery involve a period of ischaemia and reperfusion to the liver which initiates an inflammatory cascade resulting in liver and remote organ injury. Bucillamine is a low molecular weight thiol antioxidant that is capable of rapidly entering cells. Our previous studies have shown that bucillamine reduces warm ischaemia reperfusion injury in liver.

Hypothesis- Bucillamine may reduce warm ischaemia reperfusion injury by reducing neutrophil activation.

Aim- To use a well described model of liver ischaemia reperfusion to determine the effect of Bucillamine administration on neutrophil adhesions and cytokine production. Neutrophil activation is explored as possible mechanism.

Materials and Methods- Effect of bucillamine was studied in a rat model of liver ischaemia-reperfusion injury with 45 minutes partial (70%) ischaemia and 3 hours reperfusion. Liver injury was assessed by serum transaminases (AST and ALT) and propidium iodide staining of apoptotic hepatocytes on intravital microscopy. Leukocyte adhesions were assessed on Intravital microscopy. Cytokine response was assessed by measuring serum CINC-1 levels.

Results- The model produced a significant liver injury with elevated Transaminases and an acute inflammatory response. Bucillamine reduced the liver injury as indicated by a reduced AST (932±200.8 vs. 2072.5±511.79, $p<0.05$) and ALT (861.4±262.63 vs. 2079.25±322.33, $p<0.05$). The number of apoptotic cells at the end of 3 hours of reperfusion was also significantly lesser in the Bucillamine group ($p >0.001$). Serum CINC-1 levels were found to be lesser in animals given Bucillamine with a very significant difference at 24 hours post reperfusion ($p<0.001$). There also was significantly lesser neutrophil adhesion in the Bucillamine group at the end of 3hours of reperfusion.

Conclusions- Bucillamine therapy reduces the deranging effects of warm ischaemia reperfusion y inhibiting neutrophil activation.

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Publications:

1. Bucillamine improves hepatic microcirculation and reduces hepatocellular injury following liver warm ischaemia reperfusion injury

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Abstract

Liver transplantation and resection surgery involve a period of ischaemia and reperfusion to the liver which initiates an inflammatory cascade resulting in liver and remote organ injury. Bucillamine is a low molecular weight thiol antioxidant that is capable of rapidly entering cells. The effect of bucillamine was studied in a rat model of liver ischaemia- reperfusion injury with 45 minutes partial (70%) liver ischaemia and 3 hours reperfusion and at 24 hours reperfusion. Controls included ischaemia reperfusion (I/R) only, sham and bucillamine alone (without ischaemia reperfusion). Liver injury was assessed by serum Transaminases (AST and ALT). Sinusoidal blood flow and hepatocyte apoptosis measured by Intravital microscopy.
The hepatocellular injury of I/R produced a markedly elevated serum AST which was reduced with bucillamine (2072.5±511.79 vs. 932±200.8, $p<0.05$) at 3 hours reperfusion. Bucillamine treatment with I/R also increased parenchymal blood flow (RBC velocity 242.66±16.86 vs. 181.11±17.59, at the end of 3 hours reperfusion) and reduced hepatocyte necrosis/apoptosis at 3hours as well as 24 hours($p>0.001$). Bucillamine reduces the hepatocellular injury of liver ischaemia reperfusion and improves parenchymal perfusion.

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2. The attenuation of Liver warm ischaemia Reperfusion Injury by Bucillamine: through decreased Neutrophil activation and modulation of Bax/ Bcl-2.

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Abstract:

Background- Liver transplantation and resection surgery involve a period of ischaemia and reperfusion to the liver which initiates an inflammatory cascade resulting in liver and remote organ injury. Bucillamine is a low molecular weight thiol antioxidant that is capable of rapidly entering cells. We hypothesised that Bucillamine may act by replenishing glutathione levels and hence reduce neutrophil activation, modulate Bax/ Bcl-2 expression thus attenuating effects of liver warm ischaemia reperfusion injury.

Methods- Effect of bucillamine was studied in a rat model of liver ischaemia- reperfusion injury with 45 minutes partial (70%) liver ischaemia and 3 hours reperfusion. Liver injury was assessed by measuring serum transaminases (AST and ALT) and liver histology. Oxidative stress was quantified by measuring F_2 isoprostane and glutathione levels. Leukocyte adhesion was assessed by Intravital microscopy and inflammatory Cytokine response by measuring serum CINC-1 levels. Bax and Bcl-2 expression was measured by RT-PCR.

Results- The model produced a significant liver injury with elevated transaminases and an acute inflammatory response. Bucillamine reduced the liver injury as indicated by reduced AST (932±200.8 vs. 2072.5±511.79, $p<0.05$). Bucillamine reduced Bax expression, serum CINC-1 levels and neutrophil adhesion; and upregulated Bcl-2. However, Bucillamine did not affect tissue glutathione levels nor the levels of oxidative stress as measured by plasma and hepatic F_2 isoprostane levels.

Conclusions- Bucillamine reduces liver warm ischaemia reperfusion by inhibiting neutrophil activation and modulation of Bax/Bcl-2 expression.

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