Study of the effect of Remote ischaemic preconditioning (RIPC) on the early and late phase of hepatic ischaemia reperfusion injury and the role of haemoxygenase in RIPC.

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DECLARATION CONCERNING THESIS PRESENTED FOR THE DEGREE OF MD

I, Niteen Tapuria of University Department of Surgery, Royal Free & University College Medical School, Hampstead Campus, Pond Street, London NW3 2QG solemnly and sincerely declare, in relation to the thesis entitled:

Study of the effect of remote ischaemic preconditioning (RIPC) on the early and late phase of the hepatic IR and the role of haem oxygenase in RIPC.

(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.

Signature: Date:

Dedication

I have been very fortunate to have an extremely supportive family. I dedicate this thesis to my parents, who built the foundations upon which I based my values and judgement. I also dedicate this thesis to my wife Archana and my lovely son Jai.

Abstract

Reperfusion following ischaemia 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 Injury (IRI). Ischaemic Preconditioning (IPC) is an adaptive response in which tolerance to prolonged ischaemia is induced in a target organ by prior brief periods of ischaemia. Benefits of IPC have been demonstrated in experimental models and in preliminary human clinical trials. In remote ischaemic preconditioning (RIPC) brief ischaemia involves a remote organ. RIPC has been demonstrated to reduce warm liver I/R injury in an experimental model by our research group and clinical evaluation is ongoing. The effect of RIPC on the late phase of I/R and its mechanism have not been investigated.

This thesis evaluates the effect of RIPC on both the early and late phases of liver warm I/R injury with the hypothesis that beneficial effects are induced by haemoxygenase-1(HO-1), a free radical scavenger which is involved in degradation of haem and production of the vasodilator CO. 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. RIPC was performed with four cycles of 5 min ischaemia and 5 min reperfusion of the right hind limb before sustained ischaemia. Pyrrolidine dithiocarbamate (PDTC) and Zinc Protoporrphyrin (ZnPP) were administered to induce and block haem oxygenase synthesis. Changes to the microcirculation, leucocyte adherence and apoptosis were assessed by intra-vital microscopy. Hepatocellular injury was assessed by standard liver function tests. HO-1 protein was demonstrated by immunohistochemistry (IHC) and measured by Western blot. RIPC improved liver sinusoid perfusion, reduced leucocyte adherence and apoptosis in both the early and late phases of IRI.

Hepatocellular injury was reduced. RIPC increased HO-1 production in the liver, particularly in hepatic macrophages, as demonstrated by IHC. PDTC treatment (HO-1 inducer) reproduced the protective effect of RIPC whereas HO-1 inhibition with ZnPP abolished the protective effect.

The response to HO-1 induction and inhibition indicate that HO-1 has a key role in the protective effect of RIPC. Establishing the inducing agent for HO-1 may lead to new pharmacological approaches to preconditioning and the protection of the liver from IR injury. Studies on RIPC and liver warm I/R using HO-1 knockout mice would clarify the pathways involved in RIPC.

Acknowledgements

The experimental work was done in the animal laboratory in the University Department of surgery at the Royal Free Hospital, University College London. I am grateful to my supervisors Prof B.R. Davidson and Prof. Alexander Seifalian for their supervision, help, guidance and constant encouragement during my study. I am most grateful to Professor Barry Fuller whose advice helped me in planning my experiments. I am thankful to Professor M.C. Winslet for supporting me and providing necessary assistance for me to complete my research.

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Thesis Description

Chapter 1- Remote ischaemic preconditioning a novel protective method from ischaemia reperfusion injury- a review.

an overview of the pathophysiology of ischaemia reperfusion injury, protective strategies are discussed and the evidence and underlying mechanisms of remote ischaemic preconditioning have been discussed (review article).

Chapter 2- materials and methods-

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 3 - Study of microvascular and hepatocellular changes in the early phase of hepatic IR (3 hrs) and the effect of RIPC on hepatic IR.

This chapter describes preconditioning of the liver by remote hind limb ischaemia in an experimental rat model of warm hepatic IR, microvascular, neutrophil endothelial interactions, histological, biochemical changes & hepatocellular death seen in hepatic IR and the impact of preconditioning on these changes.

Chapter4- The effect of HO-1 inhibition in modulating the protective effect of remote preconditioning in the early phase of hepatic IR injury (3 hrs).

This study demonstrates the role of RIPC induced hepatic haemoxygenase expression and its effect on hepatic microcirculatory changes in hepatic IR as demonstrated by intravital microscopy in a real time manner. The study demonstrates the effect of inhibition of haemoxygenase by Zinc protoporphyrin on remote ischaemic preconditioning hepatic microcirculation in hepatic IR.

Chapter5 - New insights into the late phase of hepatic IR and the effect of RIPC on hepatocellular and microvascular changes after 24 hrs of reperfusion- recovery experimental model.

This chapter describes the hepatic microcirculatory disturbances, biochemical, histological, neutrophil endothelial interactions and hepatocyte death in the late phase of hepatic IR and the effect of RIPC on these changes in the late phase of hepatic IR.

Chapter6- Study of the spatiotemporal expression of HO-1 in the early and late phase of hepatic IR and RIPC+IR by immunohistochemistry.

This chapter describes the spatial distribution of haemoxygenase in hepatic IR in the early and late phase and effect of RIPC and compared HO expression in different groups.

Chapter7- Quantification of HO-1 expression in the early and late phase of hepatic IR and RIPC+IR by Western blot analysis.

This study quantifies the expression of haemoxygenase by western blots and compares the relative density of HO-1 expression in all animal groups.

Chapter 8- Modulation of Neutrophil activation by RIPC Induced inhibition of Cytokine-induced neutrophil chemoattractant in the early and late phase of hepatic IR.

This chapter investigates the modulatory effects of RIPC on serum CINC levels in the early and late phase of hepatic IR and correlates with HO expression. CINC is a neutrophil chemoattractant responsible for activation and recruitment of neutrophils in the inflammatory response. Neutrophils are key to IR injury and hence modulation of neutrophil activation is of prime importance.

Chapter 9 Mitochondrial function in the early and late phase of hepatic IR, modulation by RIPC and correlation with biochemical, histological and hepatocellular injury markers.

This chapter investigates the role of mitochondria and cytochrome c in early and late IR injury and investigates modulatory effects of RIPC on mitochondrial cytochrome c release.

Chapter 10 Summary and discussion of thesis.

References appear at the end of the thesis.

Publications and abstracts from the thesis are enclosed after references of the thesis

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Abbreviations

IR- Ischaemia reperfusion injury

RIPC- Remote ischaemic preconditioning

PDTC- Pyrridoline dithiocarbamate

ZnPP- Zinc protoporphyrrin

IR-24- 24 hour reperfusion injury

RIPC+IR- RIPC prior to reperfusion injury.

HO- Haemoxygenase

NO- Nitric oxide.

NOS- Nitric oxide synthase

CO- Carbon monoxide.

IVM- Intravital microscopy

PKC- Protein kinase c.

List of publications and presentations based on the research work in the thesis.

Presentations

1. New insights into the late phase of hepatic IR – **Oral presentation at the International HPB association meeting, Bombay – Feb 2008.**

2. Remote ischaemic preconditioning modulates hepatic microcirculation through haemoxygenase expression in an animal model of warm hepatic IR- **Oral presentation at the American HPB, Las Vegas (Basic science section), April 2007.**

3. Remote preconditioning modulates hepatic microcirculation to ameliorate hepatic ischaemia reperfusion injury in a rat model- **poster presented at the IHPBA- September 2006**

Publications

1. Effect of remote ischaemic preconditioning (RIPC) on hepatic microcirculation and function in a rat model of hepatic ischaemia reperfusion injury- **Niteen Tapuria, Sameer Junnarkar, Brian Davidson- published** HPB (Oxford). 2009;11(2):108-17**.**

2.Remote ischaemic preconditioning – a novel method – **Niteen Tapuria, Y Kumar , Brian Davidson- published -** J Surg Res. 2008 Dec;150(2):304-30.

Abstracts

1. New insights into the late phase of hepatic IR- **Niteen Tapuria, Sameer, Alexander M Seifalian, Brian R Davidson- HPB journal 2008, IHPBA supp.**

2. Remote ischaemic preconditioning modulates hepatic microcirculation through haemoxygenase expression in a rat model of warm hepaticIR- **Niteen Tapuria, B. R. Davidson- HPB journal, 2007, AHPBA supplement**

3.Remote ischaemic preconditioning modulates hepatic microcirculation to ameliorate hepatic ischaemia reperfusion injury in rat model-**Niteen Tapuria, Sameer Junnarkar, Wenxuan yang, A.M. Seifalian, BR Davidson-Abstract published in HPB Journal- August 2006-IHPBA Edinburgh.**

Chapter 1

Remote ischaemic preconditioning – A novel protective method from ischaemia reperfusion injury.

(A REVIEW)

Introduction

1.1 What is ischaemia reperfusion injury and when does it happen?

The restoration of blood supply to organs after a certain period of no flow ischaemia results in parenchymal damage referred to as reperfusion injury. The critical ischaemia period , is dependent on the organ and is 15-20 minutes (Jaeschke & Farhood 2002) in the liver and kidney, 2.5 hours in skeletal muscle(Eckert & Schnackerz 1991;Larsson & Hultman 1979;Tountas & Bergman 1977) whilst in the brain ischaemia for more than 5 minutes leads to considerable neuronal death and infarction. Reperfusion following periods exceeding the critical ischaemia period results in endothelial and parenchymal injury. The liver is resilient to hypoxic injury. Low flow ischaemia found in haemorrhagic shock (MAP-40mmhg for 120 min) followed by restoration of normal flow does not lead to activation of Kupffer cells, generation of free radicals and associated reperfusion injury in the initial resuscitation period(Jaeschke & Farhood 2002). This is because most kupffer cells are located in the periportal region and haemorrhagic shock is characterized by ischaemia in the pericentral regions with sinusoidal perfusion failure in the pericentral region as a result of which most kupffer cells are not affected by pericentral hypoxia.

1.2 Pathophysiology of ischaemia reperfusion injury (IRI) (Fig 1.1*)*

Following a period of ischaemia tissues adapt to anaerobic metabolism (Carden & Granger 2000). Restoration of blood supply results in oxygen supply in excess of the requirements which leads to activation of macrophages in the vasculature and consequently generation of super oxide radicals also referred to as reactive oxygen species (ROS) causing oxidative stress. The key event in the initial phase of reperfusion injury is activation of macrophages which are the primary source of extracellular ROS. CD 4 lymphocytes are also activated and serve to recruit

macrophages. Endothelial cells and parenchymal cells are activated leading to release of free radicals originating from intracellular mitochondria, xanthine oxidase system, NADH oxidase system and iron redox system. There is an imbalance between endothelial nitric oxide and super oxide radicals 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 in the early phase serves to prime macrophages and neutrophils for release of ROS(Jaeschke et al. 1993). Complement 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. Thus macrophages and neutrophils are the key cells in the initial phase and and late phase of IRI respectively.

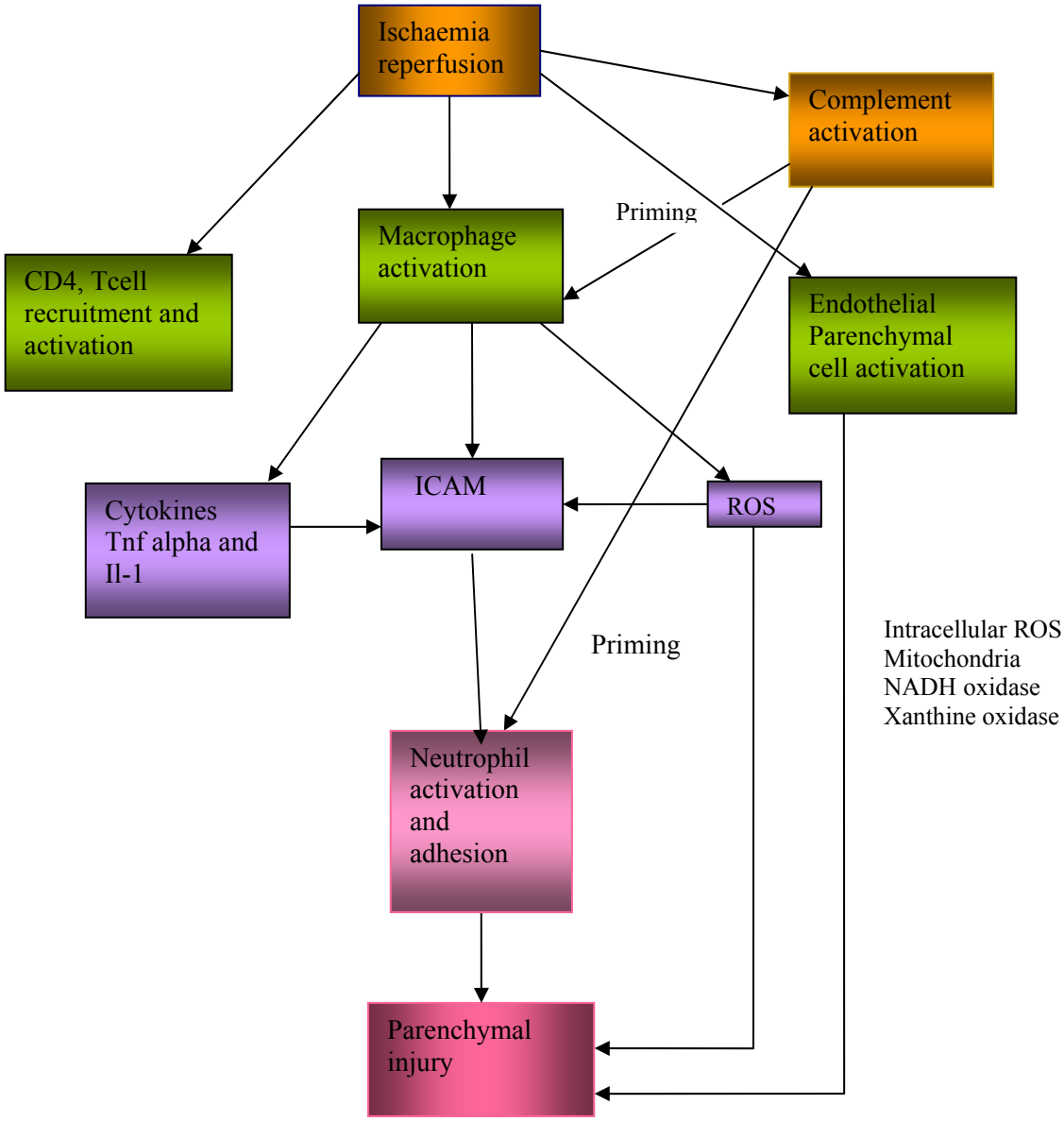
1.3 The clinical relevance of ischaemia reperfusion injury.

IRI often happens following transplantation of organs, major organ resections and trauma. IRI following transplantation can lead to primary nonfunction of the implanted organ (<5%), primary dysfunction (10-30%) and also multiple organ dysfunction syndrome. (MODS) resulting in morbidity and mortality in adult and paediatric transplants (Clavien, Harvey, & Strasberg 1992;Margarit et al. 2000;Pokorny et al. 2000).In hypovolaemic shock resuscitation leads to IRI in brain, gut, pancreas (Yamakawa et al. 2000) which results in more extensive tissue

infarction. . IRI results in adipocutaneous and musculoskeletal flap necrosis as well as non-functioning microvascular flaps.

Hepatic ischaemia reperfusion injury and its clinical relevance.

In the liver shock resuscitation reduces its tolerance to subsequent warm ischaemia (pedrizet). In major organ resections inflow occlusion (Liu et al. 1996) and subsequent restoration of blood supply causes reperfusion injury. This can lead to postoperative organ insufficiency. Ischaemia reperfusion injury is also associated with chronic rejection due to arteriosclerosis(Fellstrom et al. 1998) caused by IRI. In the context of liver transplants IR leads to nonfunction or dysfunction of transplanted organs in 10% cases. This contributes to the shortage of organs for liver transplantation due to higher susceptibility of marginal livers to the ischaemic insult. Hence it is highly essential that protective strategies aimed at minimising ischaemia reperfusion injury and maximising organ usage are developed. Hepatic ischaemia reperfusion injury may lead to acute renal failure {Lee et al, lab invest 2009}. Histological assessment of renal tissue showed increased endothelial cell apoptosis and renal tubular disruption after hepatic IR. While kupffer cells and sinusoidal endothelium are the key cells in the cold phase of hepatic IR, warm IR injury causes hepatocyte parenchymal injury through neutrophil activation and ROS release. Protective strategies aimed at reducing kupffer cell activation, sinusoidal endothelial damage, neutrophil activation and effects of free radicals released after reperfusion need to be developed in order to reduce IR injury and organ dysfunction. One of the protective strategies is ischaemic preconditioning which is discussed in the next section.



(Figure above) 1.1 Diagramatic representation of ischaemia reperfusion injury

1.4 Ischaemic preconditioning

Protective strategies have been developed for protection of organs from ischaemia reperfusion injury which are referred to as organ preconditioning. Preconditioning is only a method by which the target organ is conditioned prior to the ischemic insult in

order to reduce the extent of injury. Preconditioning could be mechanical or pharmacological. Mechanical preconditioning is either direct or remote (indirect).

In direct ischemic preconditioning a brief direct ischemic insult to the target organ followed by reperfusion results in tolerance to subsequent insults of ischaemia. Ischaemic tolerance is induced by regulation of endothelial function, blood flow and decreased macrophage as well as neutrophilic activity. This results in decreased endothelial injury and eventually decreased parenchymal injury. Direct ischemic preconditioning has been investigated as a surgical tool for many years (Murry, Jennings, & Reimer 1986) Although direct IPC does reduce reperfusion injury (Arai et al. 1999;Peralta et al. 1996;Yin et al. 1998) as well as its systemic consequences (Fernandez et al. 2002;Peralta et al. 2001) its main disadvantage is direct stress to the target organ and mechanical trauma to major vascular structures which have limited its clinical application.

Remote preconditioning is a novel method where ischaemia followed by reperfusion of one organ is believed to protect remote organs either due to release of biochemical messengers in the circulation or activation of nerve pathways resulting in release of messengers which have a protective effect. This protects target tissue without direct stress. Remote preconditioning was first demonstrated in myocardium (McClanahan et al. 1993) by McClanahan in 1993. He found that ischaemia in the kidney followed by reperfusion protected myocardium from ischaemia and reduced infarct size. In animal models brief ischaemia reperfusion of the limb, gut, mesenteric or kidney reduces myocardial infarct size. In humans skeletal preconditioning has been used for myocardial protection with the beneficial effect being attributed to regulation of endothelial protection(Kharbanda et al. 2002).

1.5 Remote organ injury and systemic inflammatory response following IRI

In addition to the local effects of IRI several studies have shown evidence of remote organ injury following ischaemia reperfusion(Fantini & Conte 1995;Horie & Ishii 2001;McCarter et al. 2004;McCarter et al. 2004;Schmeling et al. 1989;Weinbroum et al. 1997;Weinbroum et al. 1999;Yassin et al. 2002). Due to ischaemia reperfusion injury a systemic inflammatory response is created which results in remote organ injury as shown in animal models of limb ischaemia reperfusion where prolonged limb ischaemia reperfusion leads to lung, liver and kidney dysfunction. Similar evidence has been provided in hepatic ischaemia reperfusion and mesenteric reperfusion models. Due to systemic cytokine release and neutrophilic activation there is multi organ dysfunction following reperfusion injury. However the progress of remote organ injury decreases over a period of three hours from the start of reperfusion and this time point corresponds to the endogenous expression of haemeoxygenase which confers a protective effect on remote organs(Wunder et al. 2004).In another study ischemic preconditioning of the hind limb by 3 cycles of 10min ischaemia followed by 10 min reperfusion prior to 4hrs of left lower limb ischaemia followed by 2hrs of reperfusion reduced lung injury on histology, neutrophilic infiltration, myeolperoxidase activity, plasma TBARS levels indicating lower oxidative stress in the preconditioned group(IPC+IR) as compared to IR group(Olguner et al. 2006).

1.6 Evidence of remote preconditioning.

1.6.1 Intra cardiac regional preconditioning

The very first evidence of this can be traced to 1993 when Przyklenk conducted regional myocardial preconditioning in an experimental dog models(Przyklenk et al. 1993). In this model the circumflex coronary artery was occluded 4 times for 5 minutes followed by 5 minutes reperfusion prior to 1 hour of sustained left anterior

descending coronary artery occlusion and a significant reduction in myocardial infarct size was seen as compared to nonpreconditioned groups. Nakano et al questioned the existence of remote preconditioning as in an experimental Langendorff model they showed two cycles of 5 min occlusion of a branch of coronary artery followed by 5 min of reperfusion prior to global cardiac ischaemia protected the myocardium supplied by the artery but not the rest of the myocardium against sustained ischaemia(Nakano et al. 2002;Przyklenk et al. 1993). However their experiments were in a rabbit model and they used a different preconditioning protocol. Moreover their argument is not supported by any other studies. Hence evidence for non existence of remote preconditioning in other species cannot be concluded from their study.

Author/reference	**Site of preconditioning stimulus**	**Site of index ischaemia**	**model**	**Endpoint**	**Organ protection**	**Proposed mechanism**
Intracardiac preconditioning						
Przyklenk et al 1993	Circumflex occlusion	Lad occlusion	Dog	Infarct size	Reduced infarct size	Not identified
Nakano et al 2002	Cardiac ischaemia	Total LV:global ischaemia	Rabbit	Infarct size	Reduced infarct size	Not applicable
Tanaka et al 1998	Cardiac ischaemia	Remote myocardial preconditioning	Rabbit	Infarct size	Reduced infarct size	Heat shock proteins
Gho et al 1996	Cardiac ischaemia	Cardiac ischaemia	Rat	Infarct size	Reduced infarct size	?Neurogenic pathway, ?humoral

Table no 1.1- Studies on intraorgan regional preconditioning.

1.6.2 Role of stretch in intracardiac preconditioning

Stretch of myocardial muscle has been shown to reduce infarct size in the heart (Domenech & Macho 1998) (Ovize, Kloner, & Przyklenk 1994). Acute volume load followed by coronary artery occlusion for 1 hour and reperfusion for 4.5 hours

significantly reduced myocardial infarct size. Injection of Glibaclide (stretch ion blocker) into the atrium before volume overload blocked the protective effects. These observations supported the argument that stretch acts as a trigger of the remote preconditioning stimulus. Contrary to this Weinbrenner et al(Weinbrenner et al. 2004a) showed that infrarenal aortic occlusion (IOA) was linked to an increase in mean arterial pressure(13%) however, myocardial infarct size was only reduced when IOA was followed by reperfusion. Their data showed that the increase in MAP was inadequate to induce significant myocardial stretch and cardiac preconditioning. Dickson et al in a model of isolated perfused hearts which excludes pressure/stretch related effects showed transfer of effluent from preconditioned hearts to have a protective effect to ischaemia in the virgin recipient hearts. Clearly experimental evidence is in favour of protective substances released into the blood stream which confer protection on other organs. Future experiments targeted at clearly demonstrating the role of stretch in preconditioning and the underlying mechanism are needed.

In addition to stretch other non ischemic stimuli such as ventricular pacing have been shown to activate KATP channels, modulate ionic fluxes and protect the myocardium from IR injury(Petrishchev et al. 2001;Verdouw et al. 1996). However experimental evidence in support of these studies is inadequate and future studies are needed to evaluate the underlying mechanisms.

1.6.3 Interorgan preconditioning

Remote preconditioning is not confined to within an organ and can be transferred from one organ to another. Liauw et al in an experimental model of limb ischaemia demonstrated protection of remote skeletal muscle.

1.6.4 The effect of cerebral ischaemia on the heart

Spontaneous ischaemic events in the brain have been shown to induce adaptation of the heart to ischaemia(Tokuno et al. 2002). Tokuno et al were the first to demonstrate in a mouse model decreased myocardial infarction in mice with neurological signs of disease. Brain ischaemia induced by bilateral ligation of internal carotid arteries 24 hours prior to global myocardial ischaemia reduced myocardial infarct size, improved myocardial performance and attenuated increase in left ventricular end diastolic pressure following reperfusion. There was no evidence of brain damage except for transient neurological patterns(Tokuno et al. 2002).Importantly this study demonstrated that ischaemia rather than reperfusion stimulated signalling pathways which led to remote protection.

Preconditioning by cerebral ischaemia						
Author/reference	**Site of preconditioning stimulus**	**Site of index ischaemia**	**model**	**Endpoint**	**Organ protection**	**Proposed mechanism**
Tokuno et al (2002)	Cerebral ischaemia	Cardiac ischaemia	Mouse isolated heart	Infarct size	Reduced infarct size	iNOS and NO
Verdouw et al 2001	Cerebral ischaemia	Cardiac ischaemia	Pig	Infarct size	No change in infarct size	Norepinephrine

Table no 1.2- Studies on RIPC by brief cerebral ischaemia.

1.6.5 The effect of renal and infrarenal ischaemia on remote organs

Five studies showed that brief renal ischaemia reduced the size of infarct resulting from myocardial ischaemia(Ates et al. 2002;Gho et al. 1996;Pell et al. 1998;Singh & Chopra 2004;Takaoka et al. 1999)

The first evidence of interorgan remote preconditioning dates back to 1993 when **Mc Clanahan** and his team carried out experiments on a rabbit model and showed that a 10 min period of renal ischaemia was as effective as a 5 min period of coronary artery occlusion in preconditioning the heart(McClanahan, Nao, Wolke, Martin, Metz, & Gallagher 1993).Gho et al in **1996** (Gho et al. 1996) showed that brief periods of remote organ ischaemia (mesentery and kidney) were as effective in producing cardiac protection as direct cardiac preconditioning. On causing MAO (mesenteric occlusion) for 15 min and reperfusion prior to 60 min CAO (coronary artery occlusion) there was a decrease in myocardial infarct size. 15 min RAO (renal artery occlusion) prior to 60 min CAO reduced infarct size under hypothermic conditions but not normothermic conditions demonstrating that the brief renal ischaemic preconditioning protocol is ineffective by itself. The onset of myocyte injury is associated with ATP depletion and breaks in sarcolemmas which are linked to intracellular acidosis and raised phosphocreatinine levels. Takoaka et al showed by NMR-spectroscopy that brief renal ischaemia (10 min) followed by reperfusion of 20 min in rabbits led to decrease in myocardial infarct size on histology, attenuated depletion of ATP, preserved myocyte PH and improved recovery of myocardial phosphocreatinine as well as ATP levels during the subsequent reperfusion period.. Pell et al(Pell et al. 1998) in a rabbit model showed that RAO(10 min) followed by reperfusion(10min)prior to CAO for 30 min followed by 2hrs of reperfusion reduced infarct size. Singh et al (Singh & Chopra 2004) in a rat model showed that preconditioning by four cycles of 5 min RAO followed by 10 min reperfusion prior to CAO for 30 min followed by 2hrs reperfusion reduced myocardial infarct size. The study by Gho et al demonstrated that Ganglion blockade abolished the protective effects of remote preconditioning but not of direct cardiac preconditioning suggesting

a different pathway of signal transduction in both methods. Takoaka et al showed that the protective effects of RIPC from kidney were similar to direct myocardial preconditioning.

These studies collectively demonstrate that brief periods of RAO are necessary for preconditioning and brief periods of reperfusion are necessary for preconditioning. Preconditioning was not seen in permanent arterial occlusion suggesting that infarct size limitation was due to washout of a protective substance during reperfusion. Both rat and rabbit models demonstrated the benefit of preconditioning. The extent of myocardial protection by RAO was more in Takoaka's study as compared to McClanahan although both used rabbit models possibly due to differences in core temperatures of the animal models. This remained unclear as McClanahan did not report core body temperature in his study.

1.6.6 Influence of temp, blood pressure, baroreflex and heart rate on RIPC

Hypothermia has been shown to contribute to preconditioning in previous studies. {Yuan, 2004; Bolling, 2001; Hale, 1999}. McClanahan demonstrated remote preconditioning of the heart from brief RAO but did not report core body temperature and hypothermia could be a factor in preconditioning.Gho et al demonstrated preconditioning of myocardium by brief RAO (Gho, schoemaker, sharma)but they observed surgical manipulation to be associated with a fall in core body temperature in their experimental model suggesting the role of hypothermia in preconditioning.Gho et al demonstrated that (Gho et al. 1996) preconditioning by RAO for 15 min prior to CAO for 60 min was effective only under hypothermic conditions. Hypothermia enhanced preconditioning by brief CAO prior to CAO for 60 min but no enhancement of preconditioning by MAO was demonstrated by GHO et al. These findings were supported by Pell et al who demonstrated that MAO was

effective at low and high temperatures while RAO was effective only at low temp in preconditioning of the heart. The RAO stimulus was below threshold under normothermic conditions and hypothermia enhanced its protective efficacy while MAO was near its maximal protective efficacy and hence hypothermia did not enhance its effects. These studies demonstrate that the role of temperature critically depends upon the experimental protocol and model chosen and the modification of protective pharmacological/physiological preconditioning stimuli by temperature.

Fall in blood pressure may lead to poor flow in the target organ and exacerbate the preconditioning ischaemic insult thus enhancing the preconditioning effect. However Gho et al observed that Hexamethonium(Gho et al. 1996) (autonomic ganglion blocker)was associated with a drop in blood pressure and heart rate .This was seen in all groups with no significant difference in infarct size and hence the contribution of drop in blood pressure to the preconditioning ischaemic stimulus was ruled out. The vasopressor response seen on preconditioning by MAO could potentially lead to myocardial stretch and preconditioning but the effect of preconditioning was abrogated on ganglion blockade despite persistence of the rise in B.P on MAO ruling out any preconditioning effects of rise in blood pressure.

The data from Weinbrenner's (Weinbrenner et al. 2002)experiments supported the argument against any contribution of rise in mean arterial pressure or stretch to the protective effect as the rise in MAP alone did not reduce infarct size. The stretch stimulus was of inadequate strength (increased MAP by 13%) and duration (only 2 min) to trigger preconditioning in comparison to the external stimulus required in an experimental setting(Ovize, Kloner, & Przyklenk 1994). They also found a drop in heart rate which was attributed to baroreceptive changes following rise in MAP on

IOA. However baroreflex in no way contributed to the protective effect since the drop in heart rate was universal in all groups.

Weinbrenner et al (Weinbrenner et al. 2002)found a rise in lactate following IOA but the rise was transient and its decline rapid in comparison to the period(10 min) of reperfusion required for remote protection indicating the unlikely role of lactate in triggering protection. Moreover additional animal studies have shown no benefit of lactate in protection of the heart(Doenst et al. 1996). In IPC studies(Weiss et al. 1996) a decrease in lactate levels has been found indicating its unlikely role in mediation of protection .

1.6.7 Role of mesenteric preconditioning

The beneficial effect of mesenteric preconditioning on remote organs

Six studies provide evidence to support remote ischaemia preconditioning from mesentery.Shoemaker et al (Schoemaker & van Heijningen 2000b) in an experimental rat model showed that brief mesenteric occlusion (15 min)followed by (10 min)reperfusion prior to 60 min CAO and 180 min reperfusion resulted in decreased myocardial infarct size on histology. In a similar rat model GHO et al showed 15 min MAO followed by 10 min reperfusion to be as effective as 15 min of CAO followed by 10 min reperfusion prior to 60 min CAO and reperfusion.

Two studies by Wolfrum et al in an experimental rat model showed that 15 minutes of mesenteric ischaemia followed by 15 minutes of reperfusion prior to 30 min CAO followed by 150 min reperfusion reduced myocardial infarct size on histology. Liem et al(Liem et al. 2002a) showed that brief mesenteric artery occlusion (MAO15min) followed by reperfusion preconditioned the intestine and reduced myocardial infarct size. Wang et al (Wang et al. 2001) showed that MAO(30 min) followed by

reperfusion and induction of myocardial ischaemia 24 hours later (30 min of CAO and 180 min of reperfusion) reduced myocardial infarction. They also showed that remote preconditioning reduced myocardial MPO (myeloperoxidase activity) which is a marker of systemic inflammatory response.

Patel et al in a rat model showed that a single period of 15 min MAO followed by 10 min reperfusion was more effective than three cycles of 5 minutes of ischaemia followed by 5 min reperfusion prior to 30 min of ischaemia and 2hrs of reperfusion although both reduced myocardial infarct size significantly on histology.

Tang and Xiao worked on protective effects of mesenteric artery occlusion and showed that reducing infarct size and myocardial creatinine kinase levels(Tang et al. 1999;Xiao et al. 2001) . Experiments by Petrischev (Petrishchev et al. 2001) and Vlasov et al supported evidence for remote mesenteric preconditioning however in Vlasov's(Vlasov, Smirnov, & Nutfullina 2001) experiments the effect of remote preconditioning was less in comparison to direct preconditioning.

These studies collectively support the evidence for remote preconditioning by MAO and reperfusion. All studies demonstrated an effective preconditioning protocol with brief MAO and only one study (Patel) demonstrated that a single cycle of RIPC was more effective than multiple cycles. Only one study (Vlasov) showed that direct ischaemic preconditioning is more effective than RIPC and one study (Wang et al) demonstrated the existence of late phase of mesenteric preconditioning. Histology is a common endpoint in all studies but MPO activity or myocardial creatinine kinase levels were not evaluated in all studies. Most studies did not find any significant haemodynamic alterations except Wolfrum et al who showed that MAP increased on mesenteric occlusion and decreased on reperfusion with a gradual return to baseline in This was supported by data from Wang's study (Wang et al. 2001). None of the

studies measured ventricular function. Future studies aimed at clarifying the functional importance of mesenteric RIPC, comparison of preconditioning protocols to establish the ideal protocol and clinical application are needed.

Preconditioning by mesenteric ischemia						
Author/reference	**Site of preconditioning stimulus**	**Site of index ischemia**	**model**	**Endpoint**	**Organ protection**	**Proposed mechanism**
Gho 1996	Mesenteric and renal	Cardiac ischemia	Rat	Infarct size	Reduced infarct size	Neurogenic, humoral
Schoemaker et al (2000)	Mesenteric ischemia	Cardiac ischemia	Rat	Infarct size	Reduced infarct size	Bradykinin mediated and neuronal pathway
Liem et al (2002)	Mesenteric ischemia	Cardiac ischemia	Rat	Infarct size	Reduced infarct size	Increased interstitial adenosine levels; afferent autonomic nerve stimulation, activation of myocardial adenosine receptors
Wang Y.P. et al (2001)	Mesenteric	Cardiac ischemia	Rat	Infarct size	Reduced infarct size	Role for iNOS
Wolfrum (2002)	Mesenteric	Cardiac ischemia	Rat	Infarct size	Reduced infarct size	Myocardial PKC activation through both neuronal and bradykinin dep humoral pathway.
Wolfrum 2005	Mesenteric	Cardiac ischemia	Rat	Infarct size	Reduced infarct size	Calcitonin gene related peptide.
Patel at al 2002	Mesenteric	Cardiac ischemia	Rat	Infarct size	Reduced infarct size	Endogenous opiods
Tang et al	Mesenteric	Cardiac ischaemia	Rat	Infarct size	Reduced infarct size	Capsaicin sensitive sensory nerves
Xiao et al (2003)	Mesenteric	Cardiac ischaemia	Rat	Infarct size	Reduced infarct size	Capsaicin sensitive sensory nerve and NOS
Petrishcev et al	Direct cardiac and remote mesenteric ischaemia	Cardiac ischaemia	Rats	Infarct size	Reduced infarct size	NO unlikely mechanism.

Verdouw et al	Mesenteric ischemia, renal ischemia and ventricular pacing	Cardiac ischemia	Pig	Infarct size	Reduced infarct size	Ventricular pacing by activation of katp channels. Mesenteric ischemia effective at low 31 and highcore 36 degree Celsius Renal ischemia only at high temp .-36 degrees Celsius.
Vlasov et al (2004)	Intestinal ischemia	Heart and intestine	Rat	Infarct size	No myocardial protection. Intestinal adaptation	NO in direct preconditioning but not in remote preconditioning.
Liem at al (2004)	Mesenteric ischemia	Cardiac ischemia	Rat	Infarct size	reduced infarct size	Mesenteric ischemia Adenosine dependent pathway
	Mesenteric ischemia	Cardiac ischemia	Rat	Infarct size	Reduced infarct size	Inflammatory gene suppression.

Table no 1.4- Studies on RIPC by brief mesenteric ischaemia.

1.6.8 The protective effects of prior limb ischaemia on remote organs

Nine studies have shown the beneficial effects of brief limb ischaemia in preconditioning the heart.

Oxman first showed that hindlimb ischaemia reduced reperfusion cardiac tachyarrythmias(Oxman et al. 1997). A brief period of in vivo limb ischaemia (10 minutes of ischaemia) followed by reperfusion (10min) in a rat model led to cardioprotection. From this study it appeared that a systemic stress response was associated with catecholamine release due to brief limb ischaemia. Kharbanda conducted studies in both a pig model and a clinical setting. Four 5 minute cycles of IRI of the hind limb reduced myocardial infarct size and improved ventricular function in pigs. Hind limb ischaemia showed a protective effect in reducing endothelial injury in humans (Kharbanda et al. 2002;Oxman et al. 1997). Ischaemia reperfusion of the upper limb blunted its vasodilatory response to acetylcholine however remote brief ischaemia of the contralateral arm attenuated decrease in ipsilateral neutrophil activation following IR. Gunaydin et al showed that preconditioning by two cycles of 3min upper limb ischaemia(tourniquet pressure 300 mm hg) separated by 2min of reperfusion in patients undergoing coronary bypass showed an equal rise in CPK-MB in both preconditioned and control groups, however there was a significant increase in LDH in the preconditioned group with levels of myocardial lactate and lactate efflux twice as high compared to the control group providing biochemical evidence for maintenance of myocardial anaerobic glycolysis in the preconditioned group. From a clinical perspective RIPC as shown by Kharbanda et al maybe useful in patients undergoing coronary angioplasty for reducing myocyte and endothelial injury while RIPC demonstrated by Gunaydin et al maybe useful in patients undergoing cardiopulmonary bypass where preconditioning

by transient upper limb ischaemia can be used to protect the myocardium against subsequent ischaemia when the aorta is clamped. Patients with intermittent claudication may have a better tolerance to myocardial ischaemia due to preconditioning by brief limb ischaemia and consequently have a longer window period for thrombolytic therapy to salvage ischaemic myocardium. Studies by Vlasov(Petrishchev et al. 2001) showed that both brief limb ischaemia and cardiac ischaemia reduced myocardial infarct size. A significant reduction in NADH diaphorase and LDH activity was seen in the ischaemic zone in IR group which was attenuated by both RIPC and direct IPC. In addition an increase in NADH diaphorase activity was seen in intact cardiomyocytes of the preconditioned groups as compared to IR group animals. This provided evidence that both remote and direct preconditioning induced some form of metabolic activity and ischaemic adaptation in both ischaemic and intact cardiac myocytes. This is supported by evidence from Gunaydin who showed attenuated LDH activity due to anaerobic glycolysis in preconditioned myocardium. In a mouse model Li et (Li et al. 2004) al used six cycles of 5 min hind limb ischaemia followed by 5 min reperfusion and showed a significant reduction in myocardial infarct size. Expression of NF-KB proteins from both limb skeletal muscle after preconditioning and myocardium suggested the induction of protective signals in the limb being transferred to the heart and leading to ischaemic adaptation.

Kharbanda's group carried out further studies to characterise the time course of protection from RIPC and the existence of two phases of preconditioning. There was evidence of an early period of protection followed by reappearance of a phase of protection 24-48 hours later called the second window of preconditioning. Observations by Li et al showed that Hind limb preconditioning protected left

ventricular function with the effect being maximal 24 hours after preconditioning, which supported the existence of a late phase of preconditioning as seen in studies by Kharbanda and Moses. Konstantinov et al showed increased expression of myocardial heat shock protein genes after limb preconditioning resulted in myocardial cytoprotection. Kristiansen in a rat model of hind limb preconditioning showed that 4 cycles of 5min hind limb ischaemia followed by 5min of reperfusion in the donor significantly reduced coronary IR in donor hearts on implantation in the recipient. Konstantinov showed that preconditioning of the recipient by a similar preconditioning protocol of 4cycles of 5min hind limb ischaemia reduced myocardial infarct size in the donor heart. In contrast weinbrenner showed in a model of single cycle of infrarenal aortic occlusion 15 min followed by 10 min reperfusion prior to 30 min of CAO and 30 min of reperfusion reduced myocardial infarct size(Weinbrenner et al. 2004).

Seven Studies have demonstrated the beneficial effect of skeletal preconditioning on adipocutaneous, muscle and cremasteric muscle flaps as well as remote skeletal tissue(Addison et al. 2003;Kuntscher et al. 2002;Kuntscher et al. 2002;Kuntscher et al. 2002;Kuntscher et al. 2003;Kuntscher et al. 2003;Moses et al. 2004).

Remote preconditioning has a protective effect on flaps reducing flap necrosis which is of particular benefit in patients with irradiated tissues, smokers and obese patients. This protective effect is more pronounced in the late phase of preconditioning .These studies showed remote ischemic preconditioning to be associated with better microcirculation, decreased leukocyte endothelial sticking and endothelial dysfunction as well as better capillary blood flow with terminal arteriolar dilatation (Wang et al. 2004). Similar evidence of remote skeletal protection following three

cycles of hindlimb ischaemia of 10 minutes followed by reperfusion of 10 minutes in a pig model was provided by Moses et al(Moses et al. 2004)

Four studies have shown Hind limb ischaemic preconditioning to be beneficial in protection of the lung and the brain (Harkin et al. 2002; Vlasov, Korzhevskii, & Poliakova 2004; Xia et al. 2003).

Harkin et al (Harkin et al. 2002)in an experimental porcine model showed that brief preconditioning of the lower limb prior to prolonged IRI of the lower limbs protected against lung dysfunction secondary to limb IRI . Limb preconditioning reduced plasma cytokine levels, ameliorated impaired gas exchange and oxygen transport, reduced the elevation in pulmonary arterial pressure and vascular resistance, reduced pulmonary oedema and decreased lung tissue myeloperoxidase activity. In clinical settings of surgery of the lower limbs there is release of cytokines after prolonged ischaemia leading to ARDS and limb preconditioning could protect against lung dysfunction found with ARDS.In a different model of local lung IRI Waldow(Waldow et al. 2005) showed that remote limb preconditioning reversed the detrimental effects of lung IRI on lung function and attenuated pulmonary hypertension and impaired gas exchange. Thus both models of RIPC were effective in protecting the lung. Harkins demonstrated that preconditioning of the hind limb prior to limb IR injury reduced cytokines (IL-1 and Interleukin-1β) and activated neutrophils, SIRS and lung dysfunction. In contrast Waldow et al showed a reduction in plasma Interleukin-1β levels and lung tissue macrophage counts in the RIPC group to sham levels but IL-6 levels were not reduced to sham levels and release of ROS and activation of circulating primed granulocytes was not affected by preconditioning. In Waldow's experimental model systemic preconditioning by repeated hind limb

ischaemia protects again acute IR injury of the lung but not against all indices of systemic inflammation.

Xia et al showed that RIPC by 3 episodes of 5min occlusion and 5 min reperfusion of the iliac artery preserved lung function and prevented a rise in pulmonary vascular resistance as well as arterial pressure following myocardial reperfusion injury. This has important clinical applications in cardiac surgery as coronary IR has deleterious effects on the lung which is a major cause of mortality in these patients. In beating heart surgery it is not possible to directly clamp the aorta and therefore direct cardiac preconditioning is not possible. Under such circumstances preconditioning from a remote organ such as the limb may be useful as shown by Xia et al.

The brain showed ischaemic adaptation to remote preconditioning with decreased brain swelling as well as improved blood flow (Vlasov, Korzhevskii, & Poliakova 2004).

Most models of limb preconditioning supported the effectiveness of multiple brief cycles of limb ischaemia followed by reperfusion conversely Weinbrenner et al showed a single cycle to be more effective than multiple cycles and prolongation of length of the single cycle led to more effective preconditioning.

RIPC by limb ischaemia						
Author/ reference	**Site of preconditioning stimulus**	**Site of index ischaemia**	**model**	**Endpoint**	**Organ protection**	**Proposed mechanism**
Oxman et al (1997)	Hind limb	Cardiac ischaemia	Rat	Reperfusion arrythmias	Decreased arrythmias	Nor epinephrine
Birnbaum et al (1997)	Gastroenemius and reduced flow	Cardiac ischaemia	Rabbit	Infarct size	Reduced infarct size	Not identified
Liauw 1996	Gracilis of ipsilateral limb	Gracilis of contralateral limb	Rat	Muscle necrosis	Reduced by 60%	Not investigated
Kharbanda et al (2002)	Hind limb	Cardiac ischaemia	Pig	Infarct size	Reduced infarct size	Suggested involvement of adrenergic pathway and blockade by reserpine.
Gunaydin et al (2002)	Upper limb	Cardiac ischaemia (Cardiopulmonary bypass)	Human	LDH,CK release	Not conclusive	Enhanced anaerobic glycolysis
Xia et al 2004	Iliac artery	Cardiac ischaemia	Sheep	Pulmonary function and pulmonary vascular resistance	Lung protection And decreased pulmonary vascular resistance.	KAtp channel – pulmonary vascular dilatation and myocardial protection
Kharbanda et al 2002	Upper limb	Upper extremity(contralateral arm)	Human	Endothelial dysfunction	Skeletal protection	Not identified
Addison et al	Hind limb	Global skeletal protection	Pig	Ischemic necrosis	Skeletal protection	Opioid delta 1 receptors
Kuntscher et al 2003	Hind limb	Adipocutaneous flaps	Rat	Flap necrosis	Protection	NO
Kuntscher et al	Hind limb	Rat cremasteric muscle flaps	Rat	Flap necrosis	Protection	NO
Kuntscher et al	Hind limb	Flap ischaemia	Rat	Flap necrosis	Flap Protection	?
Liem et al	Hind limb		Rat		Skeletal muscle protection	?mechanism

Moses et al 2004	Hind limb		Rat	Flap necrosis	Skeletal muscle protection	KATP channels(early phase)
Wang	Hind limb, femoral A	Cremaster flap	Rat	Flap necrosis	Reduced flap necrosis	**?mechanism**
Liem at al 2002(check up)	Check (Hind Limb)	Heart	Rat	Infarct size	Interorgan preconditioning of heart	Adenosine
Weinbrenner (2002)	Infrarenal	Cardiac ischaemia	Rat	Infarct size	Reduced infarct size	Opioid receptors and PKC ; humoral pathway.
Harkin et al (2002)	Hind limb	Hind limb reperfusion injury	Porcine	Pulmonary artery pressure. PO2(alv)-PO2(art) Lung MPO activity and weight.	lung protection	Decreased circulating IL-6, circulating primed phagocytes and pulmonary neutrophil infiltration.
Guohu Li (2004)	Hind limb	Cardiac ischaemia	Mice	Infarct size	Reduced infarct size Delayed protection	NFkB and iNOS
Konstantinov June 05	Hind limb	Cardiac ischaemia in transplanted heart	Rat	Infarct size	Reduced infarct size	KATP channels modulation in recipient
Konstantinov Nov 2005	Hind limb	Cardiac ischaemia	Mouse	Infarct size	Reduced infarct size	Inflammatory gene suppression
Chen X.G. Oct 2005	Hind limb	Cardiac ischaemia	Rat	Infarct size	Reduced infarct size	NO
Moses Dec 2005	Hind limb	Skeletal muscle	Rat	infarction	Reduced skeletal infarction	K ATP, Late phase

Loukogeorgakis Aug 2005	Hind limb	Endothelial limb injury	humans	Flow mediated dilatation	Endothelial function Protected	Autonomic nervous system
Chen YS Jun 2005	Hind limb	Cardiac ischaemia	Rat	Infarct size	Reduced infarct size	Free radicals
Waldouw 2005	Hind limb	Lung IRI	Pigs	Lung functions	Protection of lung functions	Cytokine IL-6
Kristiansen 2005	Hind limb	Cardiac ischaemia in explanted heart.	Rat	Infarct size	Reduced infarct size	K ATP channels
Zhang 2006	Hind limb	Cardiac ischaemia	Rat	Infarct size	Reduced infarct size	Opioid receptors and Katp channels
Dave K.R. 2006	Hind limb	Brain ischaemia	Rat	Neuronal damage	Increased neuroprotection	Not investigated
Kanoria 2006	Hind limb	Hepatic ischaemia	Rat	Liver function hepatic blood flow	Reduced IRI Improved liver function	?adenoisne, NO
Lai 2006	Hind limb	Hepatic ischaemia	Rat	Liver function	Reduced IRI , improved liver function	Haemoxygenase(HO-1)
Cheung 2006	Hind limb	Cardiac ischaemia	Human	Trop I Lung function Cytokines	Reduced Trop I Improved lung function	Reduced cytokine levels

Table no 1.5- Studies on RIPC by brief limb ischaemia

1.6.9 The protective effect of brief hepatic ischaemia and myocardial ischaemia on remote organs.

Four studies showed the protective effects of brief myocardial and hepatic ischaemia on remote organs (Ates et al. 2002;Brzozowski et al. 2004;Brzozowski et al. 2004;Peralta et al. 2001). Both brief Hepatic and myocardial ischaemia have protective effects on the stomach (Brzozowski et al. 2004;Brzozowski et al. 2004). In an experimental rat model Brzozowski et al showed that two 5 min episodes of hepatic/myocardial ischaemia followed by 10 minutes of reperfusion each was as effective as direct gastric preconditioning in reducing gastric erosions as well as increasing gastric blood flow following sustained gastric IR. Ates et al demonstrated in a rat model that brief hepatic ischaemia (10 minutes) prior to 45 min of ischaemia in the left kidney in was associated with better creatinine clearance as well as improved sodium fractional excretion 24 hours after preconditioning. They showed reduced mitochondrial swelling, basement membrane detachment on electron microscopy , reduced renal tubular swelling, necrosis , Tnf -α levels, decreased lipid peroxidation(TBARSlevels) (Ates et al. 2002) and a relatively rapid decline in LDH levels in the remote preconditioned group as compared to IRI group. This study provides evidence of preservation of ultrastructural, histopathological and mjmbiochemical renal function in the RIPC group and supports data for beneficial effects of brief hepatic ischaemia on remote tissues.

Pre conditioning by hepatic ischaemia						
Author/reference	**Site of preconditioning stimulus**	**Site of index ischaemia**	**model**	**Endpoint**	**Organ protection**	**Proposed mechanism**
Ates et al (2002)	Brief Hepatic ischaemia	Renal ischaemia	Rat	Renal blood flow and creatinine clearance	Decreased Renal ischaemia	Adenosine ,Bradykinin, KATP, possible neuronal reflex
Peralta et al (2003)	Hepatic ischaemia	Kidneys,lungs and other organs	Rat	Organ failure	Protection of remote organs	Inhibition of Tnf – alpha induced selectin up regulation.
Brzozowski T et al (2004)	Hepatic /cardiac	Gastric ischaemia reperfusion injury	Rat	Gastric lesions and blood flow	Protection of the stomach	Cyclo-oxygenase 1 and cyclo-oxygenase-2, sensory nerve activation and release of CGRP, No, inhibition ofIl-1 and TnF alpha.
Brzozowski T et al (2004)	Hepatic/cardiac	Gastric ischaemia	Rat	Gastric lesions	Protection	Brain gut axis, sensory and vagal activation,CGRP release.

Table no 1.6- Studies on RIPC by brief hepatic ischaemia

1.7 Does ischaemic preconditioning tolerance induce cross tolerance to RIPC?

In an experimental rat model Liem et al demonstrated that 2cycles of CAO of 15 min ischaemia followed by 15 min reperfusion preconditioned the myocardium prior to 60 minCAO and reduced infarct size. This effect of IPC was abrogated on use of 8-sulfophenyl theophylline (adenosine receptor blockade) prior to IPC suggesting the role of adenosine in IPC. However 4cycles of 15min CAO followed by 15 min reperfusion rendered the myocardium tolerant to IPC and subsequent preconditioning was ineffective in reducing myocardial infarct size (Liem et al. 2004). This was associated with loss of cardiac interstitial adenosine .However RIPC with two cycles of MAO 15min in tolerant myocardium was effective in reducing myocardial infarct size suggesting alternate signalling pathways. This data suggests that repetitive brief ischaemia of same duration may render tissue tolerant to preconditioning however in

the clinical setting patients with unstable angina are unlikely to develop such tolerance to adenosine since angina is of varying severity and duration. However exogenous adenosine is effective in ameliorating ischaemia reperfusion even in those who develop tolerance as shown by liem et al. Thus remote preconditioning may be useful in situations where direct preconditioning is ineffective due to adenosine tolerance.

1.8 Remote preconditioning of Trauma.

The role of remote trauma in preconditioning has been addressed in a recent study by Ren et al (Ren, Wang, & Jones et al 2004). They demonstrated that carotid artery catheterisation (remote nonischemic vascular surgical trauma) aggravates myocardial ischaemia however abdominal incision (remote non ischemic nonvascular surgical trauma) reduces myocardial infarct size following cardiac IR This effect is more in the early phase of remote preconditioning (80% reduction in infarct size) and less in the late phase of preconditioning (40% reduction in infarct size). Remote preconditioning of trauma (RPCT) unlike ischemic preconditioning or remote ischemic preconditioning does not involve ischemic insults in order to initiate preconditioning in remote organs. There is both an early and late preconditioning phase in RPCT. The underlying mechanism of RPCT is unclear. RPCT further reduces myocardial infarct size in TNF-α knockouts supporting the argument that TNF-α does not mediate remote preconditioning of trauma (Ren, Wang, & Jones et al 2004). The role of adenosine in RIPC of the heart and noradrenaline in IPC has been shown. Therefore adenosine activity, sympathetic neuronal activity and catecholamines are potential mediators of RPCT .Since norepinephrine is involved in cross signalling with PKC {Minatoguchi et al, 2003}, NO {Costa et al, 2001} modulates release of of norepinephrine from skeletal muscle in ischaemia and both

PKC and NO play a role in remote preconditioning one may speculate the role of catecholamines, PKC and NO pathways in RPCT. Further studies in animal models using sympathetic blockade, knockouts of NO and PKC and ganglion blockade prior to RPCT are needed to pinpoint the candidate mechanism and elucidate the pathway of signal transmission.

Author/reference	**Site of preconditioning stimulus**	**Site of index ischaemia**	**model**	**Endpoint**	**Organ protection**	**Proposed mechanism**
Xiaoping Ren et al (2004)	1.RPCT (Vascular, ischaemia 2.RPCT (Nonvascular, Nonischemic) 3.Direct IPC	Cardiac ischaemia	Mouse	Infarct size	1. Increased infarct 2. Reduced infarct 3. Reduced infarct	1. TNF-α 2 ?Adenosine, NO, catechol 3. TNF-α

Table no 1.7- Studies on remote preconditioning of trauma.

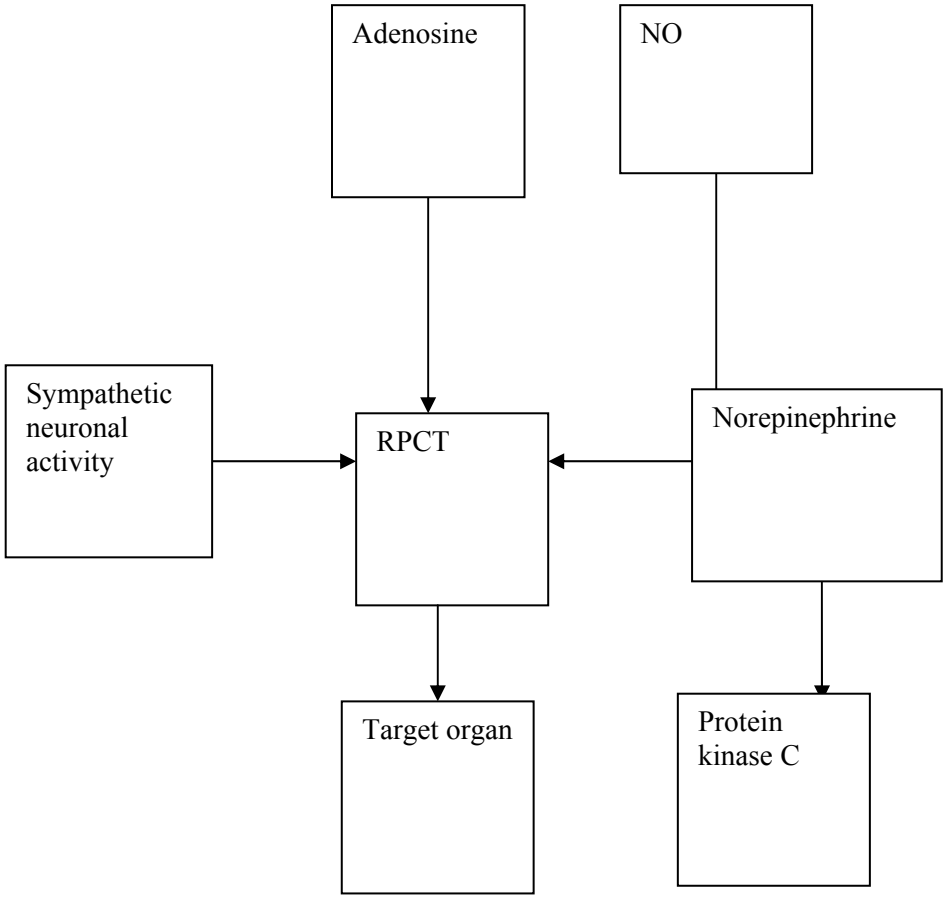


Figure 1. 2- Remote preconditioning of trauma.

1.9 Transferred preconditioning

Ischaemic preconditioning of the heart followed by transfer of coronary effluent from the preconditioned heart to the recipient heart proved beneficial in protecting the recipient heart from ischaemia reperfusion injury (Dickson et al. 2002;Dickson et al. 1999;Dickson et al. 1999;Dickson et al. 2001). The mechanism of transferred preconditioning was via opioids such as Metencephalins and not due to epinephrine or adenosine release into the effluent. Naloxone blocked opioid receptors and abrogated the beneficial effect of transfer of coronary effluent from the preconditioned heart. Coronary effluent from preconditioned hearts is also effective in preconditioning segments of jejunum via opioid receptors and KATPase channels (Dickson et al. 2002).Mesenteric ischemic tolerance induced by pre-treatment of small bowel

segments with coronary effluent leads to quicker recovery of contractile function of small bowel following reoxygenation and reperfusion.

Transferred preconditioning						
Author/reference	**Site of preconditioning stimulus**	**Site of index ischaemia**	**model**	**Endpoint**	**Organ protection**	**Proposed mechanism**
Dickson et al (1999)	Coronary effluent of donor following cardiac ischaemia	Cardiac ischaemia	Rabbit	Infarct size		Opioid receptors
Dickson et al (1999)	Whole blood transfusion following cardiac and renal ischaemia	Cardiac ischaemia	Rabbit	Infarct size	Reduced infarct	Opioid receptors and Katp channel
Dickson et al (2001)	Coronary effluent following cardiac ischaemia	Cardiac ischaemia	Rabbit	Infarct size	Reduced infarct	Metenkephalins
Dickson et al (2002)	Coronary effluent following cardiac ischaemia.	Mesenteric ischemic tolerance	Rabbit	Jejunal peristalsis	Protection of intestine Yes	Metenkephalins

Table no 1. 8- Studies on transferred preconditioning.

Remote microvascular preconditioning						
Author/reference	**Site of preconditioning stimulus**	**Site of response**	**model**	**Endpoint**	**Organ protection**	**Proposed mechanism**
Mabanta 2006	Proximal Microvascular network	Distal microvascular network	Microvascular network	Local response	Microvascular	Katp channels

Table 1.9 - Studies on remote microvascular preconditioning.

1.10 Mechanisms and the role of candidate compounds in the process of remote ischaemic preconditioning:

Adenosine, NO, TNF-α, opioids, bradykinins, Protein kinase C, CGRP, cyclo-oxygenase, KATP channels, capsaicin, heat shock proteins, norepinephrine are all involved in the mechanism of remote ischemic preconditioning. These substances are released as a response to stress and act via the neuronal or humoral pathway to produce organ protection. The pathways involved are different in response to different ischemic stimuli and often overlap. The evidence for role of each of these compounds, the mechanisms by which they act and the pathways involved are reviewed.

1.11 Direct preconditioning and evidence for different phases of the adaptive response. (Fig 1.3)

In direct preconditioning there is evidence for different phases of ischemic adaptation and protection(Bolli 2000). These phases were first identified in the heart and subsequently in other organs. In the heart the early phase protects against infarction

but not against myocardial dyskinesia(Bolli 2000) whereas the late phase protects against both. The early phase begins soon after reperfusion and lasts for up to 3 hours in ischemic preconditioning whereas the late phase starts 12-24 hours later(Kuzuya et al. 1993). The early phase is independent of protein synthesis and is due to release of endogenous substances which stimulate post translational modifications in proteins whereas the late phase is stimulated by release of endogenous substances which lead to synthesis of new proteins and altered gene expression . This is referred to as the second window of protection (SWOP)(Yamashita et al. 1998). The effects of the acute phase are short lived lasting for a 3-4 hours whereas the effects of the delayed phase are longer, lasting for 48-96 hours or sometimes for weeks(Nandagopal, Dawson, & Dawson 2001) (Marber et al. 1993).Both phases in all organs result in reduced cytokine release, neutrophil recruitment and microcirculatory disturbances. This is associated with decreased energy requirements; reduced ATP depletion, decreased ionic fluxes across the cell membrane, reduction of free radical production and preservation of intracellular redox potential. In addition there is decreased reperfusion induced apoptosis and preservation of mitochondrial integrity at the cellular level. The protective effect of the delayed phase of preconditioning is less compared to the early phase and mechanistically different(Bolli 2000). In early PC several endogenous substances have been implicated as triggers such as adenosine, noradrenaline ,opioids, bradykinin , free oxygen radicals(Cohen, Baines, & Downey 2000) as well as angiotensin, endothelin and acetylcholine (Yellon et al. 1998). Receptor mediated signalling mechanisms are involved. Protein kinase C(PKC) (Yellon et al. 1998), tyrosine kinase , mitogen activated protein kinases serve as signalling pathways and eventually converge on mitochondrial Katp channels as well as calcium channels to prevent ATP depletion and reduce calcium influx into cells(Yellon et al. 1998).

In the late phase of PC, synthesis or post translational modifications of Heat shock proteins, antioxidant enzymes and iNOS have been postulated(Yellon et al. 1998).Adenosine is involved in the late phase in protection against ischaemia and acts as a trigger(Downey, Liu, & Thornton 1993) for inducing SWOP. The protection is triggered by Adenosine, ROS, NO, Opioids and Catecholamines and subsequent pathways involve activation of kinases which lead to transcription of new proteins and culminate in formation of mediators such as inducible nitric oxide synthase (iNOS), HO-1, HSP which undergo postranslational modifications leading to the protective response.

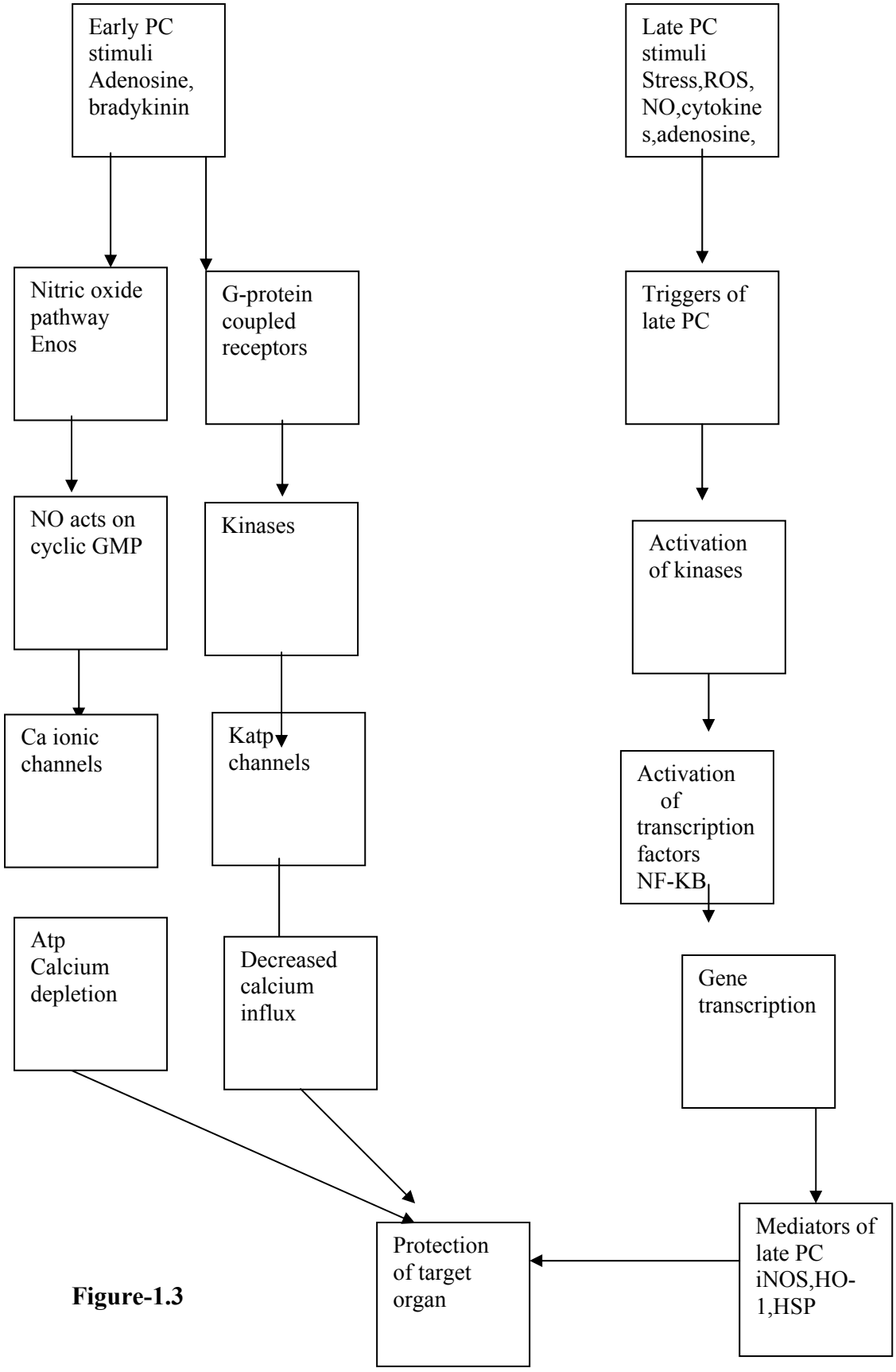


Figure-1.3

Diagram to show the phases of preconditioning.

1.12 Evidence for different phases of adaptation in remote preconditioning.

Eight studies have shown two phases of protection(ischemic adaptation) in RIPC.(Oxman et al. 1997; Pell et al. 1998; Schoemaker & van Heijningen 2000; Wang et al. 2001; Wolfrum et al. 2002) These studies showed the existence of both phases in all organ systems in animal models and in recent human studies

1) Two studies(Pell et al. 1998;Singh & Chopra 2004;Takaoka et al. 1999) showed brief MAO induced early and delayed preconditioning in the heart 2) Li et al showed a pronounced reduction in myocardial infarct size 24 hrs after hind limb RIPC(Li et al. 2004b) 3) Four studies demonstrated late RIPC in remote skeletal muscle following skeletal muscle preconditioning(Kuntscher, Kastell, Altmann, Menke, Gebhard, & Germann 2002;Kuntscher, Kastell, Sauerbier, Nobiling, Gebhard, & Germann 2002;Kuntscher et al. 2002) 4) One study showed ischemic adaptation of the brain to hindlimb ischaemia after 24 hrs of preconditioning.(Vlasov, Korzhevskii, & Poliakova 2004) 5) One study showed late phase of RIPC in the heart and liver due to HSP expression 6)The first clinical application of RIPC in children undergoing cardiac surgery on bypass showed that RIPC was most effective in myocardial protection if applied 24 hours prior to the coronary ischemic insult.(Cheung et al. 2006)

1.13 Evidence for the Pathway of transduction of remote preconditioning

1.13.1 Evidence for the Pathway of transduction from brief renal ischaemia

Four studies demonstrated the pathway from renal RIPC. Gho showed that remote myocardial protection was associated with brief periods of reperfusion of the remote organ however persistent ischaemia without reperfusion did not protect the heart.(Gho et al. 1996e) This was supported by three studies(Pell et al. 1998;Singh & Chopra 2004;Takaoka et al. 1999) which showed that reperfusion was essential for

myocardial preconditioning. However, Gho showed the protective effect of RAO was abolished by giving hexamethonium (ganglion blocker) suggesting the role of neurogenic pathway in conferring protection to distal organs.

1.13.2 Evidence for pathway of transduction from mesenteric ischaemia

Four Studies(Liem et al. 2002;Patel et al. 2002;Wang et al. 2004;Wolfrum et al. 2002) showed that reperfusionis required for preconditioning supporting the humoral pathway for transduction of the protective effect. Prior ganglion blockade abolished the protective effect suggesting the simultaneous involvement of the autonomic neurogenic pathway. Liem et al showed that brief MAO and reperfusion followed by subsequent permanent MAO reduced infarct size significantly more than brief MAO alone and this was abolished by ganglion blockade with hexamethonium. These results showed that brief periods of MAO are cardioprotective but not optimal as their effect was enhanced by further permanent occlusion and reperfusion of virgin intestine was necessary for activation of the neurogenic pathway.

1.13.3. Evidence for pathway of transduction of protective effect from limb ischaemia

Four studies demonstrated the role of the neurogenic pathway in transduction of RIPC from the limb. In an animal model Oxman(Oxman et al. 1997) showed rise in plasma catecholamine levels on preconditioning which was abolished by autonomic nerve blockade with reserpine. Loukogeorgakis et al showed the role of the autonomic nervous system in transduction of the protective signal. However they were unable to define the component of the autonomic system and demonstrate the specific pathway. Birnbaum demonstrated RIPC by electrical stimulation of gastroenemius muscle in conjunction with brief limb ischaemia in a rabbit model (Birnbaum, Hale,

& Kloner 1997) and Kharbanda showed RIPC from the limb was abolished by sympathetic nerve blockade with reserpine. In contrast six studies demonstrated rise in plasma levels of nitrates, opioids, free radicals and catecholamines(Addison et al. 2003;Chen et al. 2005;Chen et al. 2005;Oxman et al. 1997;Weinbrenner et al. 2004) (Kanoria et al. 2006) following RIPC providing evidence in support of a humoral pathway. In Weinbrenner's study(Weinbrenner et al. 2004) the protective effect was only seen in groups who had a period of reperfusion after ischaemia in comparison to those who had no reperfusion or reperfusion at the onset of ischaemia. (Takaoka et al. 1999; Weinbrenner et al. 2002) Interestingly the protective effects of **infrarenal aortic occlusion** were not abolished by ganglion blockade. They showed that simultaneous aortic occlusion along with coronary occlusion did not confer protection indicating that preconditioning had to be prior in order to allow for the substance released to reach the heart. These findings demonstrate the release of protective substances into the circulation. Autonomic nerve blockade with hexamethonium did not abolish RIPC(Chen et al. 2005) and two studies showed cardioprotection in a rat cardiac transplant model(denervated heart) suggesting the role of blood borne factors in preconditioning.(Konstantinov et al. 2005; Kristiansen et al. 2004)

Whether the neurogenic pathway was activated locally in the mesenteric, renal or skeletal beds or by release of mediators into the circulation (humoral pathway) and subsequent stimulation of sensory afferent fibres is unclear from these studies. The need for reperfusion to confer remote protection and rise in plasma levels of catecholamines, adenosine, neuropeptides, cytokines or free radicals suggests that these substances may activate neuronal pathways after release into circulation. It seems that both the neurogenic pathway and humoral pathway have some element of overlap and are not mutually exclusive**.** Measurement of interstitial levels of

mediators and sensory afferent nerve activity prior to and after preconditioning and specific blockade of synthesis and release of the mediators would help clarify their role in activation of neuronal pathways. Blockade of neuronal pathways and end receptors would help define the predominant pathway for transduction of preconditioning in different organ systems.

1.14 Role of Nitric oxide

Studies in direct IPC have demonstrated the protective effect of Nitric oxide on microcirculation.(Albrecht et al. 2003;Bauer et al. 1997;Gauthier, Davenpeck, & Lefer 1994;Kosonen et al. 1998;Kosonen et al. 1999;Kosonen et al. 2000;Kubes & McCafferty 2000;Langle et al. 1995;Lefer & Lefer 1996;Peralta et al. 1996;Peralta et al. 2001;Wink et al. 1993;Wink & Mitchell 1998) Nitric oxide is a free radical produced from L-arginine by the enzyme Nitric oxide synthase which has three forms eNOS (endogenous NOS), neuronal NOS(neuronal NOS) and iNOS (inducible NOS).(Albrecht et al. 2003; Kubes & McCafferty 2000) Of these eNOS and nNOS are constitutively expressed while iNOS is produced in response to cytokines and NF-kb. eNOS has a protective effect on microcirculation and always produces NO in small amounts which predominates in the circulation producing a protective effect on the microcirculation.

Evidence in remote ischaemic preconditioning

Six studies have demonstrated the role of NO(Kuntscher, Kastell, Altmann, Menke, Gebhard, & Germann 2002;Kuntscher, Kastell, Sauerbier, Nobiling, Gebhard, & Germann 2002;Kuntscher et al. 2002;Kuntscher et al. 2003;Kuntscher et al. 2003;Vlasov, Korzhevskii, & Poliakova 2004) in remote preconditioning of skeletal muscle, intestine, brain and heart. Kuntscher has shown a beneficial effect by hind

Limb preconditioning and protection of muscle flaps in the late phase of remote preconditioning. He showed that the protective effect was due to release of NO in the circulation by virtue of inducing hind limb ischaemia 24 hours prior to the ischemic insult in the contra lateral limb. Subsequently Kuntscher et al also showed that NO has a role in acute ischemic preconditioning after inducing hindlimb ischaemia.(Kuntscher, Kastell, Altmann, Menke, Gebhard, & Germann 2002; Kuntscher, Kastell, Sauerbier, Nobiling)

Wang(Wang et al. 2001) et al showed induction of myocardial iNOS in the late phase of preconditioning following intestinal ischaemia and reduction in myocardial infarct size as well as MPO activity(marker of neutrophilic activation). Blockade of NO activity abrogated the decrease in myocardial infarct size and MPO activity suggesting that NO inhibits neutrophil infiltration.

Tokuno et al showed that spontaneous brain ischemic events 24-48 hrs prior to cardiac IR injury reduced myocardial infarct size.(Addison et al. 2003; Tokuno et al. 2002) iNOS knock out mice did not show the beneficial effects of remote preconditioning. Vlasov demonstrated that NO has a role in direct IPC of the intestine and late phase of RIPC of the intestine but its role in the early phase of RIPC of the intestine remains unproven (Vlasov, Smirnov, & Nutfullina 2001) Vlasov et al (Vlasov, Korzhevskii, & Poliakova 2004)showed recovery of brain blood flow at 120 minutes after reperfusion and reduced cerebral oedema in the late phase of remote ischemic preconditioning (hindlimb) due to iNOS induced cerebral ischemic adaptation. However the early phase of preconditioning had no effect on brain blood flow or oedemas. A recent study has shown expression of NF-kβ due to brief limb ischaemia and NF-kβ induced iNOS 24 hours later which reduced myocardial infarct size.(Li et al. 2004) Deletion of iNOS genes in knock out mice abolished the

beneficial effect. This observation is supported from studies of direct IPC which have demonstrated that cytokines induce iNOS production. Conversely Petrischev showed that NO is not involved in remote preconditioning(Petrishchev et al. 2001) since the non-specific NO blocker L-NNA did not abolish increase in NADPH diaphorase activity in intact cardiomyocytes following limb/mesenteric remote ischaemia.(Petrishchev et al. 2001) Moreover attenuation of decrease in NADPH diaphorase activity in ischaemic tissue by preconditioning was not abrogated by NO blockers.

These studies demonstrate the role of NO in acute RIPC of the heart and muscle but its role in acute preconditioning of the intestine and brain remains unproven. IR injury impairs endothelial function in the initial phase primarily by impairment of formation and bioavailability of NO which may explain lack of effect of the early phase of RIPC in the brain and intestine. This may also be explained by different protocols of preconditioning used for remote preconditioning in different organs and different criteria for assessment of preconditioning in the heart & muscle (cytoprotective effects) as against endothelial function in the intestine and brain. Therefore similar outcome measures are needed to investigate the role of NO.

What induces NO?

In a mouse model increased NF-κß expression in the limb following RIPC induced NF- κß and iNOS in the heart suggesting the role of cytokines in the signalling pathway for induction of NO(Li et al. 2004). In a rat model, brief hepatic ischaemia increased gastric CGRP, increased (Xiao et al. 2001) reduced gastric mucosal flow, reduced gastric mucosal erosions and this effect was abolished by L-NAME (NO blocker) suggesting that CGRP induces NO release and subsequent vasodilatory effects and increased flow. Evidence suggests release of ROS, cytokines and NO into

the venous effluent following intestinal ischaemia reperfusion(Horton & White 1993) activates nuclear factor-kB and protein kinase C.(Downey & Cohen 1997) These signalling pathways induce iNOS(Kelly, Balligand, & Smith 1996) in the target organ. Peralta has shown adenosine increases NO formation and this effect is blocked by adenosine antagonists.(Peralta et al. 1999)

Pathway

The data from the studies discussed shows that brief periods of ischaemia reperfusion in RIPC induce NO pathways. Li et al showed that RIPC by hind limb ischaemia increased NF-kβ in skeletal muscle and heart and induced ischemic adaptation in the heart by iNOS formation. In view of the short half life of NO (5 seconds) it is unlikely for NO to be produced in the remote organ and reach the target organ to confer protection by the blood stream. Chen et al(Chen et al. 2005) showed that RIPC of the limb reduced myocardial infarct size through NO production. Since hexamethonium (autonomic ganglion blocker) did not abrogate reduction in myocardial infarct size it seems that NO pathways act through the blood stream. In an animal model of warm hepatic IRI Davidson's group showed increased hepatic venous plasma nitrates/nitrites(Kanoria et al. 2006) and amelioration of hepatic IR following hind limb RIPC . This provides further support for the argument in favour of bloodstream being the NO pathway. Clearly further clarification of this is needed by measuring systemic venous plasma nitrate/nitrite levels and plasma arginine levels (NO precursors) after RIPC. Also knockout models of NOS would be specific for investigating the role of NO pathways in preconditioning.

Is NO a trigger or mediator?

Tokuno demonstrated reduced myocardial infarct size following induced brief brain ischaemia without an increase in cardiac iNOS however; the protection was abolished

in NOS knockouts suggesting the role of NO as a trigger. Blockade of NO prior to RIPC by L-NAME abolished myocardial protection supporting the role of NO as a trigger.(Chen et al. 2005) Increased iNOS production 24 hours after RIPC would suggest its role as a mediator and future studies by blocking NO after RIPC are needed to demonstrate its role as a mediator.

Effector mechanism

IPC studies have demonstrated that NO modulates microvascular perfusion through its vasodilatory effect(Jaeschke et al. 1996) and through its anti-inflammatory actions including inhibition of stellate cell activation,(Rockey & Chung 1995) neutrophil adhesion,(Gauthier, Davenpeck, & Lefer 1994;Harbrecht et al. 1997;Lefer & Lefer 1996) platelet aggregation. NO plays a key role in initiating and maintaining preconditioning. The early phase of preconditioning mediated by eNOS is through generation of cGMP(Lochner et al. 2002) as shown by Lochner et al in the **myocardium**(Gidday et al. 1999) and subsequently inhibition of cAMP levels as well as reduction in energy demands. The late phase is protein synthesis dependent and is through activation of PKC, NF-kβ and transcription of iNOS.(Nandagopal, Dawson, & Dawson 2001a) In the **liver** NO mediates preconditioning by inhibitory actions on endothelin, (Peralta et al. 1996) activation of adenosine A2 receptors and subsequent NO formation.(Peralta et al. 1999) NO also has been shown to confer protection against cold ischaemia(Yin et al. 1998) of liver. NO inhibits apoptosis of cells by inhibition of caspase activity, TNF alpha and upregulation of Bcl-2.(Kim et al. 1998;Kim et al. 2000;Kim et al. 1998) NO has a protective effect on **intestinal** microcirculation such as scavenging of oxygen free radicals, maintenance of normal vascular permeability, inhibition of smooth muscle proliferation, reduction of leukocyte adherence to the mesenteric endothelium, prevention of mast cell activation

and platelet aggregation. (Guo et al. 1996; Kosonen et al. 1999; Lefer & Lefer 1996; Wink et al. 1993) Kubes et al(Kubes & McCafferty 2000) have demonstrated in the intestine that eNOS has a protective effect on intestinal mucosa while increased iNOS has been shown to increase mucosal apoptosis by generation of free radicals such as peroxynitrate.(Suzuki et al. 2000; Wu et al. 2002) However recent studies have shown that the production of peroxynitrate may be associated with loss of eNOS rather than increased iNOS production.(Cuzzocrea, Zingarelli, & Caputi 1998) This observation suggests a dichotomous role for NO in IR injury with small quantities of NO produced by eNOS reducing IRI while excessive NO due to iNOS causing deleterious effects.

1.15 Adenosine

Evidence

Adenosine is an extracellular molecule which is both a trigger and mediator of IPC as demonstrated from past studies.(Downey, Liu, & Thornton 1993) Adenosine is a hormone widely distributed in human tissues. Adenosine production occurs in myocytes, endothelial cells and vascular cells. During ischaemia of the heart ,brain and the kidney, the imbalance between oxygen supply and demand results in net breakdown of adenosine triphosphate (ATP) and release of adenosine which can increase upto 50 fold {Mullane, 1995 }

Three studies have shown the role of adenosine in RIPC of the heart by RAO and MAO. 1) Pell et al(Pell et al. 1998), rabbit model, RIPC by RAO (10 min), reperfusion (10min) prior to CAO 30 min, reperfusion 2hrs reduced myocardial infarct size by 46% and increased plasma adenosine levels. 2) Takaoka,(Takaoka et al. 1999) rabbit model, RIPC by RAO 10 min, 20 min reperfusion before 40 min CAO reduced infarct size, increased plasma adenosine levels and 8-SPT (adenosine blocker) abolished the

protective effects.3) Liem at al, RIPC by single cycle of MAO (15 min) and reperfusion prior to CAO increased plasma adenosine levels and reduced myocardial infarct size.

Two studies demonstrated the role of adenosine in RIPC of skeletal muscle

Adenosine release has been shown to be an effector molecule in skeletal muscle IPC.(Pang et al. 1997) 1) RIPC increased adenosine plasma levels and the protective effect was partially blocked with reserpine. 2) Prior adenosine blockade(Addison et al. 2003) did not completely abolish latissmus dorsi flap protection by RIPC (limb ischaemia, 3 × 10 min cycles) however adenosine blockade (8-SPT) and free radical scavenger MPG (mercaptopropionyl glycine) completely abolished RIPC effect suggesting that adenosine plays a partial role in RIPC.

Trigger or mediator

In a rabbit model adenosine blockade prior to RIPC abolished cardioprotection demonstrating its role as a trigger of RIPC(Pell et al. 1998). Adenosine blockade after RIPC before reperfusion abolished cardioprotection suggesting its role as a mediator of RIPC also.

Pathway

Following ischaemia induced ATP breakdown adenosine crosses the cell membrane and enters into the interstitial space by simple diffusion. From the interstitial space it escapes into the intravascular space by paracellular washout (slow, 10% under physiological conditions). It would seem that in RIPC adenosine produced in the remote organ would reach its target organ by the bloodstream, however it has a half life of 0.6-1.5 seconds as it is rapidly taken up by endothelial cells, red blood cells and pericytes which contain nucleoside transporters and are responsible for rapid degradation of adenosine(Horie & Ishii 2001) making it unlikely for adenosine to

reach its target by the circulation and there are no studies in RIPC which demonstrate modulation of adenosine degradation or activation of nucleoside transport inhibitors**.** Two studies showed a rise in plasma adenosine levels following preconditioning suggesting that reperfusion and release into circulation is required for adenosine induced RIPC**.**(Pell et al. 1998; Takaoka et al. 1999)

One study (Liem et al. 2002) supported a neurogenic pathway for adenosine in RIPC. They demonstrated abrogation of preconditioning of the heart by brief cycles of MAO and reperfusion due to prior ganglion blockade. To further clarify the pathway they demonstrated that intramesenteric infusion with adenosine mimicked the effects of brief MAO and prior ganglion blockade abolished the protective effects of RIPC. Ganglion blockade after reperfusion did not abrogate the protective effects but adenosine receptor blockade abolished myocardial protection. These observations suggest that adenosine acts locally to stimulate afferent nerves in the mesenteric bed which in turn activate adenosine receptors in the heart. This argument is supported by data demonstrating lack of preconditioning following intraportal or intracaval infusion of adenosine and effectively excludes effects of adenosine spillover during mesenteric infusion which could potentially stimulate adenosine receptors in the liver and contribute to myocardial protection.

Effector mechanism

8-SPT (adenosine receptor blocker) abolished RIPC by MAO. 8-SPT was given after reocclusion of mesenteric artery(Liem et al. 2002) preventing adenosine access to the mesenteric bed. This suggested the presence of adenosine receptors on the heart whose effect was blocked by 8-SPT.

Does adenosine modulate end effectors K_{ATP} channels?

In RIPC two studies (RAO reduced myocardial infarct size, rabbit model)(Pell et al. 1998;Singh & Chopra 2004;Takaoka et al. 1999) demonstrated that selective mitochondrial KATP channels blockers(5-HD) abolish the protective effect. The action potential of sarcolemmal K_{ATP} channels was unaffected. Modulation of K_{ATP} channels reduced ATP depletion, preserved intracellular PH, and enhanced recovery of ATP and phosphocreatinine levels during reperfusion (Takaoka et al. 1999). Decreased acidosis reduced intracellular Ca accumulation and myocardial infarct size. These observations are supported by IPC studies(Pang et al. 1997) and suggest that KATP channels serve as end effectors in adenosine pathways.

IPC studies have shown that adenosine mediates both the early and late phases of preconditioning via different endorgan receptors. In the heart adenosine acts via A1 receptor. Adenosine receptors mediate antiadrenergic effects indirectly by reducing C-amp levels. The A1 receptor mediated effects involve activation of Protein kinase C, Tyrosine kinases,(Dana et al. 2000) Heat shock protein and MAPK. Kinases modulate end effector mitochondrial KATP channels. A2 receptors act in the hepatic microcirculation as shown by Peralta et al(Peralta et al. 1999) A2 receptors are linked to vasodilatation and antiplugging effects of adenosine. Activation of these receptors and their effects are due to endothelium dependent and independent mechanisms, indirectly through release of NO and through direct relaxation of vascular smooth muscles. A3 receptors are found in myocytes.

The effects of adenosine include vasodilatation,(Horie & Ishii 2001) inhibition of leukocyte adhesion, neutrophil and platelet function {Bouma, 1997 ;Keller, 1991 }

and free radical production{Nolte, 1991 ;Cronstein, 1995 ;Cronstein, 1994 }. These have to be clearly demonstrated in Future studies in RIPC.

1.16 Cytokines

NF-KB

Li et al in a mouse model showed RIPC (6 cycles brief hind limb ischaemia) prior to myocardial IR reduced infarct size. IRI activated NF-kβ in hearts but prior RIPC attenuated activation of NF-kβ in IR.(Li et al. 2004) However RIPC in sham animals demonstrated NF-kβ activation in both the limb and hearts. This study demonstrates a dual role for NF-kβ. While excessive NF-kβ activation in IR injury has deleterious effects and increases infarct size, activation of NF-kβ following limb preconditioning led to an adaptive response in the heart, increased ikβ (inhibitory KB) expression which attenuated NF-Kβ activation following sustained IR injury and reduced myocardial infarct size , attenuated decrease in left ventricular developed pressures (LVDF) and increase in left ventricular end diastolic pressures (LVEDP) on reperfusion . In NF-kβ and iNOS knockout models the decrease in LVDF was attenuated but preconditioning did not confer any additional benefit. NF-kβ and iNOS knockouts had less severe increase in LVEDP but no additional attenuation was conferred by preconditioning.

NF-kβ is induced by reactive oxygen species and subsequently acts through activation of kinases in the preconditioning response. Preconditioning may downregulate the inflammatory response during reperfusion as NF-kβ activation increases its own inhibitor Ikβ (Tahepold) or it may act through a mediator such as iNOS as suggested by data from this study.

TNF –α

Increase in TNF–α levels following hepatic IR injury led to remote organ injury (lung and kidney) and IPC of the liver reduced both TNF–α levels and remote organ injury as demonstrated by Peralta et al. This study suggested that TNF–α may have a role in remote preconditioning. In RIPC two studies have investigated the role of TNF–α. Ates et al showed raised TNF–α levels following renal IR which were reduced by preconditioning with brief hepatic ischaemia and associated with improved renal function as compared to IR groups. RIPC by brief hepatic ischaemia prior to gastric IR (30min) attenuated plasma TNF-α levels in a rat model.(Brzozowski et al. 2004) Ren et al showed in a rat model of cardiac IR reduced myocardial infarct size following IPC and in TNF–α knockout mice. The protective effect of the early phase of IPC was not abolished in TNF–α knockouts but that of the late phase of IPC was abolished in knockouts suggesting that late IPC is TNF–α dependent. Both the early and late phase of RPCT (Abdominal incision prior to cardiac IR) in TNF–α knockouts further reduced myocardial infarct size suggesting that remote preconditioning of trauma was mechanistically different from TNF–α ablation.(Ren, Wang, & Jones 2004). Clinical efficacy of cardioprotective strategies maybe maximised by using a combination of RPCT and TNF–α ablation. This study also showed that blockade of NF-KB and TNF-α was additive suggesting NF-KB to be involved in the signalling pathway of TNF–α. The mechanism of TNF-α induced RIPC and IR is unclear and future studies are needed to study the mechanism and signalling pathways involved.

IL-6

In lung IR injury secondary to remote limb ischaemia reperfusion, blockade of interorgan inflammatory mediators such as cytokine Il-6 and levels of primed neutrophils by prior limb preconditioning confers protection with no significant

difference in levels of TNF-α. Conversely lung protection against local IRI by remote brief limb ischaemia is mechanistically different and entails incomplete blockade of SIRS mediators such as IL-6 and circulating primed neutrophils and complete suppression of Il-1β which is an early mediator of reperfusion injury. Therefore despite incomplete blockade it is likely that remote preconditioning reduces expression of adhesion molecules and neutrophilic infiltration in the lung.

IL-1β

RIPC has been shown to confer protection on the remote organ in lung dysfunction due to limb IR and local IR by blockade of IL-1β.

The role of IL-1β was demonstrated by Harkin et al who clearly showed an increase in IL-1β in limb IR which led to lung injury and deterioration of lung function. In sustained limb IR, limb IPC protected lung function from remote IR injury by blocking the cytokine IL-1β. Similar rise in IL-1β was shown by Waldow in IRI of the lung. RIPC of the limb ameliorated the rise in IL-1β and conferred protection on the lung. Thus IL-1β has been shown to have a role as an interorgan mediator of IR injury and as a mediator of local IRI.

1.17 Protein kinase C

Evidence

Wolfrum showed that protein kinase C (PKC) was responsible for reduction in myocardial infarct size after mesenteric ischaemia reperfusion and blockade of PKC by a highly selective inhibitor chelerythrine prevented reduction in infarct size. They showed an increase in myocardial Protein kinase C following RIPC and blockade of Protein kinase c abolished the protective effect. Weinbrenner showed RIPC by infrarenal aortic occlusion for 15 min followed by reperfusion reduced myocardial infarct size which was abolished by chelerythrine.

Pathway of transduction

There is evidence that myocardial PKC undergoes activation following ischemic stimuli in IPC.(Downey et al. 1994; Downey & Cohen 1997; Liu, Ytrehus, & Downey 1994) This leads to conversion of the cytosolic PKC to particulate PKC fraction thereby increasing the ratio of the particulate to cytosolic fraction**.** Subsequent activation of mitochondrial PKC receptors results in activation of tyrosine kinases and Map kinases which lead to opening up of mitochondrial potassium receptor K_{ATP} channels. Mitochondrial K_{ATP} channels serve as end effectors in modulation of mitochondrial energy flow and preservation of mitochondrial membrane integrity. Both humoral and neuronal pathways have an important role in PKC mediated RIPC. Wolfrum et al demonstrated increased plasma bradykinin levels following mesenteric ischemic preconditioning which activated myocardial PKC and blockade of bradykinin receptors with bradykinin antagonist HOE 140 abolished myocardial protection. Ganglion Blockade with hexamethonium did not alter the cytosolic to particulate ratio of PKC but prevented activation of PKC. These observations suggest that bradykinin induced PKC activation is a prerequisite for cardioprotective effect of RIPC, activation of PKC is a decisive step in conferring cardioprotection and both the bradykinin dependent humoral pathway as well as neuronal pathway are essential for PKC activation.

1.18 Bradykinin

Evidence

Shoemaker et al were the first to demonstrate that mesenteric preconditioning induced increased endogenous bradykinin levels which had a remote preconditioning effect on the heart and reduced infarct size following coronary infarction. Blockade of bradykinin receptors was associated with increased myocardial infarct size. However

bradykinin blockade in non preconditioned animals did not influence infarct size and in the absence of preconditioning there was no change in basal bradykinin levels (Wolfrum).

What is the pathway?

A combined sensory neurogenic and humoral pathway is strongly suggested in the bradykinin mediated RIPC (Shoemakers study). Following mesenteric ischaemia reperfusion there is local release of bradykinins which stimulates the sensory afferent nerves projecting on efferent nerves to the heart which in turn precondition the heart. Bradykinin receptors B2 are involved in sensory nerve stimulation and bradykinin receptor antagonists HOE-140 (Hoechst-140) abolish the protective effect. This study also showed that Bradykinin receptor blockade led to loss of protection in both direct and remote IPC but ganglion blockade abolished protection only in remote preconditioning. Thus direct preconditioning is associated with blood borne kininogens in contrast to the complementary effect of humoral and neurogenic pathways in RIPC. Bradykinins activate intracellular transduction of PKC as demonstrated by Wolfrum et al however the pathway downstream of kinases remains unclear. Although modulation of K_{ATP} channels is suggested but this needs to be clarified in future studies.

1.19 Catecholamines

Toombs et al showed IPC by 5 min of CAO, 10 min reperfusion in rabbits prior to 30 min CAO and 120 min reperfusion reduced infarct size.{Toombs, 1993} In reserpinised rabbits subsequent IPC failed to reduce infarct size suggesting the role of noradrenaline release in IPC. In RIPC two studies provided evidence to support the role of catecholamines. Oxman et al(Oxman et al. 1997) showed an increase in cardiac norepinephrine release and increased baseline plasma levels after limb

preconditioning due to a systemic stress response which was partially abolished by reserpine due to depletion of catecholamine stores. In reserpinised animals the antiarrhythmic effect of RIPC was blocked. Kharbanda et al showed that sympathetic blockade abolished RIPC resulting in increased myocardial infarct size. Verdouw et al demonstrated that increase in cardiac interstitial norepinephrine levels following ischaemia was cardioprotective but RIPC by cerebral ischaemia was ineffective due to inadequate increase in norepinephrine levels. The role of catecholamines in RPCT is only speculative and future studies are needed to clarify this.

Pathway and effector mechanism

In direct IPC endogenous catecholamines are known to act upon cardiac adenoreceptors stimulating myocardial protein kinase eventually leading to preconditioning of the heart.(Hu & Nattel 1995) In RIPC the likely pathway seems to be the sympathetic nerve pathway as reserpine abolished protection. It is also possible that catecholamines released into the circulation may act on sympathetic nerve endings in target organs however this needs to be further clarified by measurement of plasma catecholamine levels and use of adrenergic receptor blockers. The postreceptor mechanisms are unclear and the role of catecholamines in activation of kinases as well as modulation of K_{ATP} channels as end effectors needs to be clearly demonstrated in future experiments.

1.20 Opioids

Evidence

IPC of the intestine (3 cycles of 8 min ischaemia, 10 min reperfusion) prior to intestinal IRI (30 min MAO, 2hrs reperfusion) reduced intestinal injury, oedema, LDH and malionaldehyde levels(markers of oxidative stress)(Zhang et al. 2001). Pre-treatment with morphine mimicked the effects of IPC and naloxone abolished the

protective effects of both IPC and morphine.(Zhang et al. 2001) The intestine and colon are rich in opioid receptors and contain opioid peptides(Zhang et al. 2001). This study demonstrated an increase in endogenous opioid peptides in the effluent collected after IPC and suggested that opioids are released in response to oxidative stress to confer a protective effect against stress. Opioid release from the stress of brief IR could result in a remote protective mechanism.

Five studies have demonstrated the role of opioids in RIPC in intestinal, skeletal muscle and heart tissue.

1) Based on the theory that opioids are released as a natural response to stress, are released irrespective of whichever organ is stressed and act widely and ubiquitously on remote organs, Patel et al(Patel et al. 2002) demonstrated in a rat model that RIPC by MAO (15min), reperfusion 10 min prior to CAO (30min) and 2hrs reperfusion reduced myocardial infarct size which was abolished by naloxone. 2) RIPC (3 cycles hind limb ischaemia (10 min), 10 min reperfusion) prior to 4hrs of muscle flap ischaemia and 48 hrs reperfusion reduced latissmus dorsi, rectus abdominis and gracilis flap infarct size. This study demonstrated the remote protection of all skeletal tissue by RIPC. 3) RIPC by 15 infra aortic occlusion (IOA) and 10 min reperfusion prior to 30 min IOA and 2hrs of reperfusion reduced myocardial infarct size in a rat model(Weinbrenner et al. 2002) 4) RIPC (3cycles of 5min femoral A occlusion, 5min reperfusion) prior to 30 min CAO and 120 min reperfusion in a rat model reduced myocardial infarct size and plasma LDH levels(Zhang et al. 2006) and naloxone abolished these effects. 5) Dickson et al demonstrated a role for opioids in transferred preconditioning. Met and leu-enkephalins were liberated from the preconditioned donor rabbit heart into the coronary effluent which subsequently elicited protection when given to virgin acceptor hearts. Addition of naloxone to the coronary effluent

abolished opioid induced protection. Based on evidence that opioids induce ischaemic tolerance in the intestine in direct IPC and the presence of abundant opioid receptors in the intestine .Dickson et al treated ischemic gut with coronary effluent from preconditioned hearts and showed that the recovery of maximal contractile force of gut after ischaemia was enhanced by opioids.

Pathway

Opioid receptors are known to be present in neuromuscular regions, especially delta receptors(Evans, Hughes, & Smith 1995) and previous studies have described the humoral action of opioid receptors in skeletal muscle by demonstrating(Evans, Khan, & Smith 1997) that B-endorphins released in the circulation stimulate glucose uptake in muscle .In RIPC (hind limb) autonomic ganglion blockade by hexamethonium did not abolish the effects of opioids(Addison et al. 2003) suggesting the blood circulation to be the likely pathway of transmission of protection in opioids induced RIPC. The need for reperfusion following IOA for cardioprotection and lack of protection in occlusion without reperfusion(Weinbrenner et al. 2002) and the evidence for transferred preconditioning in virgin acceptor hearts by opioids in coronary effluent from preconditioned hearts are observations which support a humoral pathway.

Effector mechanism

Opioids act on receptors in the target organ .This has been shown in heart, skeletal muscle and intestinal tissue.(Addison et al. 2003; Patel et al. 2002; Zhang et al. 2006) Skeletal muscle flap protection by RIPC was abolished by selective δ1 opioid receptor antagonists and myocardial protection was abolished by selective K1 receptor antagonists. δ1 receptors have been shown in all species and human cardiomyocytes.(Weinbrenner et al. 2002) Activated δ1 opioid receptors induce

effects which mimic RIPC. In transferred preconditioning leu-enkephalins activate δ1 opioid receptors to induce ischemic tolerance in myocardium or gut.(Dickson et al. 2002)

Opioid induced RIPC prior to skeletal muscle IRI reduced ATP depletion, lactate accumulation, neutrophil infiltration and myeloperoxidase activity in preconditioned skeletal muscle(Addison et al. 2003) which was abolished by opioid receptor antagonists. These observations suggest that the energy sparing effect coupled with attenuation of lactate accumulation during early reperfusion is triggered by activation of opioid receptors. This is supported by IPC studies. (Cohen et al)

Opioid receptors modulate

One study have shown the role K_{ATP} channels in the end effector energy sparing effect.(Dickson et al. 2002; Zhang et al. 2006) Coadministration of Glibenclamide to coronary effluent from preconditioned hearts abolished transferred preconditioning in ischemic gut.(Dickson et al. 2002) RIPC by κ opioid receptors in the heart modulates mitochondrial pore mobility in the post receptor mechanism.(Zhang et al. 2006)

IPC studies have shown opioid receptors to act via inhibitory G protein , activation of multiple kinases with modulation of mitochondrial and sarcolemmal K_{ATP} channels serving as final end effectors.(Zhang et al. 2006) Future studies are needed to clarify postreceptor mechanisms in RIPC and resolve receptor subtypes.

1.21 Free radicals

IPC studies have shown that Free radicals can directly activate kinases leading to transcription of protective proteins {Yue, 2002}, and free radical scavenger mercaptopropionyl glycine (MPG) abrogates the protective effects of direct preconditioning. Weinbrenner et al were the first to show that free radicals are key candidate molecules in RIPC.(Weinbrenner et al. 2004) A single cycle of 15 min of

infrarenal aortic occlusion followed by 10 min reperfusion reduced myocardial infarct size significantly MPG blocked the effects of both RIPC and a single cycle of direct preconditioning but failed to block multiple cycles of direct preconditioning. This suggested that preconditioning is a graded phenomenon with multiple cycles producing a more robust preconditioning stimulus with free radicals being only partially involved in the mechanism of protection. This study did not demonstrate the source of free radicals. Recently Patwell et demonstrated the appearance of hydroxyl free radicals in the circulation following ischaemia reperfusion of the limb and the argument that free radicals maybe produced from the ischemic limb in RIPC was supported by Chen et al who (Chen et al. 2005)showed that myocardial infarction was reduced by 4 cycles of 10 min femoral artery occlusion-reperfusion was associated with elevation in whole blood free radical counts upto 2 hours following RIPC and since ROS levels were significantly low compared to IRI group this data suggests the role of low dose ROS in inducing preconditioning. In addition a period of reperfusion for generation and action of free radicals is needed. These findings collectively suggest that free radicals reach the remote organ via the blood stream in order to induce a preconditioning effect. Conversely in an animal model occlusion of the coronary artery before preconditioning supported a nerve pathway of transmission of the preconditioning effect(Chen et al. 2005).

Effector mechanism

Direct IPC studies have shown that reactive oxygen species (ROS) lead to release of triggers such as NO, catecholamines, adenosine and bradykinin, ROS activate intracellular kinases directly(Baines, Goto, & Downey 1997) and induce synthesis of protective proteins . Also ROS activate cytokine NF-Kβ which induces iNOS mRNA transcription 24 hours later to confer delayed protection in the target organ. None of

these pathways have been clearly demonstrated in RIPC. Chen et al showed that ROS induced elevation of heat shock protein and mitochondrial antioxidant enzymatic activity which helped maintain mitochondrial function and reduce apoptosis. However pre-treatment with MPG abrogated HSP and antioxidant activity. Further clarification of the end effector mechanism of ROS in RIPC , the source of ROS , pathway and the role of low dose ROS in maintenance of mitochondrial membrane permeability as well as prevention of apoptosis is needed. Also quantification of the levels of ROS which induce protective mechanisms need to be determined since high levels of ROS are the key elements in the initial cascade of IR injury.

1.22 KATP channels

Evidence

1) Moses(Moses et al. 2004) et al showed that RIPC (3 cycles hind limb occlusion) reduced latissmus dorsi infarct size which was abolished by nonselective blockers (glibenclamide) and selective mitochondrial channel blockers(5-HD)

2) Kristiansen et al showed RIPC of the donor heart by 4 cycles ischaemia (5min) of hind limb was abolished by blockade of nonselective and mitochondrial K_{ATP} channels and increased infarct size in donor heart on implantation. Administration of diazoxide (selective mitochondrial k channel activator) prior to explant conferred protective effects similar to RIPC suggesting that the effects of RIPC are memorised in the explanted hearts and are critically dependent on modulation of mitochondrial K_{ATP} channels.(Kristiansen et al. 2004)

3) Konstantinov et al showed that blockade of K_{ATP} channels following RIPC in the recipient increased myocardial infarct size in the implanted heart in the recipient.

4) Dickson et al showed blockade of K_{ATP} channels abolished transferred preconditioning in virgin hearts conferred by coronary effluent from preconditioned hearts

5) Mabanta et al showed that remote microvascular preconditioning is mediated by K_{ATP} channels and blockade of K_{ATP} channels abolished preconditioning.

6) Pell et al showed RIPC by 10 min RAO, 10 min reperfusion reduced myocardial infarct size and cardioprotection was abolished by K_{ATP} blockers (5-HD).

7) Addison et al showed remote skeletal protection by RIPC (3×10 min ischaemia) was abolished by 5-HD (selective mitochondrial channel blocker).

Pathway and effector mechanism

Two studies have demonstrated the modulation of IRI in denervated hearts in a transplant model and this suggests that the humoral pathway is involved in conferring protection. The role of opioids in transferred preconditioning supports the humoral pathway.

Are these K_{ATP} channels triggers of RIPC, mediators or End effectors?

Moses showed blockade of K_{ATP} channels prior to RIPC abolished protection suggesting their role in the trigger mechanism of RIPC. Blockade of K_{ATP} channels after RIPC abolished protection however blockade 10 minutes prior to ischaemia did not influence infarct size indicating a role of K_{ATP} channels in the mediation of signal transduction. This study demonstrated that the critical time period for K_{ATP} channels to remain open after triggering was 10 minutes further supporting a role in mediation of signal transduction. One study has shown the opening of mitochondrial K_{ATP} channels and ionic fluxes across mitochondrial K_{ATP} channels in the opioid pathway of RIPC(Zhang et al. 2006) and one study showed ionic fluxes across K_{ATP} channels in RIPC by the adenosine pathway.(Pell et al. 1998) These observations suggest that

K_{ATP} channels serve as end effectors in the pathway of RIPC and their role needs to be further clarified as endeffectors for other candidate molecules.

Mitochondrial or Sarcolemmal K_{ATP} channels?

All studies discussed above clearly demonstrate the role of mitochondrial K_{ATP} channels in RIPC as blockade with 5-HD (selective mitochondrial channel blocker) abolished RIPC. Pell et al showed that RIPC was abolished by 5-HD without affecting the action potential of sarcolemmal fibres or vasodilator effects of sarcolemmal K_{ATP} channels. The muscle used in Moses model was surgically denervated, not associated with changes in action potential and muscle contractility suggesting the unlikely role of sarcolemmal K_{ATP} channels in the energy sparing effect. Two studies showed K flux across mitochondrial K_{ATP} channels after RIPC (Pell and Zhang).

Pell showed that K_{ATP} channels reduce ATP depletion; maintain intracellular PH and phosphocreatinine levels in heart muscle after RIPC. Moses showed decreased neutrophilic infiltration, myeloperoxidase activity and ATP sparing effect in preconditioned skeletal muscle. These data suggest that K_{ATP} channel opening reduces the rate of ATP hydrolysis or mitochondrial ATPase activity thereby decreasing the rate of ATP depletion. Also opening of mitochondrial K_{ATP} channels decreases mitochondrial calcium load which preserves mitochondrial integrity. Mitochondrial volume is regulated by K_{ATP} channels and volume changes modify energy flow through the electron system thereby influencing energy transfer between mitochondria and cellular ATPases. These studies showed that RIPC applied in vivo exerts protection upon skeletal muscle and heart after explantation from the body and has potential beneficial effects in relation to heart transplantation, cardiopulmonary bypass, autologous skeletal muscle transplantation.

1.23 CGRP (calcitonin gene related peptide) and brain gut axis

Evidence

CGRP is a neuropeptide and principal neurotransmitter found in capsaicin sensitive sensory nerves. CGRP receptor antagonists abolished reduction in infarct size by IPC(Li et al. 1996) in a rat model suggesting the role of CGRP in mediation of IPC of the heart.

Five studies demonstrated the role of CGRP in RIPC by brief MAO. 1) Tang et al showed that brief MAO (10 min) and 2) Xiao et al showed that brief MAO (6 cycles, 4 min ischaemia, 4min reperfusion) increased plasma levels of (CGRP) and reduced myocardial infarct size, 3) Wolfrum et al showed that RIPC by MAO (single cycle, 15 min ischaemia and 15min reperfusion) increased plasma levels of CGRP following RIPC, reduced myocardial infarct size and blockade of CGRP receptors and ganglion blockade abolished RIPC. Ganglion blockade did not affect CGRP release, 4) Brzozowski et al showed decreased mucosal CGRP and blood flow in gastric IR and RIPC by hepatic/coronary ischaemia (2 cycles, 5min ischaemia, 10 min reperfusion) restored mucosal CGRP levels in gastric IR and gastric flow, 5) Brzozowski et al showed that prior treatment with CGRP receptor antagonist and prior treatment with Capsaicin induced sensory nerve deactivation abolished RIPC by MAO. Administration of exogenous CGRP in denervated animals restored RIPC effects.

What is the pathway?

An increase in plasma levels of CGRP as shown in three studies (Tang, Xiao and Wolfrum) supports a humoral pathway. However ganglion blockade abolished myocardial protection from brief MAO demonstrating a neuronal pathway for CGRP in RIPC by MAO (Wolfrum).

Capsaicin selectively depletes neurotransmitters in sensory nerves and is known to cause deactivation of sensory afferent neurons. Two studies in RIPC by MAO have shown that treatment with capsaicin prior to RIPC by MAO abolished both reduction in myocardial infarct size and increase in CGRP levels.(Tang et al. 1999; Xiao et al. 2001) Two studies in RIPC by brief hepatic artery occlusion and CAO(Brzozowski et al. 2004d;Brzozowski et al. 2004f) (brzozowski) also showed that prior treatment with capsaicin abolished the increase in gastric mucosal CGRP, increase in gastric blood flow and reduction in gastric erosions following RIPC. These observations strongly support the role of sensory neurons in RIPC. In addition Brzozowski et al showed that vagotomy abolished RIPC from brief hepatic ischaemia or CAO and reduced plasma levels of CGRP. This observation suggests that vagal efferents form a part of the effector pathway in CGRP mediated RIPC and demonstrates the role of the brain gut axis in RIPC. Finally L-name (NO blocker) abolished reduction of myocardial infarct size by CGRP mediated RIPC suggesting that CGRP acts via the NO pathway.(Xiao et al. 2001)

End effector mechanism

One study showed(Wolfrum et al. 2005) that in RIPC by MAO, CGRP activates myocardial PKC and reduced infarct size. PKC activation and myocardial protection was abolished by both ganglion blockade and CGRP receptor antagonists. These data suggest that in RIPC, CGRP acts at the cellular level by activation of kinases. Another study showed the NO pathway as discussed above and NO may be the endeffector responsible for CGRP effects. This argument is supported by past studies providing evidence for CGRP related NO release.(Xiao et al. 2001) One study showed the inhibition of cytokines (TNF-α and Il-1β) release in the target organ.(Brzozowski et al. 2004) Further clarification of the pathway downstream is needed in future studies.

1.24 Prostaglandins

Evidence

Brzozowski showed that Indomethacin (nonselective cyclo-oxygenase blockers), SC-560 (selective cox-1) and rofecoxib (selective cox-2) blockers abolished the gastric protective effects of IPC by 2 cycles of 5min celiac artery occlusion, RIPC by 2 cycles of 5min hepatic ischaemia or CAO(Brzozowski et al. 2004) Concurrent treatment with exogenous PGE2 counteracted the effects of Cox blockers and restored the hyperaemic mucosal effects of RIPC. Brzozowksi et al also showed increased gastric mucosal generation of PGE2 in IPC and RIPC. These observations suggest that increased endogenous activity of prostaglandins particularly of the COX-2 variety could be involved in preconditioning and it is known from past studies that they have a protective effect in gastric ulcers.

Pathway and effector mechanism

Brozozowski showed that functional ablation of sensory nerves by pretreatment with capsaicin abolished the protective effects in all preconditioned groups. This observation suggests the involvement of sensory afferent neurons in the pathway. Vagotomy abolished RIPC from brief hepatic/CAO suggesting the role of vagal efferents and the brain gut axis in mediation of RIPC. RIPC was associated with increased CGRP and decreased TNF-α and Il-1β expression and release suggesting amelioration of gastric IRI by prostaglandins through modulation of cytokine release and increased mucosal flow by vasodilatory effects of CGRP through NO release. These effects were attenuated by pre-treatment with capsaicin and ablation of sensory nerves known to release NO and CGRP.

1.25 Heat shock proteins (HSP) and Haemoxygenase (HO-1)

Heat shock proteins HSP28, 40,60,70,90,104 have an important physiological and pathological role. One of the most important heat shock proteins is HSP70 which has a cytoprotective role. The constitutive form of heat shock proteins is HSP73 which all cells express to some extent. The inducible form is HSP72.

Heat shock proteins expression is more in myocytes compared to micro vascular cells Enhanced heat shock protein expression may serve to protect the heart against ischaemia reperfusion, hypoxia and chemicals in addition to hyperthermia. Heat shock proteins express themselves as early as 3hours post reperfusion in the myocardium, begin to decay from 42 hrs post reperfusion and persist upto 72 hours as detected by immunohistochemistry.

Three studies in RIPC , Tanaka et al showed in a rabbit model RIPC by 4 cycles of 5min CAO and 5 min reperfusion(Przyklenk et al. 1993;Tanaka et al. 1998) increased cardiac tolerance due to increased HSP expression in the ischaemic and remote myocardium, Konstantinov showed RIPC by 6 cycles of 4min femoral artery occlusion and 4 min reperfusion increased expression of HSP73 genes(anti-inflammatory) in the heart of preconditioned mice as compared to sham(Konstantinov et al. 2005) and Chen et al in a rat model showed increased myocardial HSP expression (HSP 70) following hind limb RIPC.(Chen et al. 2005)

What induces HSP?

HSP is induced by ischaemia(Tanaka et al. 1998) and ROS.(Chen et al. 2005; Chen et al. 2005) Tanaka showed myocytes with immunoreactivity for HSP as early as 3 hours and persistence of this immunoreactivity upto 72 hrs. This suggests that HSP expression may be associated with a delayed phase of protection. Mechanisms by which ischaemia may induce HSP expression include enhanced tissue levels of

catecholamines and angiotensins which induce HSP expression, myocardial dyskinesia which induces compensatory hyper contraction of nonpreconditioned myocardium, increased workload and a modest increase in HSP in the non ischemic myocardium and elevated end diastolic pressure which leads to increased workload and HSP expression. However these need to be clearly demonstrated in experimental settings.

What is the pathway and effector mechanism?

One study showed that (Konstantinov et al. 2005) reperfusion stimulates HSP gene expression in myocardium following limb RIPC in a rat model. Chen et al demonstrated that reperfusion induced myocardial HSP expression following limb RIPC and this was abrogated by free radical scavengers (MPG)(Chen et al. 2005). These studies suggest that circulatory mediators stimulate HSP expression in remote tissue and free radicals are one of these mediators. The effector mechanism may involve scavenging of ROS and modulation of K channels but this need to be clearly demonstrated.

Haemoxygenase

Haemoxygenase (HO-1) is a form of inducible heat shock protein. It is an enzyme which mediates the rate limiting step of breakdown of haem to biliverdin, CO and iron. In this process it scavenges ROS and mitigates IRI. Previous studies have shown haemoxygenase to reduce hepatic IR and lung IR (Amersi et al. 1999;Coito et al. 2002;Fondevila et al. 2003;Katori et al. 2002).

One study showed RIPC by Limb ischaemia(Lai et al. 2006) (4cycles of 10 min ischaemia) reduced hepatic IRI and improved liver function. Haemoxygenase expression at 3hrs of reperfusion suggested its role in amelioration of IR. Zinc protoporphyrin (HO-1 inhibitor) 1 hr prior to IR abolished RIPC effects.

What is the pathway?

So far no studies have demonstrated HO-1 activity in serum following RIPC. It is likely that following RIPC peripheral breakdown of haem may lead to increased CO levels in the blood and CO has both vasodilatory and antiplugging effects in hepatic sinusoids. However no study has measured Post RIPC serum carboxyhaemoglobin levels to demonstrate this. Lai et al demonstrated no increase in HO-1 in peripheral macrophages following RIPC and therefore the role of peripheral macrophages as a pathway for HO-1 induction in the liver is unlikely. RIPC induces a low grade oxidative stress in the limb which may lead to release of ROS and cytokines into the circulation, low grade oxidative stress in the liver and increased hepatic HO-1 expression. This is hypothetical and needs to be proved by using ROS scavengers. HO-1 pathways scavenge ROS following IR, stabilise mitochondrial membrane permeability and reduce apoptosis by decreased cytochome C release as shown by previous studies. HO-1 also downregulates iNOS and inhibits NF-Kβ expression. Future studies are needed to define the pathway and effector mechanism of HO-1.

1.26 Effect of RIPC on inflammatory gene expression

The first study to look at effect of remote preconditioning on inflammatory gene expression modification in a human model showed that brief forearm ischaemia led to suppression of proinflammatory genes(Konstantinov et al. 2004) encoding proteins in circulating leukocytes, reduction in neutrophil chemotaxis, adhesion and exocytosis. Studies by Huda (RIPC MAO model) showed decreased inflammatory gene expression following brief MAO.

1.27 Clinical trials

Kharbanda et al conducted the first clinical trial in humans(Kharbanda et al. 2002) and showed that forearm ischemic preconditioning is associated with diminished

ischaemia reperfusion injury in the contra lateral arm as well as diminished endothelial injury. The mechanism was not clearly identified although it was thought to be due to a substance released in the circulation. In a recent trial in children undergoing cardiac surgery for congenital heart defects on cardiopulmonary bypass (CPB) Kharbanda et al demonstrated that four cycles of right hindlimb ischaemia (5 min) followed by reperfusion(5min) prior to CPB preconditioned the heart and reduced infarct size.(Cheung et al. 2006) Thereafter Kristiansen et al studied the effect of forearm ischaemia on cardiac ischaemia in cardiopulmonary bypass procedures and found it to be protective. This protective effect was attributed to KATP channels.(Kristiansen et al. 2004)

In humans Konstaninov(Konstantinov et al. 2004) showed diminished expression of inflammatory genes in neutrophils following limb RIPC. Since neutrophils are one of the key mediators of the late phase of IRI responsible for oxidative stress and injury to organs modulation of neutrophil activation would be of prime importance in reducing IRI.

The next page shows a tabulated summary of the studies on mechanisms in RIPC.

	Proposed mechanism	Site of index ischaemia	Site of preconditioning stimulus	Author/reference	model	Endpoint	Organ protection
1	?iNOS expression (Humoral mechanism)	Hind limb(right)cremaster flap	Hind limb(Femoral artery)	Wang W.Z. et al 2004	Rat	Flap necrosis	Flap protection
2	iNOS and NO	Cardiac ischaemia	Cerebral ischaemia	Tokuno et al (2002)	Mouse isolated heart	Infarct size	Reduced infarct size
3	NFkB and iNOS	Cardiac ischaemia	Hind limb	Guohu Li (2004)	Mice	Infarct size	Reduced infarct size Delayed protection
4	NO	Adipocutaneous flaps	Hind limb	Kuntscher et al 2003	Rat	Flap necrosis	Protection
5	NO	Adipocutaneous flaps	Hind limb	Kuntscher et al	Rat	Flap necrosis	Flap Protection
6	NO	Cardiac ischaemia	Hind limb	Chen X.G. Oct 2005	Rat	Infarct size	Reduced infarct size
7	NO	Rat cremasteric muscle flaps	Hind limb	Kuntscher et al	Rat	Flap necrosis	Protection
8	NO in direct preconditioning but not in RIPC.	Heart and intestine	Intestinal ischaemia	Vlasov et al (2004)	Rat	Infarct size	No myocardial protection. Intestinal adaptation
9	Role for iNOS	Cardiac ischaemia	Mesenteric	Wang Y.P. et al (2001)	Rat	Infarct size	Reduced infarct size
10	NO unlikely pathway.	Cardiac ischaemia	Direct cardiac and remote mesenteric ischaemia	Petrishcev et al	Rats	Infarct size	Reduced infarct size
11	Increased interstitial adenosine levels; afferent autonomic nerve stimulation, activation of myocardial adenosine receptors	Cardiac ischaemia	Mesenteric ischaemia	Liem et al (2002)	Rat	Infarct size	Reduced infarct size
12	Adenosine receptors and sarcolemmal Katp channels	Cardiac ischaemia	Renal	Pell et al (1998)	Rabbit	Infarct size	Reduced infarct size
13	Adenosine	Cardiac ischaemia	Renal	Takaoka et al (1999)	Rabbit	Infarct size	Reduced infarct size
14	?Adenosine	Cardiac ischaemia	RPCT	Xiaoping Ren et al (2004)	Mouse	Infarct size	Reduced infarct size
15	Angiotensin receptorss	Cardiac ischaemia	renal	Singh et al (2004)	Rat	Infarct size	Reduced infarct size
16	Autonomic nervous system	Endothelial limb injury	Hind limb	Loukogeorgakis Aug 2005	humans	Flow mediated dilatation	Endothelial function Protected
17	Neurogenic, humoral	Cardiac ischaemia	Mesenteric and renal	Gho 1996	Rat	Infarct size	Reduced infarct size
18	Bradykinin mediated and neuronal pathway	Cardiac ischaemia	Mesenteric ischaemia	Schoemaker et al (2000)	Rat	Infarct size	Reduced infarct size
19	Brain gut axis, sensory and vagal activation,CGRP release.	Gastric ischaemia	Hepatic/cardiac	Brzozowski T et al (2004)	Rat	Gastric lesions	Protection
20	Calcitonin gene related peptide.	Cardiac ischaemia	Mesenteric	Wolfrum 2005	Rat	Infarct size	Reduced infarct size

22	Capsaicin sensitive sensory nerves	Cardiac ischaemia	Mesenteric	Tang et al	Rat	Infarct size	Reduced infarct size
23	Cyclo-oxygenase 1 and cyclo-oxygenase-2, sensory nerve activation and release of CGRP, NO, inhibition ofIl-1 and TnF -α.	Gastric ischaemia reperfusion injury	Hepatic /cardiac	Brzozowski T et al (2004)	Rat	Gastric lesions and blood flow	Protection of the stomach
24	Cytokine IL-6	Lung IRI	Hind limb	Waldouw 2005	Pigs	Lung functions	Protection of lung functions
25	Inhibition of TnF – α induced selectin up regulation.	Kidneys,lungs and other organs	Hepatic ischaemia	Peralta et al (2003)	Rat	Organ failure	Protection of remote organs
26	Enhanced anaerobic glycolysis	Cardiopulmonary bypass	Upper limb	Gunaydin et al (2002)	Human	LDH,CK release	Not conclusive
27	Free radicals	Cardiac ischaemia	Hind limb	Chen YS Jun 2005	Rat	Infarct size	Reduced infarct size.
28	Heat shock proteins	Remote myocardial preconditioning	Cardiac ischaemia	Tanaka et al 1998	Rabbit	Infarct size	Reduced infarct size
29	Inflammatory gene suppression	Cardiac ischaemia	Hind limb	Konstantinov Nov 2005	Mouse	Infarct size	Reduced infarct size
30	Inflammatory gene suppression.	Cardiac ischaemia	Mesenteric ischaemia	Huda 2005	Rat	Infarct size	Reduced infarct size
31	K ATP channels	Cardiac ischaemia in explanted heart.	Hind limb	Kristiansen 2005	Rat	Infarct size	Reduced infarct size
32	K ATP, Late phase	Skeletal muscle	Hind limb	Moses Dec 2005	Rat	infarction	Reduced
33	KATP channel – pulmonary vascular dilatation and myocardial protection	Cardiac ischaemia	Iliac artery	Xia et al 2004	Sheep	Pulmonary function and pulmonary vascular resistance	Lung protection And decreased pulmonary vascular resistance.
34	KATP channels modulation in recipient	Cardiac ischaemia in transplanted heart	Hind limb	Konstantinov June 05	Rat	Infarct size	Reduced infarct size
35	KATP channels(early phase)		Hind limb	Moses et al 2004	Rat	Flap necrosis	Skeletal muscle protection
36	Ventricular pacing by activation of katp channels. .	Cardiac ischaemia	Mesenteric ischaemia, renal ischaemia and ventricular pacing	Verdouw et al	Pig	Infarct size	Reduced infarct size

37	Myocardial PKC activation	Cardiac ischaemia	Mesenteric	Wolfrum (2002)	Rat	Infarct size	Reduced infarct size
38	PKC and mitochondrial KATP channels	Cardiac ischaemia	Mesenteric ischaemia	Wang Y.P. et al (2002)	Rat	Infarct size	Reduced infarct size
39	Nor epinephrine	Cardiac ischaemia	Hind limb	Oxman et al (1997)	Rat	Reperfusion arrythmias	Decreased arrythmias
40	Norepinephrine	Cardiac ischaemia	Cerebral ischaemia	Verdouw et al 2001	Pig	Infarct size	No change in infarct size
41	Suggested adrenergic Pathway and blockade by reserpine.	Cardiac ischaemia	Hind limb	Kharbanda et al (2002)	Pig	Infarct size	Reduced infarct size
42	Opioid delta 1 receptors	Global skeletal protection	Hind limb	Addison et al	Pig	Ischemic necrosis	Skeletal protection
43	Opioid receptors	Cardiac ischaemia	Coronary effluent of donor following cardiac ischaemia	Dickson et al (1999)	Rabbit	Infarct size	Reduced infarct size
44	Opioid receptors and PKC ; humoral pathway.	Cardiac ischaemia	Infrarenal	Weinbrenner (2002)	Rat	Infarct size	Reduced infarct size
45	Metenkephalins	Cardiac ischaemia	Coronary effluent following cardiac ischaemia	Dickson et al (2001)	Rabbit	Infarct size	Reduced infarct
46	Endogenous opiods	Cardiac ischaemia	Mesenteric	Patel at al 2002	Rat	Infarct size	Reduced infarct size
47	Haemoxygenase	Liver ischaemia	Hind Limb ischaemia	Lai et al (2006)	Rat	Liver function	Improved liver function

Table 1.10- Studies on mechanisms in RIPC

1.28 Conclusions and Future work

Remote preconditioning is a novel method of preconditioning. Limb, mesentery and kidney are organs which can effectively be used to induce remote protection and reduce IRI. Brief periods of cardiac ischaemia confer protection on the lungs and vice versa. This may be of benefit in cardiovascular surgery and coronary bypass procedures.

Similar protection in musculoskeletal flaps and protection of the brain function needs to be evaluated in clinical trials. Adenosine, NO, PKC, Bradykinins, Catecholamines, Opioids, HO-1(HSP), Free radicals and Cytokines are the candidate mechanisms which form part of a complex cascade involved in transduction of the preconditioning effect with overlap between neurogenic and humoral pathways. The RIPC stimulus requires reperfusion providing unequivocal evidence of a humoral mediator. However there are many circulating mediators such as NO, Opioids and Adenosine which have undergone selective blockade to abrogate RIPC. The end effect of the remote stimulus

is to reduce the mitochondrial permeability in the target organ conserving cellular ATP levels and reducing apoptosis. Microarray analysis has shown transcription of anti-inflammatory genes and modulation of oxidative stress to be of prime importance in preconditioning. Application of remote preconditioning in clinical settings has so far been limited and its use particularly in organ transplantation is of interest.

Kanoria et al demonstrated improved liver function by RIPC in hepatic IRI and increased hepatic blood flow by ICG and laser Doppler flow measurements[13]. Lai et al demonstrated improved liver functions and the role of haemoxygenase in hepatic protection(Lai et al. 2006). Gustaffson demonstrated protection of hepatic function by remote preconditioning(Gustafsson et al. 2006). None of these studies addressed the in vivo microcirculatory changes seen in hepatic IR and the effect of RIPC on hepatic IR. We focused our study on the hepatic microcirculatory changes in hepatic IR and the modulation of hepatic microcirculation by RIPC. We hypothesised that RIPC modulates hepatic microcirculation in IR injury and applied intravital microscopy to investigate effects of RIPC.We also investigated the role of RIPC induced haemoxygenase (HO) expression in the liver in modulation of hepatic microcirculation in IR and the carried out the first study to investigate the effects of remote ischaemic preconditioning in a recovery model of hepatic IR after 24 hours of reperfusion. In addition we investigated the effects of HO on neutrophil activation and mitochondrial cytochrome c induced activation of caspase apoptotic pathway.

Chapter 2

Material and Methods

Summary of material and methods

Overview of end points and methods of assessment in this study

In this study we evaluated the microcirculatory changes of IR and its alteration by RIPC by applying intravital microscopy. Injection of RBC labelled with fluoroisothyocyanate enabled us to assess sinusoidal flow, RBC velocity, sinusoidal diameter and perfusion in a real time manner. The endothelial neutrophil interactions representative of the inflammatory cascade were assessed by intravital microscopy after injecting neutrophils labelled with rhodamine 6 G. Serum was used for assessing transaminase levels (markers of hepatocellular injury) and plasma for CINC levels (cytokine levels) by ELISA in all animal groups.

At the end of reperfusion (180 minutes) liver tissues were harvested and stored in formalin for histological assessment of liver tissue. Immunohistochemistry was carried out to assess expression of haemoxygenase and its spatial distribution. Snap frozen liver tissue was used for western blot assessment and quantification of haemoxygenase expression and for mitochondrial cytochrome c levels.

2.1 Experimental protocol

2.1.1 Animals and surgical procedures.

The Study was conducted under project license from the Home Office in accordance with Animals (Scientific Procedures) Act 1986. Male Sprague –Dawley rats, weighing 250-300gms 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 chew pelletes and libitium. Animals were anaesthetized with 0.5-1% isoflurane and maintained with 1-1.5l/min of N_2O and 0.5-1.0% 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).

The technique of remote ischaemic preconditioning involves using a tourniquet, which is tied around the thigh in one of the hind limbs. This tourniquet is tightened till until the manometric pressure is twice the systolic pressure and there is no audible Doppler signal in the leg. The procedure includes 5 minutes of ischaemia followed by 5 minutes of reperfusion. This will be repeated four times(Kristiansens model) in order to obtain a significant beneficial effect based on past experimental evidence in animals(Goto et al. 1995) that three or more brief periods of preconditioning are more effective than a single period in animals. At the end of the experiment the animals will be killed by exsanguination.

Polyethylene catheters will be inserted into the right femoral/carotid artery for monitoring of mean arterial blood pressure and right jugular/femoral vein for administering normal saline (1ml /100gm body weight 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 was mobilised. 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. This model facilitates splanchnic decompression. Total hepatic ischaemia can potentially lead to splanchnic congestion and cytokine release leading to false results. Ischaemia was for a period of 45 minutes followed by 3 hours of reperfusion to assess the early period of hepatic IR(Koti et al 2002) and 24 hrs reperfusion to study the late phase.

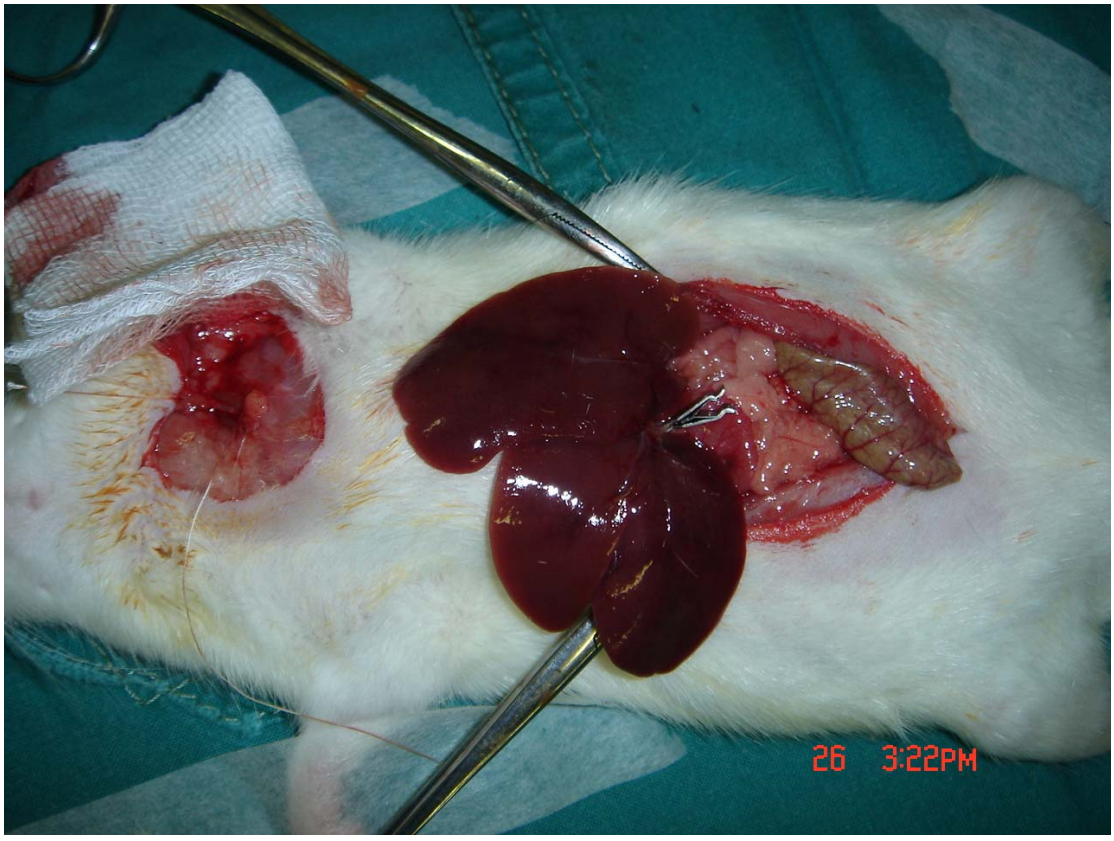


Fig 2.1 Animal model of Partial hepatic ischaemia (70%

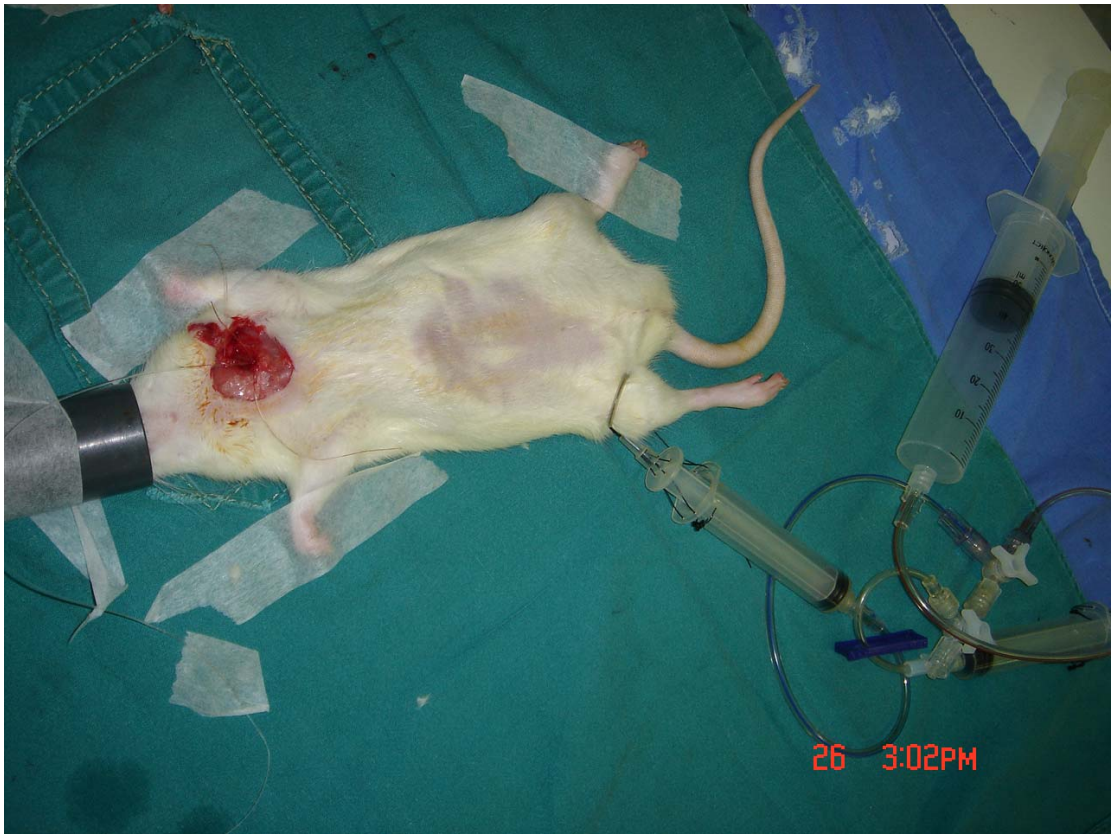


Fig 2.2 Animal model of Remote ischaemic Preconditioning

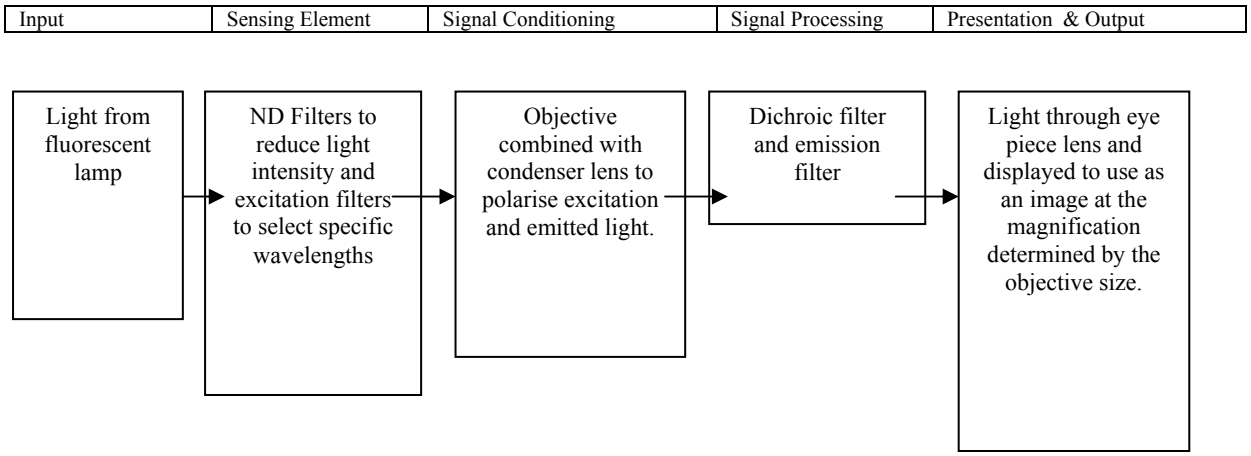
2.2 Intravital fluorescent microscopy

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) so that it could be used to examine opaque tissues{Kasten et al}.

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 2.3).

Figure 2.3 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 2.4)

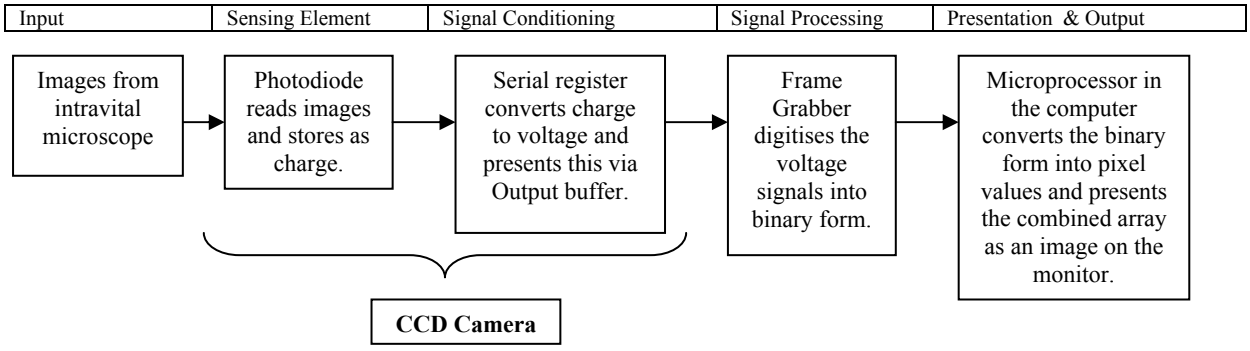


Figure 1.4 Image recording system in the intravital microscope

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 2.1. (Fig 2.5)

Table 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

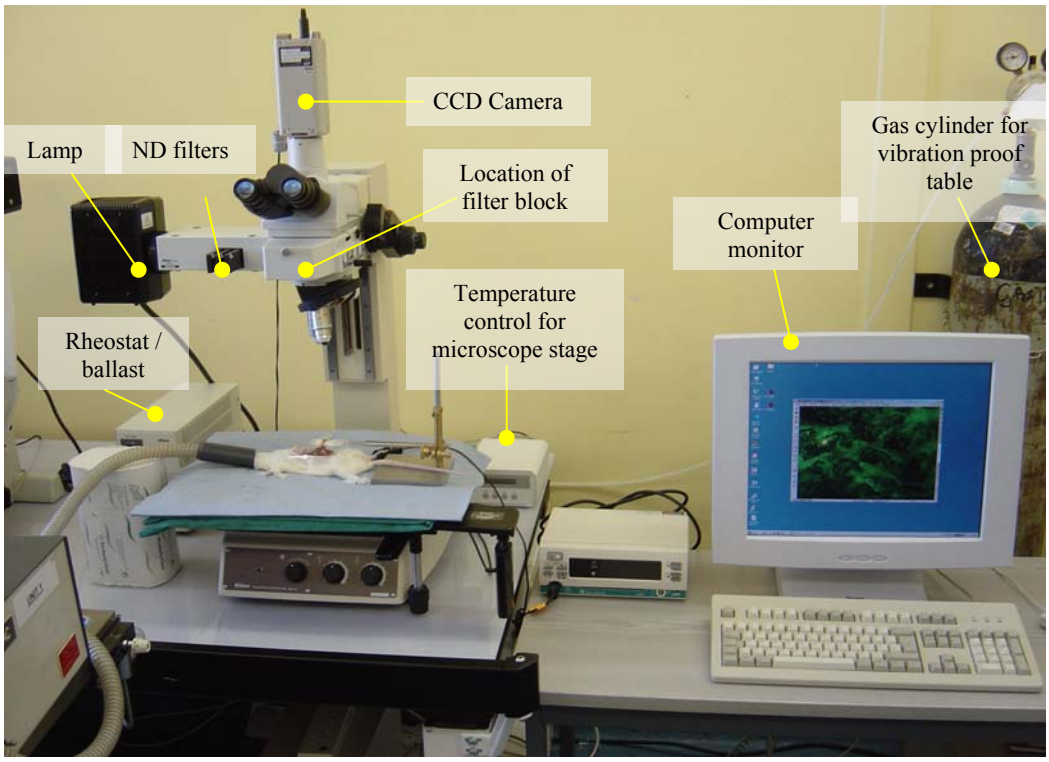


Figure 2.5 The complete intravital microscopy setup.

2.2 Principles

One of the main characteristics of the intra-vital fluorescence microscope is that it requires fluorescence. Fluorescence is the 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.

The chromophores are excited by an external light source; they absorb the 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, 1991 2 /id} – 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}.

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 fluorescence probe which produces emission light of a particular wavelength.

A dichromatic mirror allows passage of excitation wavelengths of certain values and emission wavelengths of certain values. According to Stokes' law the wavelength of emission is of lower energy and therefore longer wavelength than that of the excitation. 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 2.6

.

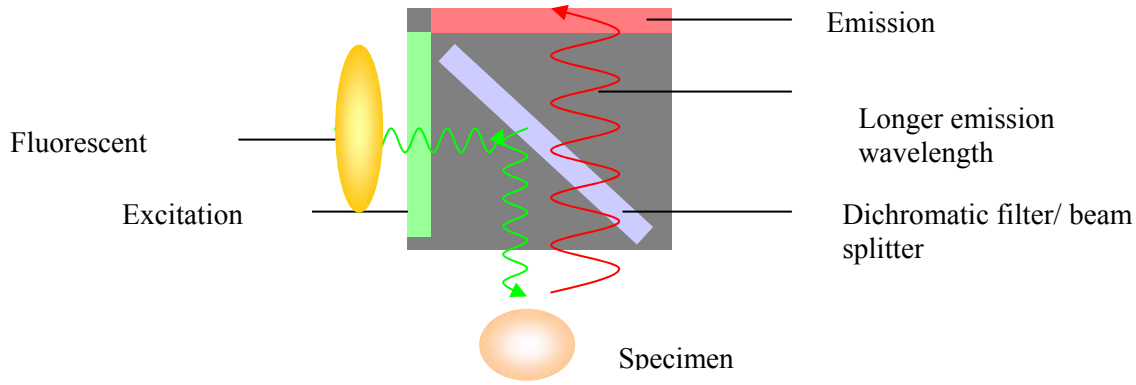


Fig 2.6 Schema of Filter Block

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 mistakenly 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.

2.3 Components

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.

2.3.2 Objectives

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 micrometers 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 et al}. 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 et al}.

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.

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. 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. In video rate CCD cameras, this process occurs at regular video-rate of 60 Hz in RS 170 format. However the JVC camera used in this study produces an image acquisition rate of 50 Hz).

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.

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.

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.

2.3.6 Preparation of FITC (Fluoro isothyocyanate) labelled RBC, Rhodamine and Propidium iodide.

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.

Preparation of Rhodamine 6G (Sigma Aldrich)

Rhodamine 6G was prepared by dissolving in normal saline and the dose given was 0.3mg /kg i.v. Dose used was based on previous experiments by Wunder et(Wunder et al)

Preparation of Propidium iodide (Sigma Aldrich)

Propidium iodide was dissolved in normal saline and administered at the end of the experiment intrarterially before termination of the animal to assess the number of dead hepatocytes. The dose used was 0.5 kg/ml based on previous experiments by Potter et al.

2.3.7 Intravital videofluorescence microscopy

The animals were maintained under anaesthesia with isoflurane and oxygen .Their temperature was maintained by a warm mat which was regulated by thermostat.

The liver was exposed and placed upon a glass mount and covered with a cover slip. The liver was continuously irrigated with normal saline. A drop of saline was placed on the cover slip and the tip of the lens of the microscope was immersed in the saline drop.

A Nikon microscope attached to a video camera was used. The power of magnification was 10x and 40x.The microscopy images were transferred by the camera to the video monitor and recorded for offline analysis. Quantitative assessment of microcirculatory parameters was performed offline by frame by frame analysis of the videotaped images. Microcirculation was assessed by evaluating acinar perfusion in ten randomly chosen acini and leukocyte endothelial interaction in ten postsinusoidal venules.

RBC velocity (V)

- 0.5 ml of FITC labelled red cells suspended in glucose saline buffer solution (20mgFITC /ml of RBC) were given intravenously to assess the velocity of RBC flow. Ten randomly chosen nonoverlapping rappaport acini were assessed.

- The RBC velocity was calculated by assessing the length (L) of RBC movement (microns) in each sinusoid in subsequent frames. 25 frames were captured per second. Hence the velocity was calculated using the formula= L×25/number of frames moved.

Sinusoidal Perfusion and perfusion index (PI)

The sinusoidal perfusion index was evaluated as ratio of perfused sinusoids (Continuous (CP) + intermittent (IP)) to the amount of total visible sinusoids which includes non perfused sinusoids as well. (Scp +Sip/ Scp+Sip+Snp).

Sinusoidal diameter (D)

This was measured by assessing the length across the sinusoids and expressed in microns. {Potter et al}

Sinusoidal blood flow

The sinusoidal blood flow was calculated using the formula- Velocity (V) ×22/7× (D/2)2. {Potter et al}

2.3.8 Neutrophil adhesion

Rhodamine 6G (0.3mg/kg) was given intravenously for staining of neutrophils. Rhodamine has an affinity for binding to neutrophils and its use for visualising adherent neutrophils is well established (Wunder et al).The numbers of sticking leukocytes were counted as those stationary for a period of 30 seconds under green filter light and expressed as leukocytes/mm2.The area of the vessels was calculated using the product of diameter and length assuming cylindrical geometry (3.14×D×L).

2.3.9 Hepatocellular death

Hepatocellular death was assessed by intravenous injection of Propidium iodide (0.15mg/kg i.v) prior to termination of the animal and the number of nuclei stained with the dye were counted under the camera and expressed as number of apoptotic

cells/high power field. Propidium iodide is a drug with affinity for dead cells (oncolytic necrosis/ apoptosis) as it penetrates the cell membrane to stain dead nuclei. (Brock&Potter et al)

2.4 Data collection and statistical analysis

Data from the pulse oximeter, pressure monitor were collected through the time period of observation on a laptop. All values are expressed as mean ±standard error of the mean (SEM). Analysis of variance (ANOVA) and Bonferroni adjustment for multiple comparisons were used unless otherwise stated, where unpaired Student's *t*-test was used for statistical analysis between groups. $P< 0.05$ was considered statistically significant.

2.5 Biochemical assays

The serum was thawed and analysed on a Hitachi 747 auto-analyzer (Hitachi Ltd, Tokyo, Japan) by using commercially available enzymatic kits (Boehringer Mannheim Ltd., East Sussex,UK) for lactate dehydrogenase (LDH), aspartate aminotransferase (AST) and alanine aminotransferase (ALT).

2.6 Histological investigations

At the end of the experiment the liver was removed, 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. The paraffin sections were dewaxed by immersing them in xylene three times for duration of 2 minutes each followed by a gap of 2 minutes. Subsequently the sections were immersed in 100% OP twice followed by 74%OP once and thrice in water for 2 minutes each. Sections were stained in haematoxylin 5 mins and then differentiated in 1% acid alcohol. Blue in tap water .Stained with 1% Eosin 5mins and de-Hydrated in alcohol and cleared in Xylene & mounted in DPX.

Assessment by histology was done using modified Suzuki criteria

Numerical assessment	0	1	2	3	4
Sinusoidal congestion	None	Minimal	Mild	Moderate	Severe
Vacuolation	None	Minimal	Mild	Moderate	Severe
Necrosis	None	Single cell	30%	60%	>60%

2.7 Evaluation of spatiotemporal expression of haemoxygenase by immunohistochemistry.

2.7.1 Controls

Positive control section for each antibody was used and included in every run. The positive control used was recombinant HSP 32 obtained from abcam laboratories .The negative control used was normal rabbit serum instead of the primary antibody (recommended dilution, 1/4000).

2.7.2 Antigen retrieval – in order to retrieve the antigen the following steps are essential-

Blocking endogenous peroxidase.

Slides were immersed in 0.3% hydrogen peroxide in methanol (for a full rack of slides this is 4ml H_2O_2 in 400ml of methanol) for 30 minutes.

Blocking non specific background.

Non-specific protein binding sites were blocked with serum from goats because goat serum contains antibodies which bind with the non specific antigenic binding sites thus preventing them from binding secondary antibody.One drop of blocking solution (50-100µl) was placed on each section and left for 1 hour.

Blocking endogenous avidin-biotin

Microwave /autoclave pre-treatment enhances avidin biotin activity in immunostaining sections. Avidin biotin activity was blocked.

2.7.3 Primary antibodies.

Primary antibodies used were anti rabbit polyclonal IgG for HO. All primary antibodies were diluted with PBS (1:200) 50-100µl for each section. This antibody chosen was a polyclonal antibody which detected HO-1. The incubation period was overnight.

2.7.4 Secondary antibodies.

Goat serum (anti rabbit monoclonal IgG) was used as secondary antibody because this would specifically bind to the polyclonal rabbit antobody The dilution used was 1:50 , duration of incubation was 45 minutes and the positive reaction was identified by ABC kit (Santa Cruz biotechnology, Santa Cruz, Ca with peroxidase as substrate and 3.3' diaminobenzidine tetrachloride as chromogen was used.

2.7.5 PBS and antibody diluents

PBS (0.01M) containing 0.1% bovine serum albumin and 0.1% sodium azide is used as a diluent for all immunoreagents except those containing peroxidase (since azide inhibits peroxidase and would prevent the immunoreaction becoming visibly labelled). Dilute peroxidase conjugates in plain PBS. The purpose of adding azide is to prevent contamination with fungi etc. The purpose of the BSA is to provide a high concentration of "inert" protein to compete with the diluted antibody for non-specific binding sites on the walls of the storage vessel. It may also absorb any anti-albumin that may be present and might produce unwanted staining.

2.7.6 Method of HO-1 immunohistochemistry and controls.

The method used for HO-1 immunohistochemistry has previously been used (polka and Van Noorden et al).Positive control section for each antibody was used and included in every run. The positive control used was recombinant HSP 32 (abcam

lab). The negative control used was normal rabbit serum instead of the primary antibody (recommended dilution, 1/4000).

Reliability of technique

The technique used is the indirect immunoperoxidase technique well described in the literature (Nakane and Pierce 1967). Although it is a sensitive technique to identify HO protein and its distribution antibodies used are not highly specific and may cross react with other antigens. The technique does not help quantify HO expression.

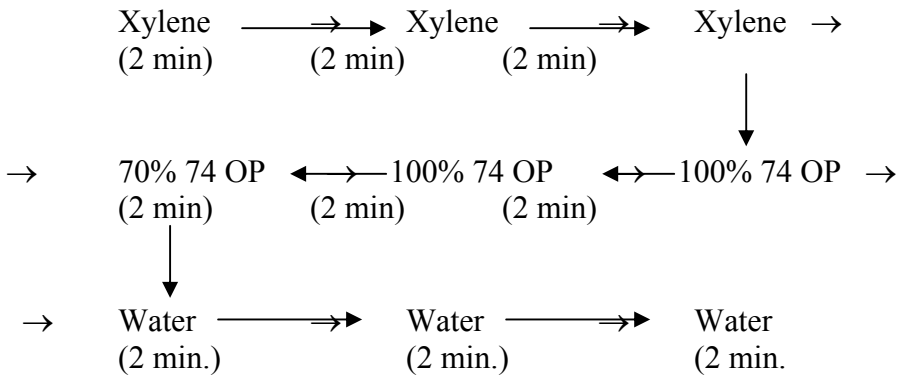
2.7.7 Immunostaining procedure

The paraffin fixed slides were dewaxed in several changes of xylene and then rehydated through absolute alcohol, 90%alcohol, 70%alcohol to distilled water or deionised water (1-2 minutes in each solvent). Mercury pigment was removed with iodine. In order to retrieve antigen the slides were immersed for 30 min in a solution of 0.3% H2O2 (4ml of H2O2 per 100 ml) in methanol to inactivate endogenous peroxide. The slides were rinsed in phosphate buffer solution (PBS). This was followed by blocking of non-specific background with 100 ml of normal rabbit serum (diluted 1:30 with PBS) for 30 minutes. The serum was drained off onto paper tissue. The primary rabbit polyclonal antibody was diluted in PBS containing 0.05% bovine serum albumin and 0.01% sodium azide at room temperature for 2hrs. The slides were rinsed with PBS three times for 5 minutes each and then incubated for 30minutes with 50-100 µl of 1:200 diluted biotinylated antibody solution(anti rabbit IgG). This was followed by three rinses with PBS for 5 minutes each. The slides were then incubated with secondary antibody (1:50 for 45 minutes)) 50-100µl on each slide for 45 minutes Finally the sections were incubated with vectastain ABC reagent. As per kit instructions 1 drop A + 1 drop B into 5ml PBS. 50 - 100υl were put on each and left for *30 min* and then rinsed with PBS. Slides were placed in rack – 3 x 5min **DAB**

(Diaminobenzidine) for approx. *5-10* minutes 10min and counterstained with haematoxylin (approximately 40 sec). Dehydrated and mounted.

Immunostaining Steps

- The sections were dewaxed & re-hydrated



- **Antigen Retrieval**
- Endogenous peroxidase (**0.3% H_2O_2**): For 30 minutes.

  400ml MeOH + 4ml 30% H_2O_2

- Washed with PBS×3.
- 100µl of 1:30 **normal rabbit serum** on **each**. Leave for *20 min* to *2 hr (30 min)*.
- **Primary antibody** (rabbit polyclonal IgG) in optimum dilution – 50 - 100µl was applied on each section.

  Sections left for *2 hr* at room temperature / ***overnight*** **at 4^oC**
- Rinsed with PBS: sldes were placed in rack – 3 x 5min
- **Secondary antibody** (goat serum anti rabbit IgG)

  50 - 100µl of 1:200 on each slide for *45 minutes*.
- Rinsed with PBS: Place slides in rack – 3 x 5min
- **ABC**: Important: Prepare at least 30 min in advance

  As per kit instructions 1 drop A + 1 drop B into 5ml PBS

  50 - 100µl on each and leave for *30 min*.
- Rinsed with PBS: Place slides in rack – 3 x 5min

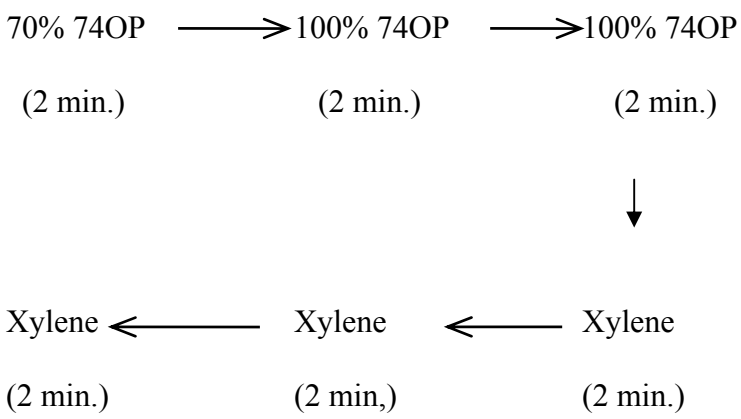
- **DAB** (Diaminobenzidine) for approx. *5-10 minutes **(10min.)***.

  PBS 400ml + DAB (50mg/ml) 4ml=1:100+30%H_2O_2 200υl=0.015%

  (Denature DAB with household bleach before disposal).

  Check development regularly (some antibodies need only a short incubation)

- Counterstained with haematoxylin (approximately 40 sec)
- Dehydrated and mounted



- Mounted with coverslip using pertex (line of pertex and drop of xylene).

The immunohistochemistry slides were studied by light microscopy. One section from each animal (6 animals in one group) was used for immunohistochemistry assessment.

2.7.8 Assessment and quantification

Paraffin embedded tissue was stained with haematoxylin.An objective scoring method was devised which assessed the intensity of sinusoidal macrophage staining graded from 0 (no positive stain) to 4 (intense and crisp brown stain). A percentage positive score was obtained by multiplying the grade with the percentage of stained macrophages. The maximum score possible was 400 (4X100%) while the minimum was 0 (0X0%).

2.8 Western blot analysis for haemoxygenase (HO-1)

Haemoxygenase protein was identified using Western blotting. Frozen samples of Liver tissue (100- 200 mg) were homogenised in TOXEX buffer (20 mM HEPES [pH

7.9], 0.35 mol/L NaCl, 20% glycerole, 1% Nonidet P-40, 1 mmol/L $MgCl_2$, 0.5 mmol/L ethylenediaminetetraacetic acid [EDTA], 0.1 mmol/L ethylene glycolbis(b-aminoethyl ether)-*N*,*N*-tetraacetic acid [EGTA], 100 mmol/L dithiothreitol [DTT], 0.1% phenylmethylsulfonyl fluoride [PMSF], 10 mg/mL aprotinin) on ice, incubated for 30 minutes, and centrifuged at 13,000 rpm for 5 minutes. For each lane, 100 mg of protein was dissolved in 10 mL of 13 sodium dodecyl sulfate loading dye and boiled for 5 minutes. A biotinylated protein marker (New England Biolabs, Schwalbach, Germany) was added. The samples were separated on a 12% sodium dodecyl sulfate–polyacrylamide gel and transferred to a polyvinylidene fluoride membrane (Immobilon-P, Millipore, Bedford, MA) by electroblotting for 2.5 hours (Semidry Trans-Blot, BioRad, Hercules, CA). The membrane was blocked in a buffer containing 20 mmol/L Tris-base (pH 7.6), 137 mmol/L NaCl, 3.8 mL 1 mol/L HCl/L, 0.1% Tween (13 TBST), and 5% low-fat dry milk powder for 1 hour and incubated with a rabbit polyclonal anti–HO-1 antibody (1:1,000 dilution; SPA 895, StressGen, Biotechnologies, Victoria, British Columbia, Canada) in 13 TBST and 5% low-fat dry milk for 2 hours at room temperature. After 3 washing steps with 1 X TBST, a secondary anti-rabbit (1:10,000 dilution; ECL-detection kit, Amersham Pharmacia, Freiburg, Germany) and horseradish peroxidase– conjugated anti-biotin antibody (1:1,000, New England Biolabs) was added and incubated for 1 hour in 1 X TBST and 5% low-fat dry milk. Following 2 washing steps with 1 X TBST and 2 washing steps with 1 X TBS, detection was performed by the ECL detection kit (Amersham Pharmacia) according to the manufacturer's instructions. After this the membrane was exposed to a digital camera as part of an electronic imaging system to visualise the proteins bound to the antibody.

Controls

Positive control used was recombinant HSp 32 manufactured by Stressgen laboratories.

Quantification and measurement

Densitometry

This was done by using Adobe Photoshop. The JPEG picture of the western blot was analysed using this software. The maximum density of the film is 255. The density of the background on the film was measured and the density of the HO band obtained for each sample was measured. The density of the background was subtracted from the density of the HO band. The value obtained was subtracted from 255 to obtain the final density of the sample.

2.9 Elisa for CINC-1

2.9.1 The assay

The Quantikine Rat CINC-1 Immunoassay is a 4.5 hour solid-phase ELISA designed to measure rat CINC-1 in cell culture supernates, serum and plasma. It contains E. coli-expressed recombinant rat CINC-1 and antibodies raised against the recombinant factor. This immunoassay has been shown to accurately quantitate the recombinant factor.Results obtained using natural rat CINC-1 showed linear curves that were parallel to the standard curves obtained using the Quantikine kit standards. These results indicate that the Quantikine rat CINC-1 kit can be used to determine relative mass values for naturally occurring rat CINC-1.

2.9.2 Principle of assay

This assay employs the quantitative sandwich enzyme immunoassay technique. A polyclonal antibody specific for rat CINC-1 has been precoated onto a microplate.Standards, controls and samples are pipetted into the wells and any rat

CINC-1 present is bound by immobilised antibody. After washing away any unbound substances, an enzyme linked polyclonal antibody specific for rat CINC-1 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells. The enzyme reaction yields a blue product that turns yellow when the stop solution is added. The intensity of the colour measured is in proportion to the amount of rat CINC-1 bound in the initial step. The sample values are read off the standard curve.

Standards used in the assay

The undiluted rat CINC-1 standard served as the high standard 500 pg/ml**.** Calibrator diluent served as the zero standard (0 pg/ml).

Control used in assay

Rat recombinant CINC in a buffered protein base was used as positive control**.**

Reagents

Rat CINC-1 microplate- one 96 well polystyrene microplate coated with polyclonal antibody specific for rat CINC-1.

Rat CINC-1 conjugate- 12.5ml of a polyclonal antibody against rat CINC-1 conjugated to horseshoeradish peroxidise with preservatives.

Rat CINC-1 Standard- 2.5 ng of recombinant rat CINC-1 in a buffered protein base with preservatives, lyophilized.

Rat CINC-1 control- 1 vial of recombinant rat CINC-1 in a buffered protein base with preservatives, lyophilized.

Assay diluent RD1W- 12.5ml of a buffered protein solution with preservatives.

Calibrator diluent RD5-4- 21ml of a buffered protein solution with preservatives.

Wash buffer concentrate- 50ml of a 25 fold concentrated solution of buffered surfactant with preservatives.

Colour reagent A-12.5 ml of stabilized hydrogen peroxide.

Colour reagent B- 12.5 ml of stabilized chromogen. (tetramethyl benzidine)

Stop solution-23ml of a diluted HCL

Plate covers- 4 adhesive strips.

Sample collection

Blood samples were allowed to clot for 2 hours at room temperature before centrifuging for 20 minutes at approximately 1000 x g. Serum samples were aliquoted and stored at -20° C.

Sample preparation

Rat serum samples were diluted 2-fold into calibrator diluent RD5-4 prior to assay. 2-fold dilution is 70 µl + 70µl calibrator diluent RD5-4.

REAGENT PREPARATION

All reagents were brought to room temperature before use.

Rat CINC-1 Kit Control - The Kit Control was reconstituted with 1.0 mL deionized or distilled water. Assay the Control undiluted.

Wash Buffer - If crystals were formed in the concentrate, the buffer was warmed to room temperature and mixed gently until the crystals were completely dissolved. To prepare enough wash buffer for one plate, 25 mL of Wash Buffer Concentrate was added into deionized or distilled water to prepare 625 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B were mixed together in equal volumes within 15 minutes of use and protected from light. 100 µL of the resultant mixture was required per well.

Rat CINC-1 Standard - The Rat CINC-1 Standard was reconstituted with 5.0 mL of Calibrator Diluent RD5-4 (care was taken not to substitute other diluents). This

reconstitution produced a stock solution of 500 pg/mL. The standard was allowed to sit for a minimum of 5 minutes with gentle mixing prior to making dilutions.

Use polypropylene tubes. 200 µL of Calibrator Diluent RD5-4 was pipetted into each tube. The stock solution was used to produce a 2-fold dilution series (below). Each tube was gently mixed but thoroughly before the next transfer. The undiluted rat CINC-1 Standard served as the high standard (500 pg/mL). Calibrator Diluent RD5-4 serves as the zero standard (0 pg/mL).

CALIBRATION

This immunoassay is calibrated against a highly purified E. coli-expressed recombinant rat CINC-1 produced at R&D Systems. The mature protein contains 72 amino acid residues and has a predicted molecular mass of approximately 7.8 kDa. Based on total amino acid analysis, the absorbance of a 1 mg/mL solution of the E. coli-expressed recombinant rat CINC-1 at 280 nm was determined to be 0.08 A.U.

Assay procedure

50 µl of assay diluent RD1W added to each well. 50 µl of standard, control or sample per well added. The plate was gently tapped for 1 minute to mix the contents and covered with adhesive strip. This was followed by incubation for 2hrs at room temperature. Each well was aspirated and washed for a total of five times with wash buffer (400µl). 100 µl of rat CINC-1 conjugate was added to each well. The plate was covered with adhesive strip and incubated for 2hrs at room temperature. The wells were aspirated and washed five times. 100 µl of substrate solution was added to each well and incubated at room temp for 30 min under protection from light. 100 µl of stop solution was added to each well. Plate was gently tapped to ensure mixing. Optical density was measured within 30 minutes using a microplate reader set to 450 nm.(correction wavelength set at 540nm or 570nm).

2. 10 Western blots for cytochrome C.

Method for Western blots for cytochrome C.

Sampling of tissues- liver tissues from six animals in each group of the early phase of hepatic IR were taken. Total of 36 samples were taken.

Tissue homogenisation and mitochondrial extraction.

Cytosol and mitochondrial enriched fractions obtained from the liver were rinsed in 0.15 mol/L KCL, scissor minced and homogenized in 3 volumes of 0.25 mol/L sucrose. Low speed centrifugation was performed to remove cellular debris and obtain whole cell lysates.The whole cell lysates were centrifuged at 500g for 10 minutes. The supernate was spun at 2600g for 10 minutes. The resulting pellet was resuspended in a buffer consisting of 20mmol/L Tris –Hcl (PH 7.4), 0.25 mol/L sucrose and 5.4 mmol/L ethylenediaminetetraacetic acid.

Western blot for cytochrome c.

Equal **a**mounts of protein loads were added onto 15% polyacrylamide gels were transferred to nitrocellulose membranes. Thereafter the membranes were treated with anticytochrome C (BD biosciences pharmingen, Franklin, Lakes, NJ, USA), anti Bax (BD biosciences pharmingen) . Positive control used was rat pc 12 cell lysate. Mean density of the band area was measured using the Scion image beta 4.02 (Scion Frederick, Md, USA) after obtaining black and white inages as Tiff files by an image scanner

2.11 Drugs used for HO induction and inhibition

PDTC (HO inducer)

Group five animals (PDTC+IR) were given Pyrrolidine dithiocarbamate (PDTC, Sigma Chemical Co, St Loius, Mo, USA) 100 mg /kg dissolved in 0.9% sodium chloride given subcutaneously 30 minutes prior to IR 30 min before ischaemia.

PDTC and sham and toxicity of PDTC

Tsuchihashi et al (2003) demonstrated in an animal model that doses in excess of 600 mg /kg killed animals but animals did not die by injection of any dose upto 600mg/kg. Doses more than 200 mg/ kg offset the beneficial effects of PDTC as

demonstrated by Liu et al (1999). Hence we chose a dose of 100 mg/ kg as used in experiments by Tsuchihashi. PDTC shows a dose dependent relation ship with regard to its beneficial effects. At doses of 100 mg/ kg PDTC induces HO in kupffer cells but does not influence sinusoidal dilatation. Hata et al showed that at doses of 150 mg/ kg PDTC induced sinusoidal dilatation in sham animals , increased HO-1 with peak mRNA levels detectable at 3 hrs after PDTC injection and then declined. HO Protein expression peaked at 24-48 hrs after injection and was dose dependent. Immunohistochemistry revealed that PDTC at higher doses induced HO-1 in both periportal kupffer cells and heatocytes in pericentral areas. Since previous experiments have shown effects of PDTC in sham we did not investigate PDTC in sham

ZnPP (HO inhibition)

Group six animals (ZnPP+RIPC+IRI) were given Zinc protoporphyrin (ZnPP Sigma Chemical Co, St Loius, Mo, USA) 1.5 mg/kg (HO-1 blocker) by intraperitoneal route 1 hour prior to RIPC and subsequent IRI.

ZnPP and sham and hepatotoxicity of ZnPP.

Previous experiments in sham animals have shown that ZnPP inhibits haemoxygenase if used in the darkness. If ZnPP is exposed to light its effect of HO inhibition is lost however, ZnPP inhibits hepatic artery dilatation when exposed to light and this potentially leads to hepatotoxicity (Greenbaum et al; 1991, zygmunt et al; 1994). Hepatoxicity can confound the experimental data and hence in order to avoid this confounding effect ZnPP was prepared in the dark and administered through a syringe covered with silver foil with minimal laboratory light. The dose used was 1.5 mg /kg (2.5μmol/kg) and this is much lower than the hepatotoxic toxic dose of ZnPP (5- 10μmol/kg) as shown by Greenbaum et al. Amersi et al (1999)

showed that ZnPP treatment in shams led to undetectable baseline HO and reduced portal flow and bile flow. Since previous experiments on shams have been detected we did not undertake experiments to investigate effect of ZnPP on sham group in our study.

2.12 Hypothesis and Aim

Our hypothesis was that remote ischaemic preconditioning from limb ischaemia may have a beneficial effect in reducing ischaemia reperfusion injury of the liver by modulation of hepatic microcirculation through haemoxygenase pathways.

Aim- To study the effect of remote ischaemic preconditioning in an animal rat model of Ischaemia reperfusion injury and the role of haemoxygenase in the mechanism of RIPC.

The next chapter focuses on investigating dynamic microcirculatory changes and hepatocellular injury in the early phase of hepatic ischaemia reperfusion injury and the effects of remote ischaemic preconditioning on hepatic IR in the early phase (3 hrs of reperfusion). Subsequent chapters describe the effect of haemoxygenase inhibition on the effects of RIPC in the early phase of hepatic IR and the effect of HO induction in modulating the early phase of hepatic IR. The next chapter describes the effects of hepatic IR after 24 hrs of reperfusion and the role of remote ischaemic preconditioning on hepatic IR in a recovery model. The final chapters describe the effect of RIPC on CINC (cytokine) in hepatic IR and the effect of RIPC on mitochondrial cytochrome c release.

Chapter 3

Study of microvascular and hepatocellular injury in the early phase of hepatic IR (3 hrs) and the effect of RIPC on hepatic IR.

3.1 Introduction

The effect of hepatic IR on hepatic microcirculation: Findings & mechanism of IR induced hepatic injury.

Menger et al (1999) have demonstrated that the nature of microvascular injury which precedes manifestation of hepatic parenchymal tissue damage includes both hypoxia due to lack of microvascular perfusion (i.e. no-reflow) and a reperfusion-associated inflammatory response, which includes the activation and dysfunction of leukocytes and Kupffer cells (the reflow paradox). No-reflow in sinusoids is thought to be caused by endothelial cell swelling and intravascular hemoconcentration, and involves also a deterioration of the balance between ET and NO. The reflow paradox is associated with: (i) the release and action of proinflammatory cytokines (TNF-alpha, IL-1) and oxygen radicals; (ii) the up-regulation of endothelial and leukocytic adhesion molecules (selectins, beta-integrins, ICAM-1); and (iii) the interaction of leukocytes with the endothelial lining of the hepatic microvasculature. Menger et al demonstrated the in vivo relevance of leukocyte-endothelial cell interactions in the manifestation of hepatic ischaemia-reperfusion (I/R) injury. Reperfusion following either 20 min or 60 min of left hepatic lobar ischaemia resulted in a significant increase in the number of stagnant leukocytes in sinusoids and adherent cells in postsinusoidal venules compared with sham-operated controls. In parallel, hepatic I/R was associated with increased serum transaminases and reduced bile flow with significant correlation to the number of adherent leukocytes in postsinusoidal venules but not with sinusoidal stasis. These findings suggested that the adherence of leukocytes to the endothelial lining of venules, rather than of sinusoids, may determine the manifestation of hepatocellular damage and liver dysfunction. Menger et al also demonstrated that 60 min of lobar ischaemia followed by reperfusion injury caused a significant decrease in

serum AST and ALT activities and reduction of bile flow which directly correlated with the extent of microcirculatory failure, ie, impairment of sinusoidal perfusion and decrease of erythrocyte flux, indicating the decisive role of microvascular

The effect of RIPC on hepatic haemodynamics.

Lai et al demonstrated improved liver functions and the role of haemoxygenase in hepatic protection(Lai et al. 2006). Gustaffson demonstrated protection of hepatic function by remote preconditioning(Gustafsson et al. 2006). Kanoria et al carried out the only study on effect of RIPC on hepatic macrodynamics demonstrating improved liver function, increased hepatic blood flow by ICG and laser Doppler flow measurements (Kanoria et al 2006). Kanoria et al showed an increase in nitrate levels in hepatic venous plasma but did not specifically investigate the role of NO in the mechanism of RIPC. None of these studies demonstrated the in vivo microcirculatory changes seen in hepatic IR and the effect of RIPC on hepatic IR. We focused our study on the hepatic microcirculatory changes in hepatic IR and the modulation of hepatic microcirculation by RIPC. We hypothesised that RIPC modulates hepatic microcirculation in IR injury and applied intravital microscopy to investigate effects of RIPC.

3.2 Material and methods

3.2.1 Animals and surgical procedures- refer material and methods, chapter 2, pg 92.

3.2.2 Animal model – refer material and methods, chapter 2, pg 92.

3.2.3 Limb preconditioning- refer material and methods, chapter 2, pg 92.

3.2.4 Experimental groups (n=6 in each group)

Four groups of animals were studied. Group one (Sham) in which animals were subjected to laparotomy only and underwent an identical experimental protocol without clamping. In group two (IRI) the animals were subjected to 45 minutes of ischaemia followed by three hours of reperfusion. Ischaemia was induced by a microvascular clamp applied across the **vascular pedicle** supplying the left and median lobes of the liver (70 % ischaemia). Group three were preconditioned immediately prior to IRI (RIPC+IRI) group. In group four (RIPC+Sham) preconditioning was done in Sham. Standard protocols for preconditioning and inducing ischaemia as described above were used.

Experimental design –

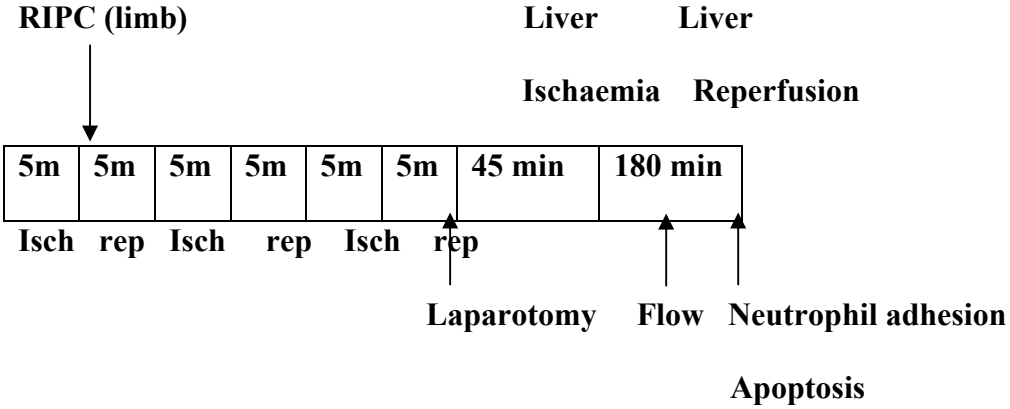


Figure 3.1

Intravital videofluorescence microscopy.

Refer material and methods- chapter 2, page 92-102.

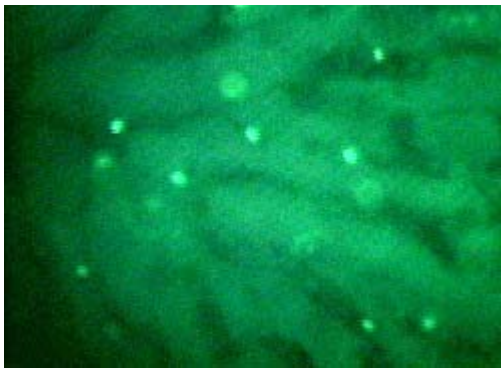


Figure 3.2 - Red blood cells labelled with FITC are seen in the hepatic sinusoids.
Velocity= Length moved/ no of frames ×25 μm/sec.
Perfusion Index= Perfusion index = (Scp +Sip/ Scp+Sip+Snp).
[Continuous perfusion (Scp) + intermittent perfusion (Sip) to the total visible sinusoids which includes non perfused sinusoids (Snp)]

RBC velocity (V)

- 0.5 ml of FITC labelled red cells prepared from rat blood suspended in glucose saline buffer solution (20mgFITC /ml of RBC) were administered intravenously to assess the velocity of RBC flow. Ten randomly chosen nonoverlapping rappaport acini were assessed at each time point and the mean value was calculated.
- The RBC velocity was calculated by assessing the distance of RBC movement (microns) in each sinusoid in subsequent frames. 25 frames were captured per second. Hence the velocity was calculated using the formula= D×25/number of frames moved.

Sinusoidal Perfusion and perfusion index (PI).

The sinusoidal perfusion index was evaluated as ratio of perfused hepatic sinusoids Continuous perfusion (Scp) + intermittent perfusion (Sip) to the total visible sinusoids

which includes non perfused sinusoids (Snp). Perfusion index = (Scp +Sip/ Scp+Sip+Snp).

Sinusoidal diameter (D)

Sinusoidal diameter was assessed by measuring the **distance** across ten randomly chosen hepatic sinusoids at each time point and the mean value was expressed in microns. **Ischaemia reperfusion injury is known to cause endothelial injury and endothelial constriction. Measuring sinusoidal diameter using intravital microscopy helped clarify the effects of IR & RIPC on sinusoidal diameter and the mechanism of increased blood flow in hepatic IR injury.**

Sinusoidal blood flow

The sinusoidal blood flow was calculated using the formula- (V) ×22/7× (D/2)2. V= Velocity, D= diameter.

Neutrophil adhesion

Rhodamine 6G (0.3mg/kg)(Wunder et al. 2002) was given intravenously for staining of neutrophils. The numbers of leukocytes adherent to the sinusoidal endothelium and venular endothelium were counted as those stationary for a period of 30 seconds under green filter light and expressed as leukocytes/mm2.The area of the vessels was calculated using the product of **circumference** and length assuming cylindrical geometry (3.14×D×L).

Hepatocellular cell death

Hepatocyte apoptosis was assessed by intravenous injection of Propidium iodide (0.15mg/kg i.v) (Brock et al. 1999) and the number of nuclei stained with the dye were counted under the camera and expressed as number of apoptotic cells/high power field seen on the video monitor.

Histology

Liver tissue was fixed in 10% formalin and embedded in paraffin in preparation for light microscopy analysis. Sections were cut at 5µ and stained with haematoxylin and eosin for histological analysis. Modified Suzuki criteria were used to describe the histological changes. The scoring used is detailed in chapter 2, pg 103-104.

Statistical analysis

All data are presented as mean and standard error of the mean (SEM).Differences were considered significant for $P<0.05$ .Comparisons between groups were performed by one way analysis (ANOVA). Bonferroni correction was applied for ANOVA.

3.3 Results

Haemodynamic parameters.

Blood pressure (MAP) (Figure 3.5)

Sham

The sham group was haemodynamically stable through all time points of observation.

IRI

In the IR injury group the baseline MAP showed an insignificant fall immediately after reperfusion from 102.00 mm hg to 81.66 mm hg.

RIPC+IRI

Preconditioning in the IR group (RIPC+IR) showed an insignificant fall in blood pressure immediately after reperfusion but the blood pressure recovered rapidly to baseline levels (30-60 min) as compared to IR (60-120 min) group.

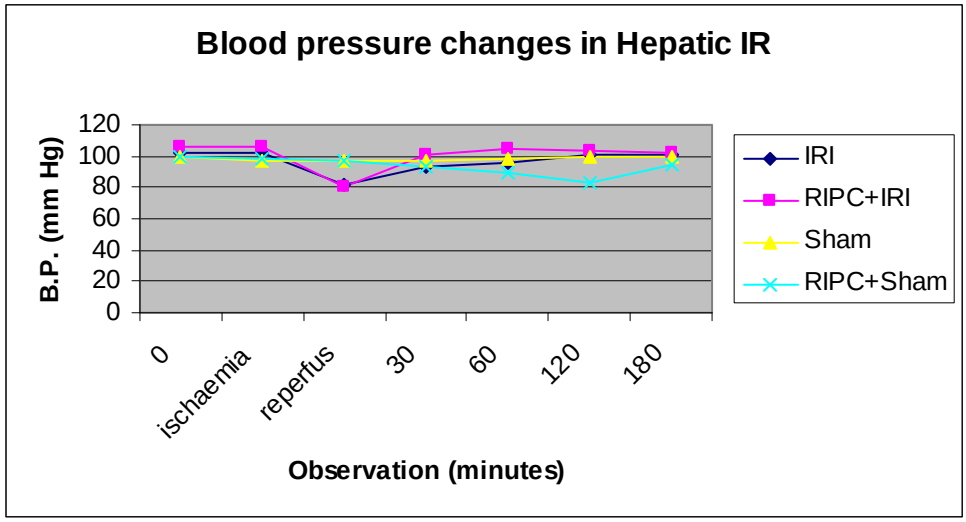


Figure-3.5

Changes in blood pressure seen in hepatic IR and effect of preconditioning. Both IR and RIPC+IR show a similar fall in blood pressure immediately after reperfusion, however RIPC prior to IR restores B.P. to baseline rapidly compared to IR only. Values expressed as mean + sem. $P<0.05$.

Pulse rate

No significant difference between IR and preconditioned groups was seen as shown graphically**.**

Oxygen saturations

No significant fall in saturations in both IR group and RIPC+IR group

Hepatocellular injury (liver transaminase levels)

The hepatic transaminase levels were lower in the preconditioned group but were not significantly different between IR injury and preconditioned group. **(Figure 3.6)**

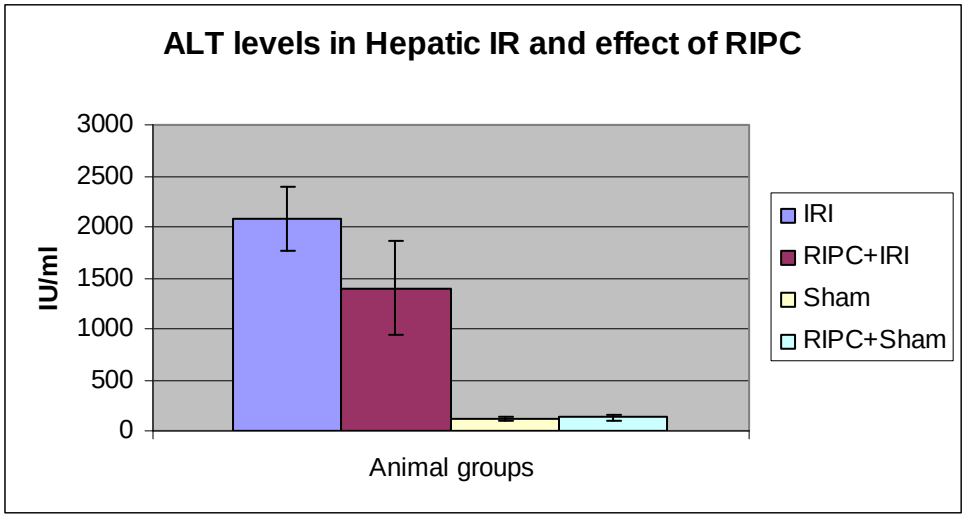


Figure 3.6 (above) graphically depicting liver transaminases in RIPC+IR/ IR.

IR was associated with significantly higher ALT values in contrast to sham ($p<0.05$). Preconditioning prior to IR injury reduced serum transaminase levels although the difference was not statistically significant. Preconditioning in Sham animals did not show any significant elevation in transaminases.

	IR	RIPC+IR	Sham	RIPC+Sham
ALT (IU/ml)	**2079± 322.3**	**1398± 460.8**	**118± 22.37**	**132± 25.49**

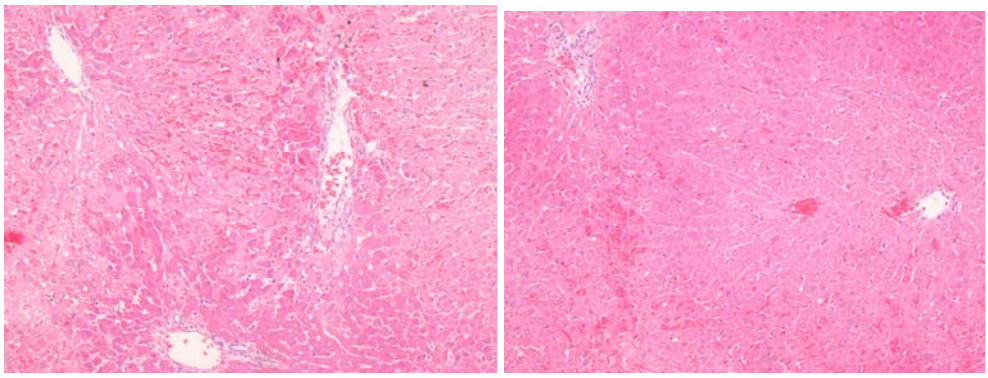
Table (above) showing liver transaminases in different animal groups.

Histological investigation

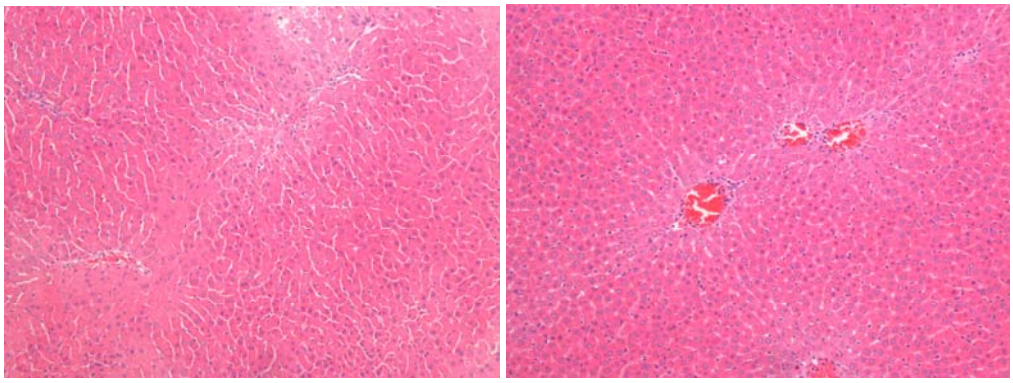
	IR	RIPC+IR	Sham	RIPC+Sham
Suzuki score	**8.83± 0.7**	**6.2± 0.58**	**4± 0.31**	**1.5± 0.34**

The modified Suzuki score was significantly reduced in the preconditioned group compared to IR group ($P<0.001$).Histological changes of IR injury were significant periportal congestion in association with severe necrosis in zones 2 and 3 and also sub capsular necrosis. The changes were diffuse. The Suzuki score was significantly reduced in RIPC+IR which showed less necrosis and damage compared to the IR

group. Sham animals revealed minimal changes. Congestion was present in both portal vein and central veins. Hepatocytes show vacuolation. RIPC+Sham animals show minimal changes, some vacuolation, but no necrosis, similar to the sham group.



IR Injury **RIPC+IR**



Sham **RIPC+Sham**

Figure (3.7) Histology a).IR- The HE section shows large areas of necrosis and sinusoidal congestion, normal residual hepatocytes noted at bottom of the frame
b).RIPC+IR-The HE section shows sinusoidal congestion, some hepatocyte vacuolation but no significant necrosis
c).Sham- The HE section reveals no significant damage but some central vein congestion.
d).RIPC+Sham- The HE section reveals congested central vein but no other significant change . Cells are viable around portal area.

Intravital microscopy results

Velocity of RBC flow

The mean RBC velocity in the sham group was constant throughout the time of observation. IRI led to decreased RBC velocity and preconditioning led to an increased RBC velocity, which was significantly higher than the IR group at all time points. **(Figure 3.8)**

Sinusoidal flow

Sinusoidal flow was also was significantly increased in the preconditioned (RIPC+IR) group as compared to IR group. **(Figure 3.9)**

Sinusoidal diameter

No significant difference between sinusoidal diameter in IR injury and RIPC+IR was seen suggesting that preconditioning does not modulate sinusoidal tone in the early phase of hepatic IR**. (Figure 3.10)**

Sinusodial perfusion

The perfusion index is the ratio of perfused sinusoids (continuous+ intermittent) to non perfused sinusoids. RIPC+IR group shows significantly increased perfusion in the sinusoids as compared to IR group. **(Figure 3.11)**

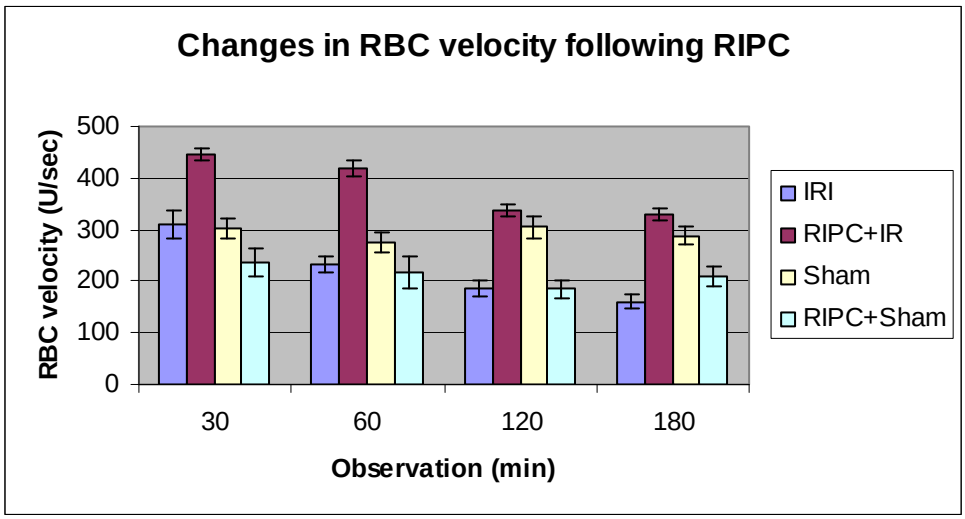


Figure 3.8 (above) Velocity of RBC flow in sham remained unchanged through 180 minutes of observation. Significant increase in velocity in preconditioned animals (RIPC+IRI) as compared to IRI at all time points of observation. Values expressed as mean $\pm$ sem. * = P<0.05 (RIPC+IR/IR).**= P<0.05 (RIPC+IR/Sham).

Sinusoidal flow

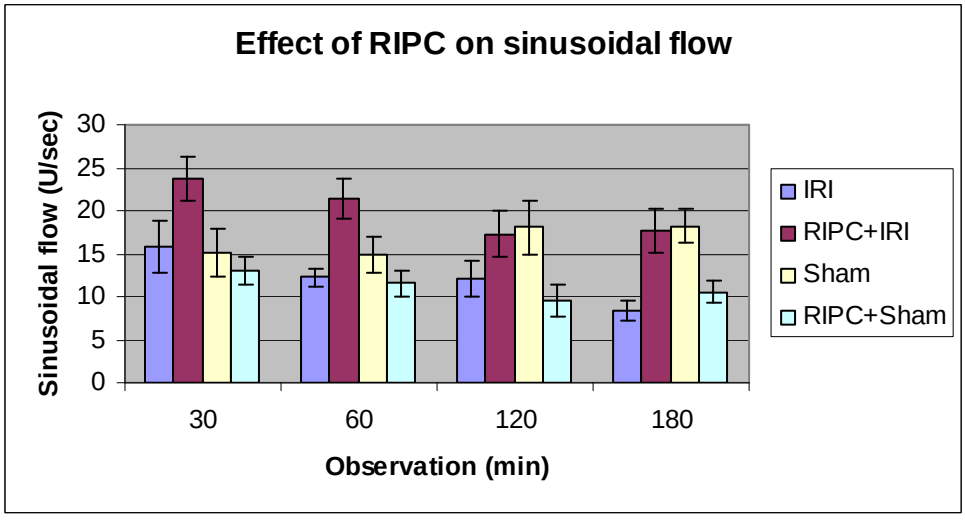


Figure 3.9 (above) Sinusoidal flow - V× (D/2)2×л = V is velocity of RBC, D is sinusoidal diameter. Significantly better flow in preconditioned animals (RIPC+IRI) as compared to non preconditioned (IR). Values expressed as mean $\pm$ sem. * = P<0.05(RIPC+IR/IR). ** = P<0.05(RIPC+IR/ Sham). ●= P<0.05 (Sham/IR).

Diameter of sinusoids

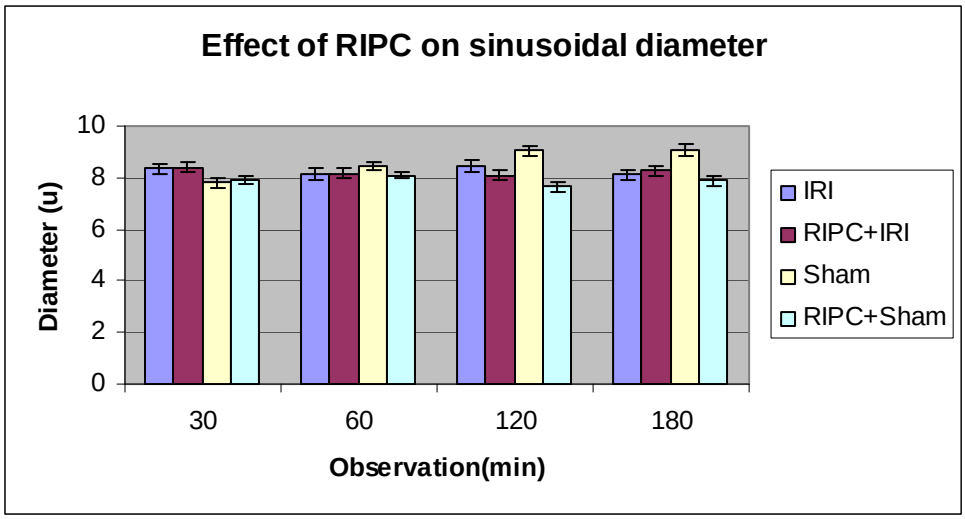


Figure 3.10 (above) - The mean diameter showed no significant difference between the RIPC+IR group and IR group. The difference between Sham and RIPC+IR and between Sham and IRI was not statistically significant.

Perfusion of sinusoids.

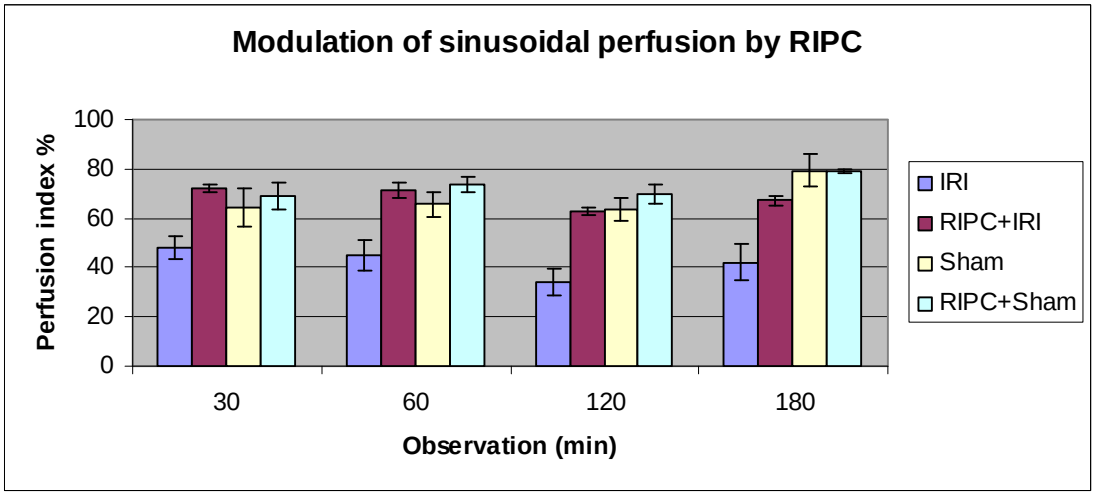


Figure 3.11 (above) Sinusoidal perfusion index -The PI remains unchanged in sham throughout 180 minutes of observation. The PI in remote preconditioned animals (RIPC+IRI) is significantly higher than non preconditioned animals (IRI). The PI in RIPC+IR is not different from Sham. Values expressed as mean ± sem. * =P<0.05.

Results

Venular neutrophil adhesion

The image (**figure 3.12**) shows adherent neutrophils to the venular endothelial wall. The number of adherent neutrophils /mm^2 was assessed by the formula described in chapter 2. Significantly reduced venular neutrophil adhesion in the RIPC+IR group compared to IR group was observed (**figure 3.13**).

Sinusoidal neutrophil adhesion

Significantly reduced neutrophil adhesion was observed in the sinusoids in preconditioned animals in comparison to hepatic IR only **(figure 3.14).**

Neutrophil adhesion

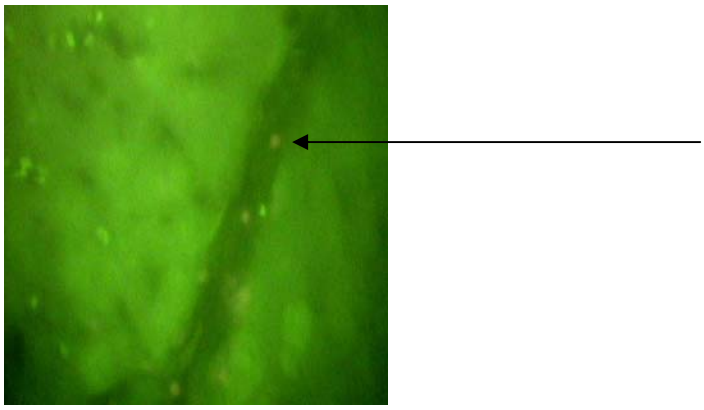


Figure 3.12 Neutrophils stained by rhodamine is seen adherent to post sinusoidal venular endothelium. The number of adherent neutrophils divided by the area of endothelial surface (л×D×L) gives the number of neutrophils/ mm2. D= sinusoidal diameter, L= length of segment along adherent neutrophils.

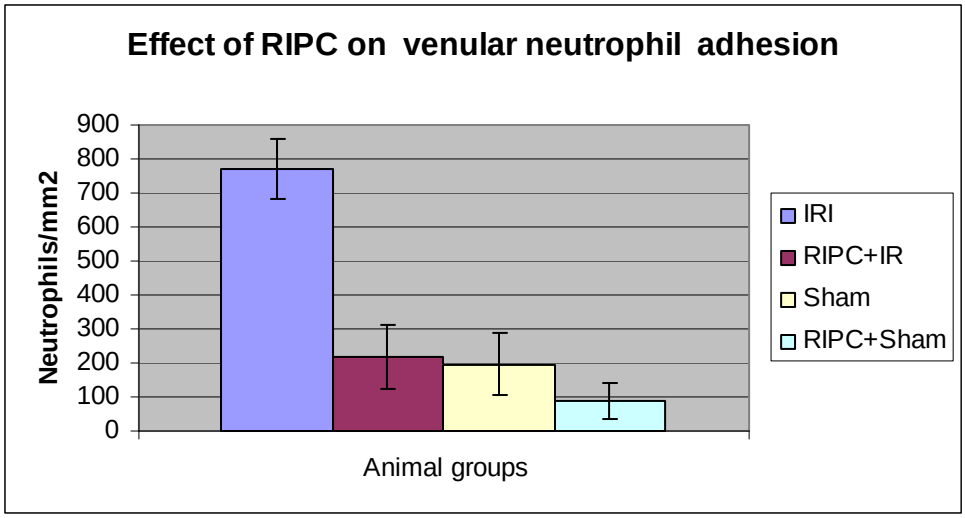


Fig 3.13- Significantly reduced venular neutrophil adhesion in preconditioned (RIPC+IRI) group compared to non preconditioned group (IRI). Values expressed as mean ± sem.

* = P<0.05 (IR/RIPC+IR), ** = P<0.05(IR/Sham),
●= P<0.05(IR/RIPC+Sham).

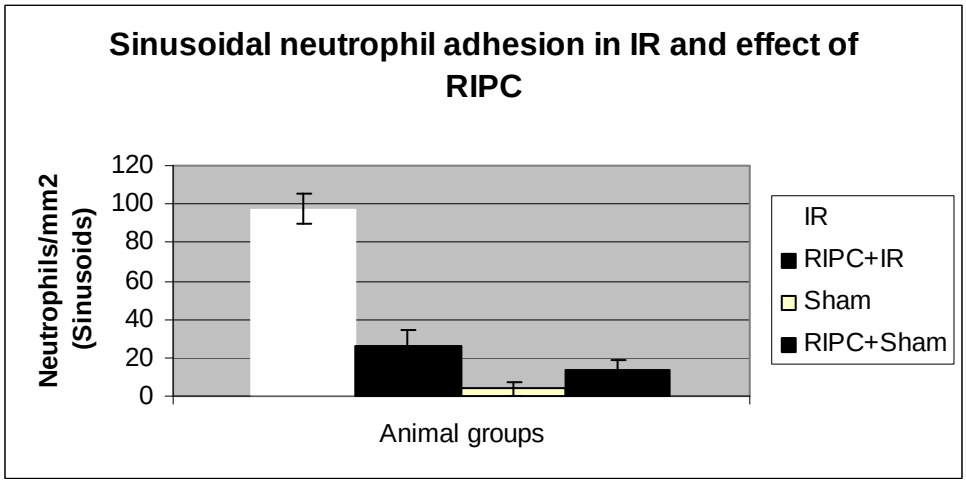
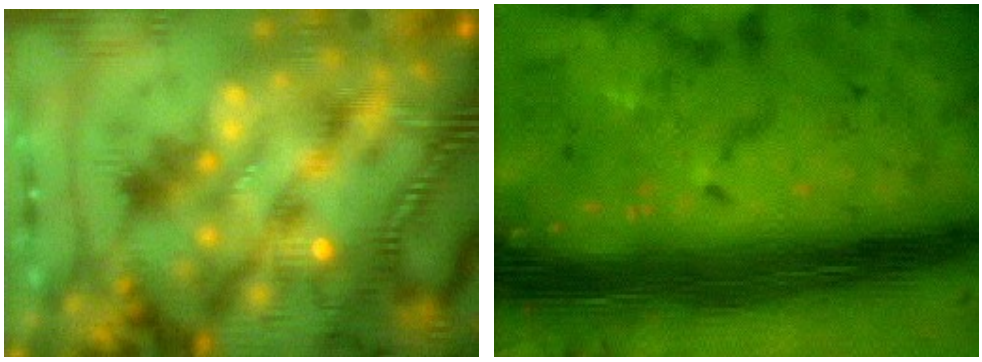


Figure 3.14 - Significantly reduced sinusoidal neutrophil adhesion in preconditioned group (RIPC+IRI) compared to non preconditioned group (IRI). Values expressed as mean ± sem. * = P<0.05 (IR/RIPC+IR), **= P<0.05(IR/Sham),
●= P<0.05(IR/RIPC+Sham).

Hepatocellular death as seen by propidium iodide under IVM



IRI (12a) **RIPC+IRI (12b)**

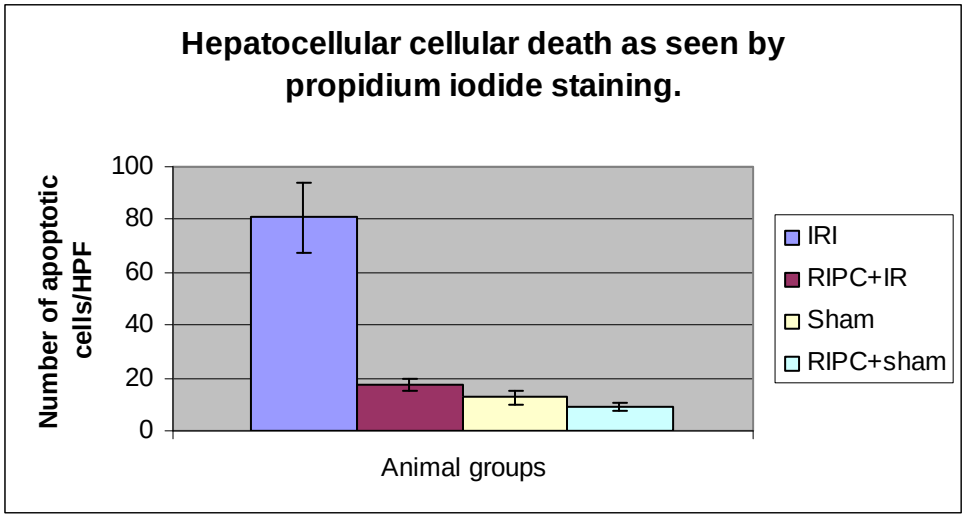


Figure 3.15

Figure a - Hepatocellular cell death in IRI 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 b - Hepatocellular cell death seen in RIPC+IR by propidium iodide staining (IVM).
Figure c- Hepatocellular cell death in preconditioned group (RIPC+IRI) is significantly less compared to non preconditioned (IRI) group. Values expressed as mean
$\pm$ sem. * = $P<0.05$ (IR/RIPC+IR), **= $P<0.05$(IR/Sham),
●= $P<0.05$(IR/RIPC+Sham).

3.4 Discussion

New findings

This study demonstrates that RIPC modulates the hepatic microcirculatory changes in IR injury to produce increased RBC velocity, sinusoidal perfusion and decreased neutrophil adhesion in the sinusoids resulting in improved hepatic microcirculation and decreased hepatocyte cell death. This suggests that RIPC results in improved hepatic microcirculation . This is the first in vivo study to demonstrate microcirculatory effects of RIPC in hepatic IR by intravital microscopy. Recently remote ischaemic preconditioning has been shown to modulate pancreatic microcirculation and reduce pancreatic IRI (Oehmann et al. 2007).

Adequacy of model

The experimental model used is a previously well described hepatic lobar ischaemiamodel of warm IR .In the model 45 minutes of partial ischaemia(70% of the liver) was induced followed by three hours of reperfusion (Koti et al. 2002). Intravital microscopy has shown that IRI significantly reduced velocity of flow and perfusion as compared to sham animals (Menger et al). Therefore the model was considered suitable to study the effects of RIPC.

Transplant vs warm hepatic IR

Since this study was limited to investigating the effects of warm IR and the effects of RIPC in warm IR a partial hepatic IR model was chosen as against a transplant model.

Global vs lobar ischaemia

Global hepatic ischaemia is an ideal model which truly reflects liver transplantation but global ischaemia can potentially lead to congestion of the intestine and cytokine release which can confound the results.To avoid this a portosystemic shunt is necessary. Global hepatic ischaemia without portosystemic shunt in a rabbit model has been described by Kanoria et al however the limitation of this model was that ischaemia could only be induced for about 25 minutes. Moreover to study the effects of hepatic IR, 70% partial hepatic IR models are well described in the literature (Menger et al 1994; Menger et al 1996).

Animal specificity

Partial hepatic IR models have been described in mice and pigs in addition to rats. In all these animal models hepatic IR is reproducible and has been used to investigate the effects of preconditioning.

Adequacy of preconditioning

RIPC was produced using a tourniquet around the Hind limb for four cycles of five minute ischaemia followed by reperfusion. Preliminary experiments showed a significant protective effect of four cycles of RIPC in hepatic IR. This technique of RIPC has previously been used in the modulation of cardiac IR injury following transplantation (Kristiansen et al 2004). Three or more cycles of limb preconditioning have been shown to be more effective than a single cycle in previous experimental studies (Addison et al. 2003; Kristiansen et al. 2004; Moses et al. 2004) and this formed the basis for four cycles of RIPC used in the current study.

Systemic haemodynamics

Shams were haemodynamically stable throughout the course of the experiments. Limb preconditioning in sham animals caused a transient insignificant fall in blood

pressure during preconditioning with rapid recovery to baseline levels suggesting no major effects of sham laparotomy, anaesthesia or RIPC alone. A transient fall in blood pressure and oxygen saturations associated with onset of reperfusion was seen in both the IR group and RIPC+IR group. However recovery of blood pressure to normal levels was rapid in the RIPC+IR group (30-60 minutes) as compared to IR only (60-120 minutes). These observations have been made in preconditioning models of lung IRI (Harkin et al. 2002;Waldow et al. 2005). Kanoria et al have demonstrated fall in blood pressure following hepatic IR in a rabbit model and rapid recovery of blood pressure in the group which was preconditioned (60 minutes) (Kanoria et al 2006).

Histological findings and limitations of the scoring system.

The RIPC+IR group showed less necrosis, vacuolation and congestion compared to IR group and objective scoring showed a significantly less score in the preconditioned group. However it was difficult to arrive at an objective score if the changes noted were focal/patchy. There were several sections with only sub capsular infarction/necrosis. Hence despite an objective system of scoring it was difficult to score these sections using the criteria. This is one of the limitations of an objective scoring system.

Hepatic microcirculatory changes.

Sham

The sham group showed constant velocity, flow and perfusion in the sinusoids over the 180 minutes of observation. This suggests a stable model for the study.

Effects of IR injury on hepatic microcirculation and neutrophil adhesion.

Our study shows a significant fall in sinusoidal perfusion and velocity of RBC flow in IRI group which was observed at all time points in our study. Increased venular

neutrophil adhesion was seen in the IR group as compared to sham group. There was an increase in sinusoidal neutrophil stasis in the IR group with the the perfusion index being significantly lower compared to sham animals.

Significance of sinusoidal perfusion

Past studies have demonstrated that the mechanisms which contribute to failure of perfusion include endothelial swelling and injury, leukocyte endothelial interaction, leukostasis, alteration in RBC flow and increased viscosity of blood (Vollmar et al. 1995;Vollmar et al. 1996;Vollmar et al. 1994;Vollmar et al. 1994;Vollmar, Richter, & Menger 1996). Reduced RBC velocity maybe secondary to free radical release producing erythrocyte damage, and deformity. This contributes to increased viscosity of blood and decreased RBC velocity (Vollmar et al. 1994). Leukocyte adhesion causes endothelial injury, red blood cell stasis, modulation of sinusoidal tone and perfusion (Vollmar et al. 1995;Vollmar et al. 1994). Perfusion failure has been shown to correlate with hepatic dysfunction, reduced bile flow and increased transaminases(Vollmar et al. 1994). .Venular adhesion of neutrophils correlates directly with hepatic dysfunction, raised serum transaminases and decreased bile flow(Vollmar et al. 1994) .

The above discussed studies have shown that reduction in RBC velocity occurs due to leukocyte adhesion, endothelial injury and perfusion impairment (No reflow phenomenon) and increased RBC damage due to free radical release during IR

Effect of IR on serum transaminases

Serum transaminases are higher in the IR group suggesting hepatocellular injury.

Effect of IR on hepatocellular death

In our study significantly increased hepatocyte cell death was seen in the IR group after 180 minutes of reperfusion as compared to sham group and RIPC+IR group.

Correlation of microcirculatory flow, neutrophil adhesion, hepatocellular death and serum transaminases in hepatic IR

This study demonstrates that decreased sinusoidal perfusion, RBC velocity and increased neutrophil adhesion are associated with increased hepatocellular injury and hepatocellular death. These findings are supported by studies from Vollmar and Menger as discussed above.

Effects of RIPC on hepatic microcirculation in IR injury (early phase of IR).

This study demonstrates that RIPC increases RBC velocity and erythrocyte flux across sinusoids, significantly reduced hepatocyte cell death, increased sinusoidal perfusion and sinusoidal flow as compared to the IR group. Interestingly there is a significant initial rise in RBC velocity and flow in the RIPC+IR group as compared to sham and IR although the initial velocities in sham and IR are not significantly different. However at 120 minutes a significant drop in velocity is seen in the IR group in comparison to RIPC+IR and sham. Hence the initial rise in velocity in the RIPC+IR group serves to maintain RBC flow in comparison to IR group. The initial molecular pathway responsible for the increased baseline velocity is unclear but it is likely to be induced by RIPC in the hepatic ischaemia period itself since the velocity of flow is seen to be high as early as 30 minutes following reperfusion in the local microvasculature.

In the RIPC+IR group perfusion is significantly better than the IR group and comparable to sham values suggesting that preconditioning prevents a fall in perfusion in hepatic IR. The significantly improved perfusion is associated with decreased serum transaminases and significantly reduced hepatocellular death.

Effect of RIPC on neutrophil adhesion in early phase of IR.

A significant reduction in both venular neutrophil adhesion and sinusoidal adhesion implies modulation of neutrophil activation by RIPC. The decrease in neutrophil activation accounts for decreased oxidative stress, IR injury and perfusion failure. Previous studies(Glanemann et al. 2003) have shown ischemic preconditioning to reduce hepatic IR induced perfusion failure and preservation of mitochondrial redox state but none have demonstrated the role of RIPC in hepatic IR

The observations in our study suggest that RIPC modulates RBC flow, neutrophil adhesion and sinusoidal perfusion. RIPC may induce protective mechanisms which protect RBC against free radical induced damage, however this needs to be further investigated.

Effect of RIPC on hepatocellular injury

The serum transaminase levels in the RIPC+IR group were lower than IRI suggesting decreased liver injury in the RIPC+IR group as compared to IR group.

Correlation of microcirculatory flow, hepatocellular injury, neutrophil adhesion serum transaminases and hepatocellular death in RIPC+IR

The findings in our study show that significantly increased RBC velocity, sinusoidal perfusion are associated with decreased neutrophil adhesion, decreased hepatocellular injury and cell death in comparison to IR.

Effects of RIPC on Sham. (RIPC+Sham group)

Remote ischemic preconditioning in the normal sham group showed reduced RBC velocity, sinusoidal flow and perfusion at 120 minutes as compared to 30 minutes after laparotomy and recovery to baseline at 180 minutes. This maybe due to initial preconditioning induced oxidative stress . Chen et al (Chen et al 2005) who

demonstrated that limb RIPC produced an increase in free radical levels in the blood which may explain the oxidative stress.

Potential pathway and mechanisms in remote ischaemic preconditioning of the liver.

Chen at al demonstrated RIPC induced NO in the heart to reduce myocardial IRI which was not abolished by neurogenic blockade (Chen Y.S et al). In another study increase in plasma free radicals following brief limb RIPC induced heat shock proteins in remote skeletal muscle (Chen X.G et al). Lai et showed increased haemoxygenase (HO-1) in hepatic macrophages following limb preconditioning the which was associated with protection of liver function (Lai et al, 2006). These data suggest that brief limb ischaemia leads to release of biochemical messengers such as free radicals in the blood which may induce oxidative stress in the remote organ and HO-1 expression. HO-1 is known to promote degradation of haem, increase CO production and scavenge free radicals and protect the liver from hepatic IR. It has been observed in animal models of ischaemia reperfusion injury of the liver and kidney that there is an increase in microsomal haem content accompanied by a decrease in cytochrome-p450 content. Haem is a source of reactive oxygen species i.e. free oxygen radicals which cause disruption of mitochondrial membranes associated with oxidative stress. Moreover it has also been seen that following reperfusion the oxygen flow to the organ is more than its requirement and hence excessive oxygen results in free radical generation under the influence of the xanthine oxidase system.The activation of the haemoxygenase system would scavenge these free radicals and prevent reperfusion injury.

Based on this hypothesis experiments were conducted by Katori et al (Katori et al 2002) and they found that overexpression of hepatic HO was beneficial in reducing

IRI. Mc Carter (Mc Carter *et al* 2004) showed that prolonged limb ischaemia reperfusion resulted in remote organ injury however, the release of endogenous haemoxygenase is involved in protection of the liver from limb ischaemia reperfusion injury. They showed that following bilateral limb ischaemia there was remote liver injury which did not progress beyond six hours from the onset of limb reperfusion .This correlated with increased hepatic haemoxygenase activity. Blockade of HO activity with chromium mesoporphyrin resulted in progression of liver injury with increased apoptosis. They also showed that adenovirus mediated gene transfer of haemoxygenase mitigated remote liver injury following limb ischaemia reperfusion. Similar experimental evidence was produced by Wunder et al who showed the role of endogenous hemoxygenase by showing decreased leukocyte endothelial interactions in the liver under intravital microscopy in animal groups who were pretreated with haemin before limb ischaemia limb reperfusion as compared to groups in whom hemoxygenase activity was blocked with chromium mesoporphyrin. Haemoxygenase (HO-1) is an inducible form of haemoxygenase which is expressed under oxidative stress such as hypoxia, ischaemia and inflammation. In vivo studies of liver have shown Ito cells, which are hepatic sinusoid associated pericytes primarily responsible for CO (derived from HO-1) mediated blood flow regulation in hepatic sinusoids(Hayashi et al. 1999;Suematsu, Wakabayashi, & Ishimura 1996). These studies formed the basis of our hypothesis that RIPC may induce HO-1 to modulate hepatic microcirculation.

Conclusion

This study demonstrates the microcirculatory effects of hepatic IR and the modulatory effects of RIPC on hepatic microcirculation as demonstrated by intravital microscopy. Improved hepatic blood flow is associated with decreased neutrophil

adhesion and cell death. The modulatory effects of RIPC could potentially be due to HO-1. The effect of HO-1 inhibition on protective microcirculatory changes induced by RIPC has not been demonstrated so far and hence we investigated by intravital microscopy the effects of HO inhibition on RIPC in hepatic IR. The next chapter focuses on the role of HO inhibition in RIPC in an animal model of hepatic IR.

Chapter 4

The effect of HO-1 inhibition in modulating the protective effect of remote preconditioning in the early phase of hepatic IR injury.

4.1 Why haemoxygenase?

Evidence for HO-1 in IR

As discussed in the last chapter it has been observed in animal models of ischaemia reperfusion injury of the liver and kidney that there is an increase in microsomal haem content accompanied by a decrease in cytochrome-p450 content. Haem is a source of reactive oxygen species i.e. free oxygen radicals which cause disruption of mitochondrial membranes associated with oxidative stress. Following reperfusion excessive oxygen results in free radical generation under the influence of the xanthine oxidase system in addition to haem.

HO the rate limiting enzyme in the degradation of haem catalyzes the oxidative degradation of haem into carbon monoxide (CO), biliverdin (which is rapidly converted to bilirubin) and free iron (Fe2+). There are three isoforms of haemoxygenase HO-1 (inducible HO) also known as heat shock protein, HO-2 (constitutive HO found mainly in brain and testis), HO-3 which is related to HO-2 but not well characterised. HO-1 is responsible for degradation of haem in senescent RBCs. Degradation of haem and formation of CO results in consumption of free radicals.Thus haemoxygenase (HO-1) enhances scavenging of free radicals and could potentially reduce hepatic IR injury by promoting haem degradation (Katori et al. 2002). Based on this hypothesis experiments were conducted by Busuttil et al and they found that the HO system was beneficial in reducing hepatic IR in animal liver transplant models. Kato (Kato et al, 2001) showed that pre-treatment of donor rat livers with cobalt protoporphyrin (COPP) reduced hepatic IR after cold preservation and reperfusion in ex vivo models with significantly increased bile flow and portal flow as compared to non treated livers. In liver transplant models pre-treatment with COPP enhanced rat survival and decreased histological severity of IR injury in the

liver as compared to nontreated rats. The beneficial effects of haemoxygenase have been demonstrated in genetically fat zucker rats (Amersi et al, 1999) with significantly decreased hepatic IR injury in steatotic livers.

Stimuli inducing HO.

Haemoxygenase (HO-1) is an inducible form of haemoxygenase which is expressed under oxidative stress such as hypoxia, ischaemia and inflammation .

Mechanism of action

The mechanism of HO system is not fully established. It plays a pivotal role in in the maintenance of antioxidant and oxidant homeostasis during cellular injury. It exerts four major beneficial effects: a) antioxidant function b) antiapoptosis c) Anti-inflammatory function d) maintenance of microcirculation. The antioxidant function relies on haem degradation, production of biliverdin, bilirubin and the formation of ferritin via FE2+. The production of CO with its vasodilatory and antiplatelet properties maintains microcirculation and may be involved in antiapoptotic mechanisms. The functions of HO-1 and CO seem to be related to their ability to modulate immune functions. (Haga et al, 1996). CO has been shown to exert i) anti-inflammatory actions ii). Regulate cGMP activity through activation of guanylate cyclise which is known to regulate endothelial-dependent vasodilatation iii). Inhibit platelet aggregation (Brune et al, 1987) i.v). CO inhibits apoptosis by activating MAPK (Otterbein et al, 2000). Endogenously generated CO rather than NO generated from iNOS (Pannen et al, 1998) has been shown to preserve sinusoidal perfusion and to limit hepatic dysfunction in a model of haemorrhagic shock in rats. HO activity maybe linked to iNOS (haem protein) (Maines et al 1997). INOS requires two haem molecules, and CO generated by HO-1 can bind to NO (Wang J et al, 1994) thus producing an inhibitory effect on iNOS.

RIPC and HO-1

Recently Lai et al (Lai et al. 2006) in a rat model of partial hepatic IR injury showed that remote ischaemic limb preconditioning confers cytoprotection and protection of liver function against IRI due to haemoxygenase expression. In a rat model of partial hepatic IR Lai preconditioned the liver by four brief cycles of prior hind limb ischaemia (10 min) followed by 10 min of reperfusion followed by hepatic ischaemia and 240 min of reperfusion. Lai et al demonstrated that RIPC reduced hepatic IR by decreased histological evidence of injury, decreased serum transaminase levels and showed increased haemoxygenase expression in the liver. They showed that haemoxygenase inhibition by zinc protoporphyrrin abolished the protective effects of RIPC. They also demonstrated that increased hepatic haemoxygenase persisted for 24 hrs in the liver and then showed a gradual decline.They did not demonstrate the microcirculatory changes in hepatic IR, the modulatory effect of remote ischaemic preconditioning on hepatic microcirculation and the effect of HO inhibition on the modulatory effects of RIPC in hepatic IR. We applied intravital microscopy to study the effect of haemoxygenase induction on the hepatic microcirculation in hepatic IR and the effect of inhibition of HO on the modulatory effecs of RIPC in hepatic IR.

Effects of PDTC

Therapeutic strategies aimed at reducing IR injury have focused both on preventing the effects of reactive oxygen species and on downregulating the signal transduction cascades related to the expression of proinflammatory genes. Pharmacologic preconditioning based on modulating endogenous protective molecules has been proposed as an alternative therapeutic intervention.

Among such agents, pyrrolidine dithiocarbamate (PDTC) has a range of biochemical activities including redox state alternation, chelation of heavy metals and enzyme

inhibition(Tsuchihashi *et al.*, 2003). PDTC was initially regarded as a potent inhibitor of nuclear factor- kappa B (NF-κB) and used as an antioxidant compound to counteract the toxic effects of free radicals(Liu *et al.*, 1999). PDTC is one of the most effective inducers of haeme oxygenase-1(HO-1), which also confers cytoprotection against oxidative stress(Hartsfield *et al.*, 1998). Hartsfield demonstrated that the antioxidant PDTC enhances HO-1 gene transcription (Hartsfield et al, 1998). HO is the rate-limiting enzyme in the conversion of haeme into carbon monoxide (CO), biliverdin (which is rapidly converted to bilirubin) and free iron (Fe^{2+})(Katori *et al.*, 2002). HO cleaves the haem ring into CO and biliverdin which is rapidly reduced to bilirubin.

Zinc protoporphyrrin (ZnPP)

Zinc protoporphyrrin is a competitive inhibitor of the enzyme HO-1 and also inhibits guanylate cyclase and depletes endogenous cGMP (Verma *et al*, 2003). Thus it inhibits the effect of CO (end product of haem degradation by HO-1) on endothelium through guanylate cyclase. ZnPP is a non-specific blocker of both HO-1 and HO-2. It has got toxic side effects on the CNS, liver at high doses (Greenbaum *et al*). ZnPP is a metalloporphyrrin which on exposure to light disintegrates and does not inhibit haemoxygenase. On exposure to light it exerts toxic effects and inhibits hepatic vasodilation.

4.2 Material and methods

4.2.1Animals and surgical procedures

Refer chapter 2, pg 92

4.2.2 Limb preconditioning

Refer chapter 2, pg 92

4.2.3Tissue and blood collection

Refer chapter 2, pg 105

4.2.4 Experimental groups (n=6 in each group)

Six groups of animals were studied. **(Observations for first four groups are based on experiments in chapter 1).**

- Group one (Sham) in which animals were subjected to laparotomy only and underwent an identical experimental protocol without clamping.
- Group two (IRI) the animals were subjected to 45 minutes of ischaemia followed by three hours of reperfusion. Ischaemia was induced by a microvascular clamp.
- Group three were preconditioned prior to IRI (RIPC+IRI) group. Protocols described above for preconditioning and inducing ischaemia were used.
- Group four sham animals were preconditioned.(RIPC+Sham)
- Group five animals (PDTC+IR) were given Pyrrolidine dithiocarbamate (PDTC, Sigma Chemical Co, St Loius, Mo, USA) 100 mg /kg dissolved in 0.9% sodium chloride given subcutaneously 30 minutes prior to IR.
- Group six animals (ZnPP+RIPC+IRI) were given Zinc protoporphyrin (ZnPP Sigma Chemical Co, St Loius, Mo, USA) 1.5 mg/kg (HO-1 blocker) by intraperitoneal route 1 hour prior to RIPC and subsequent IRI.

Selection of dose and route of administration of ZnPP and PDTC

The dose of ZnPP was selected according to the experiment described by Tsuchihashi.(Tsuchihashi *et al.*, 2003) (Kato *et al.,*2003). The route of administration was selected according to the study by Lai et al i.e intraperitoneal (Lai et al., 2006). Animals received a single dose of 1.5mg/Kg (2.5µmol/kg) of Zinc Protoporphyrin (HO inhibitor) given intraperitoneally 1 hr prior to RIPC . Our preliminary experiements revealed that higher doses caused animal death after 1-2 hrs of reperfusion and intravenous infusion caused the animals to be haemodynamically unstable soon after reperfusion with animal death. In order to obtain a haemodynamically stable model with gradual absorption of the drug through the intraperitoneal route we used the drug dosage and intraperitoneal route described above. Lai et al have described administration of ZnPP by intraperitoneal route 1 hr prior to RIPC. ZnPP was dissolved in 0.2 mol/L sodium hydroxide and diluted in 0.85% sodium chloride according to manufacturer guidelines. Ph was adjusted to 7.4. Stock solution concentration was 1mg/ml. ZnPP was prepared in a dark room to prevent disintegration and toxicity of the drug. It was administrered through a syringe covered with silver foil to avoid exposure to light.The dose for PDTC and route of administration was selected based on experiments by Tsuchihashi (Tsuchihashi *et al*., 2003). The time course and dose of PDTC used were based on previous studies by Liu *et al*(Liu *et al.*, 1999).

Experimental design

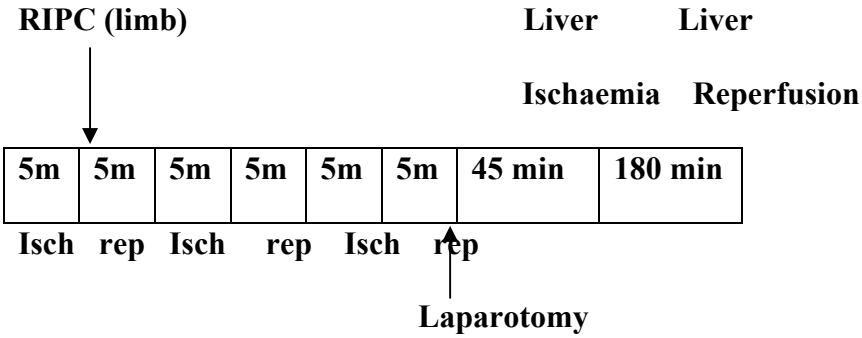


Figure (above) showing time points used in the study.

4.2.5 Intravital videofluorescence microscopy

Refer chapter 2 – material and methods, pg- 100-102

End points observed were- RBC velocity (V), sinusoidal Perfusion and perfusion index (PI), sinusoidal diameter (D), sinusoidal blood flow, neutrophil adhesion and hepatocellular death.

4.2.6 Histology

Liver tissue was fixed in 10% formalin and embedded in paraffin in preparation for light microscopy analysis. Sections were cut at 5μ and stained with haematoxylin and eosin for histological analysis. Modified Suzuki criteria (chapter 2, pg 103-104) were used to describe the histological changes.

4.2.7 Immunohistochemistry for HO-1 and western blots for HO-1

Immunohistochemistry and western blots for HO-1 were done as described in chapter 2, material and methods, pg 104-108.

4.2.8 Biochemical analysis for ALT / AST (Hepatocellular injury).

As described in chapter 2, material and methods, pg 103.

4.2.9 Statistical analysis

All data are presented as mean plus minus standard error of the mean (SEM).Differences were considered significant for $P<0.05$ .Comparisons between groups were performed by one way analysis (ANOVA). Bonferroni correction was applied for ANOVA.

4.3 Results

Haemodynamic measurements. (Fig 4.1)

RIPC+IR group showed a drop in MAP similar to IR only group however MAP recovered to baseline in RIPC+IR rapidly (30- 60 min) compared to IR (60-120min). HO inhibition caused a further drop in B.P. and delayed recovery of B.P.

There was no significant difference in pulse rate.

Hepatocellular injury (Fig 4.2)

The significant derangement of transaminases seen in on administering ZnPP to the RIPC group suggests that inhibition of HO-1 abolishes the protective effect of RIPC on the liver leading to increased hepatocellular in IR injury.

Blood pressure (MAP)

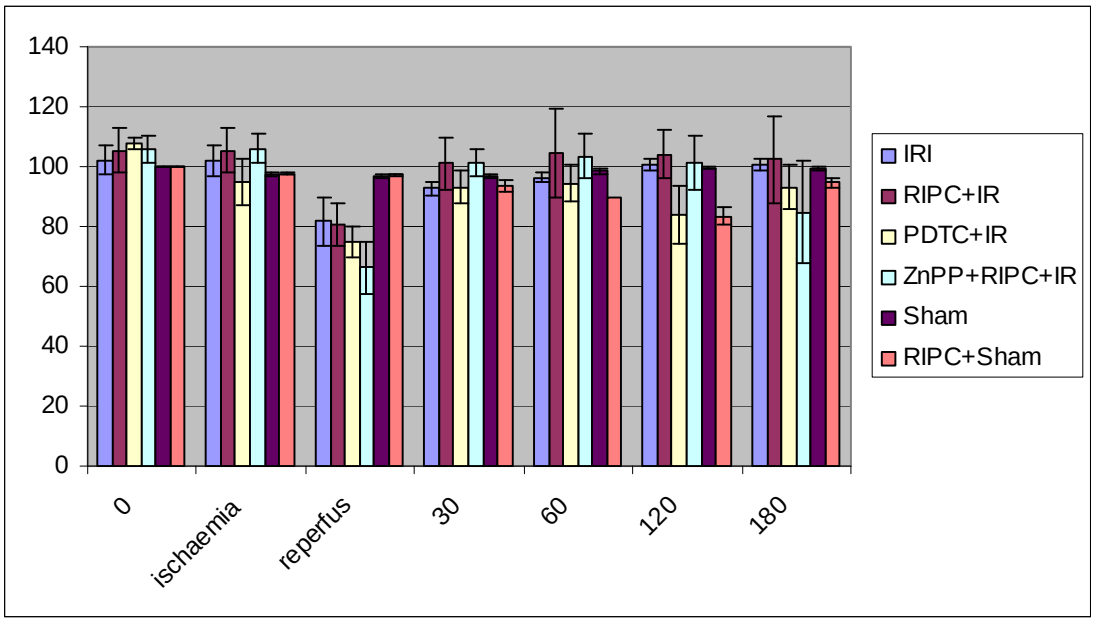


Figure 4.1 Changes in blood pressure - effect of HO inhibition on RIPC.
RIPC prior to IR restores B.P. to baseline rapidly compared to IR only. Inhibition of HO showed a greater fall in B.P but there was no difference in recovery to Baseline.Values expressed as mean + sem. P<0.05.

Serum transaminase levels

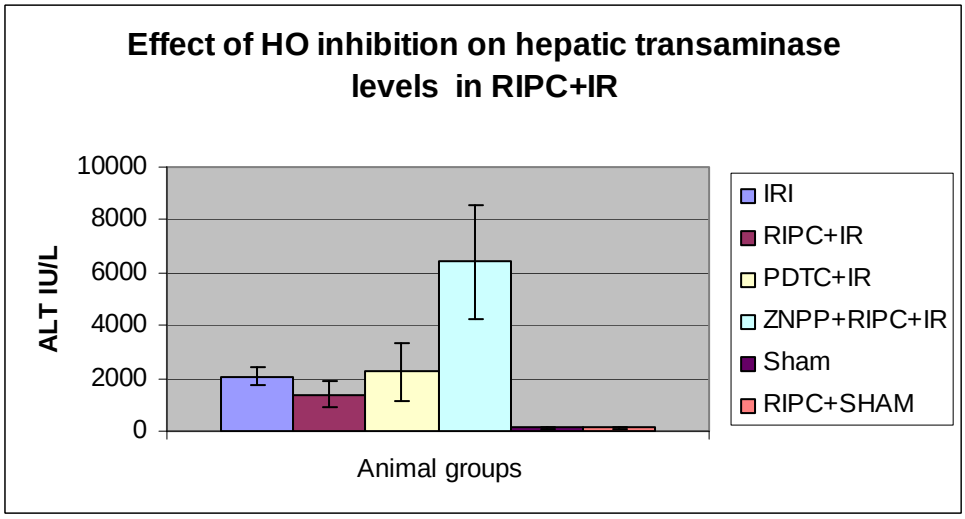


Figure 4.2 (IR/RIPC+IR, P>0.05. RIPC+IR/ZNPP+RIPC+IR, P<0.05)

Intravital microscopy results (Early phase of IR)

Velocity of RBC flow (Figure 4.3)

The velocity of flow was significantly reduced in hepatic IR (160.83 ± 12.24 μm/sec) after 180 min of reperfusion. RIPC significantly increased velocity of flow (328.04 ± 19.13 μm/sec). PDTC (HO induction) reproduced the effects of RIPC in hepatic IR (300.88± 22.109 μm/sec). ZnPP (HO inhibition) significantly reduced velocity of flow of RBC in the RIPC group (170.74± 13.43 μm/sec).

Sinusoidal flow (Figure 4.4)

The sinusoidal flow was significantly reduced in hepatic IR (8.42 ± 1.19). RIPC significantly enhanced sinusoidal flow (17.75± 2.59) and the effects of RIPC were reproduced by PDTC (17.66± 3.71). ZnPP significantly reduced sinusoidal flow in the RIPC group (9.46± 1.34).

Sinusoidal diameter (Figure 4.5)

There was no significant difference in the sinusoidal diameter between different groups.

Sinusoidal perfusion (Figure 4.6)

The sinusoidal perfusion index was significantly higher in RIPC group (67.28 ± 1.82) as compared to IR group at all time points (42.12 ± 7.28). PDTC reproduced the effects of RIPC in hepatic IR (82.33± 3.5). However inhibition of HO by ZnPP in RIPC (60.29± 1.82) showed a fall in perfusion only at 180 min of reperfusion.

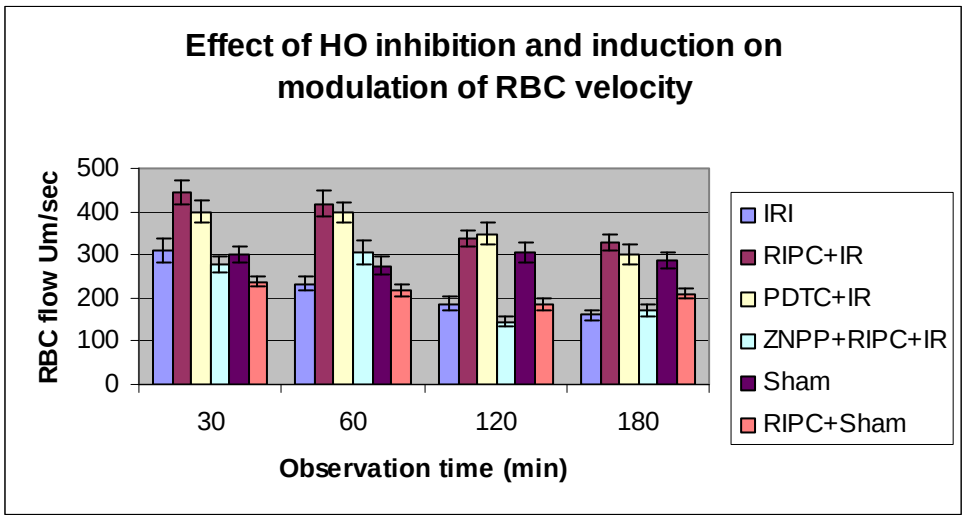


Figure 4.3 – Velocity of blood flow.

Significant increase in velocity in preconditioned animals prior to ischaemia reperfusion injury (RIPC+IRI) as compared to IRI at 30, 60 and 120 minutes of reperfusion.HO inhibition causes a significant fall in velocity of flow in preconditioned animals. Values expressed as mean ± sem. * = P<0.05 (RIPC+IR/IR). **= P<0.05 (RIPC+IR/ ZNPP+RIPC+IR).

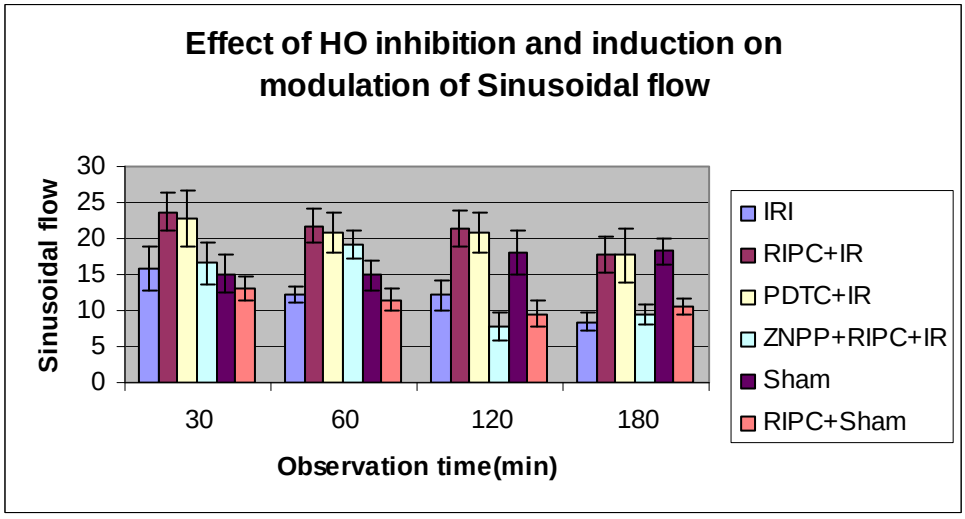


Figure 4.4- sinusoidal flow

Sinusoidal flow - V× (D/2)2×л = V is velocity of RBC, D is sinusoidal diameter. Significantly better flow in preconditioned animals (RIPC+IRI) as compared to non preconditioned (IR). HO inhibition significantly inhibits flow in preconditioned animals.Values expressed as mean ± sem. * = P<0.05(RIPC+IR/IR).
** = P<0.05(RIPC+IR/ ZNPP+RIPC+IR).

Diameter of sinusoids

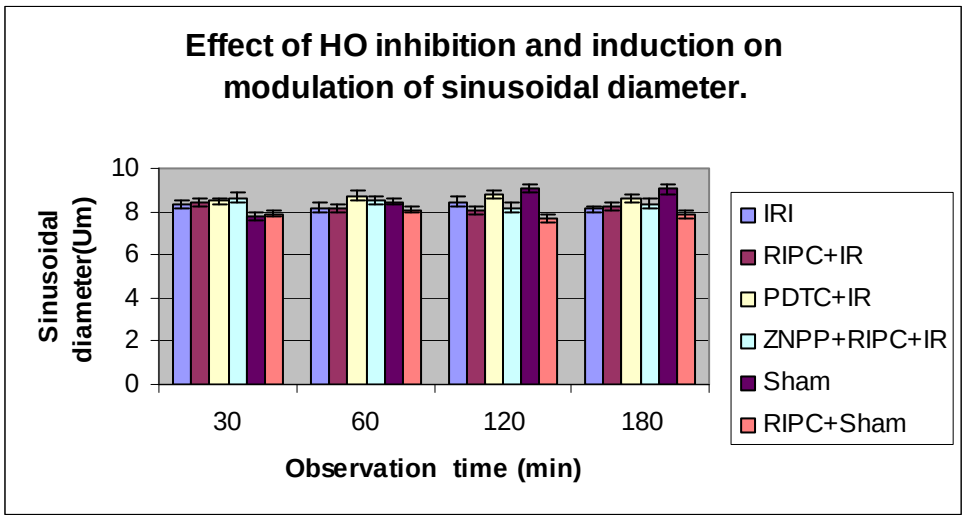


Figure 4.5 (No significant change in sinusoidal diameter seen in preconditioned animals and on inhibition of HO)

Perfusion of sinusoids

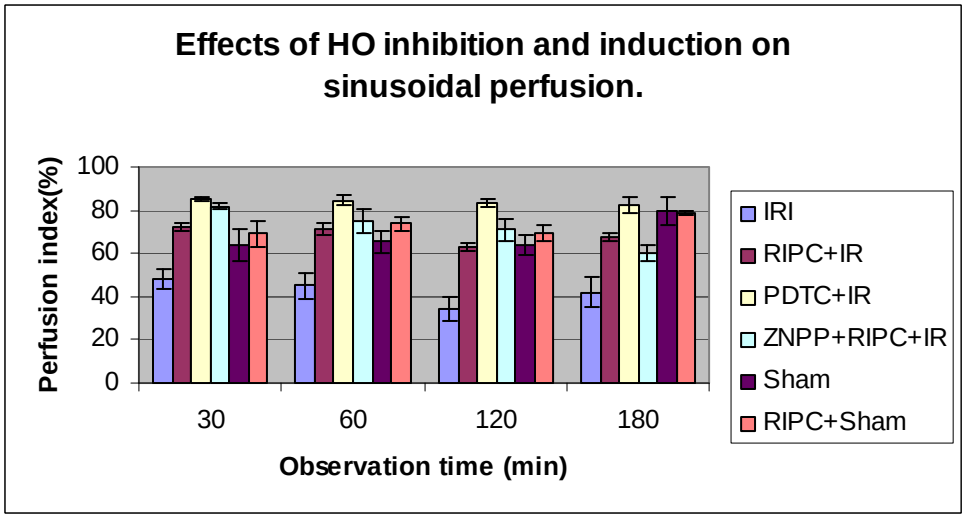


Figure 4.6

Sinusoidal perfusion index - The PI in remote preconditioned animals (RIPC+IRI) is significantly higher than non preconditioned animals (IRI). HO inhibition does not affect sinusoidal perfusion in the early stage of IR however sinusoidal perfusion declines at 180 minutes of reperfusion. Values expressed as mean $\pm$ sem. $P<0.05$.

Neutrophil adhesion in venules and sinusoids as seen by IVM.

Effect of IR

Significantly increased neutrophil adhesion in IR injury is seen in both postsinusoidal venules (769.05 ± 87.48) and sinusoids (97.4± 7.49).

Effect of RIPC+IR

Preconditioning significantly reduced neutrophil adhesion in IR injury in both postsinusoidal venules (219.66± 93.79) and sinusoids (25.69± 9.08).

Effect of PDTC+IR

PDTC significantly reduced neutrophil adhesion in both postsinusoidal venules (89.58± 58.32) and sinusoids (17.98 ± 11.01) reproducing the effects of RIPC.

Effect of HO inhibition on RIPC+IR.

HO inhibition with ZnPP significantly increased venular (589.04 ± 144.36) and sinusoidal neutrophil adhesion in preconditioned animals (121.39 ± 30.65).

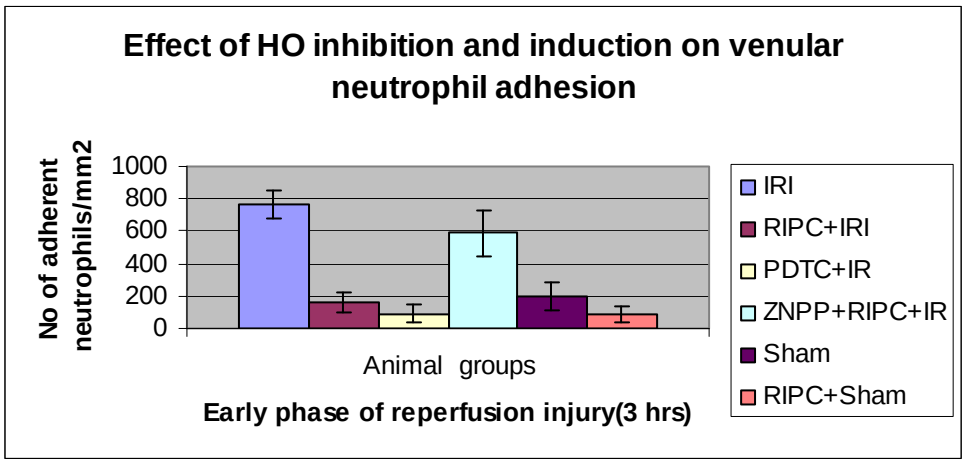


Figure 4.7 (Venular neutrophil adhesion in early phase of hepatic IR)

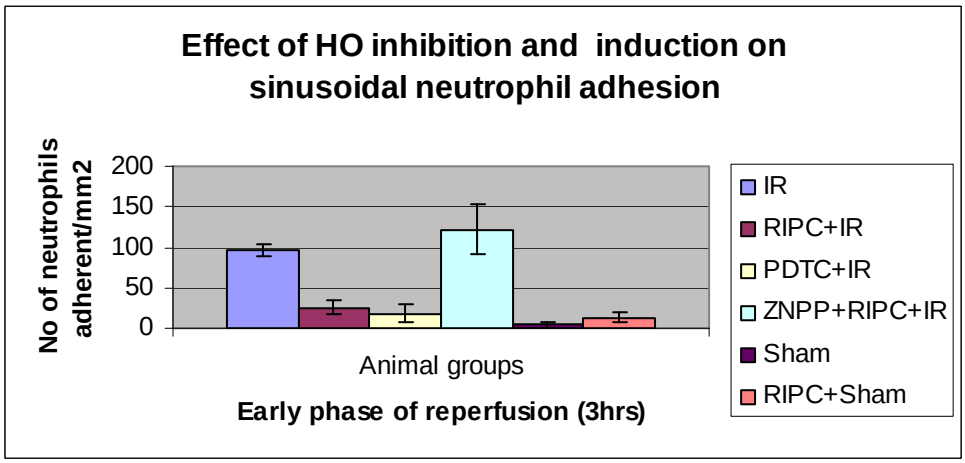


Figure 4.8 (Sinusoidal neutrophil adhesion in early phase of hepatic IR)

Significantly reduced venular neutrophil adhesion in preconditioned (RIPC+IRI) group compared to non preconditioned group (IRI). HO inhibition in RIPC+IR showed significantly increased venular neutrophil adhesion .Values expressed as mean $\pm$ sem. * = $P<0.05$ (IR/RIPC+IR), ** = $P<0.05$(RIPC+IR/ ZNPP+RIPC+IR),
Significantly reduced sinusoidal neutrophil adhesion in preconditioned group (RIPC+IRI) compared to non preconditioned group (IRI). Values expressed as mean $\pm$ sem. * = $P<0.05$ (IR/RIPC+IR), **= $P<0.05$(RIPC+IR/ ZNPP+RIPC+IR).

Hepatocellular death in IR

Significantly increased cell death was observed in IR injury of the liver (80.83 ± 13.03) Fig 4.9 a & 4.9 b.

Hepatocellular death in RIPC+IR

Preconditioning significantly reduced hepatocellular death in hepatic IR (17.35 ± 2.47) Fig 4.9a& 4.9c.

Hepatocellular cell death in PDTC+IR (HO induction)

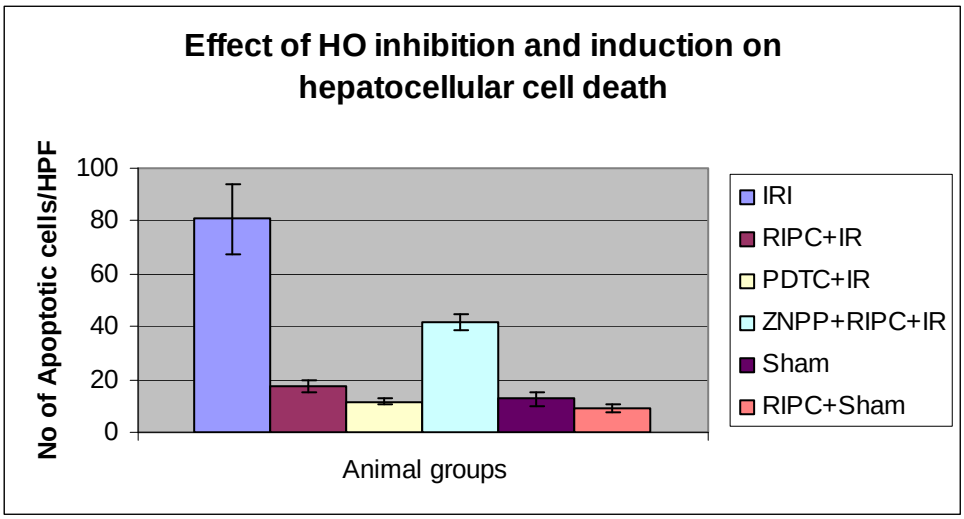
PDTC significantly reduced hepatocellular death in hepatic IR reproducing the effects of RIPC (11.66 ± 1.17) Fig 4.9 a & 4.9d.

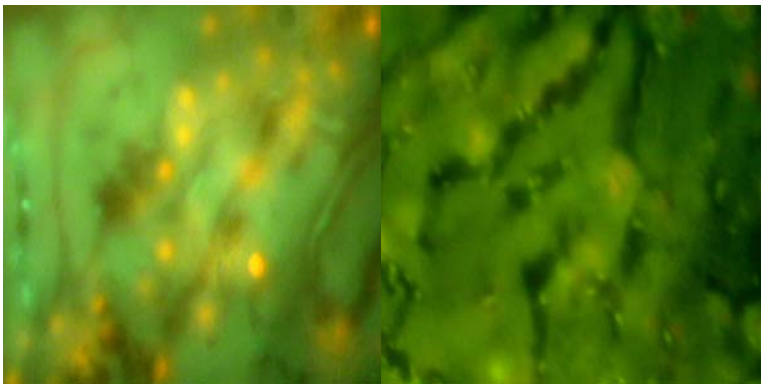
Hepatocellular death in ZnPP +RIPC+IR (HO inhibition)

ZnPP significantly increased hepatocellular death in preconditioned animals (41.33 ± 3.07) Fig 4.9 a & 4.9e.

Hepatocellular death

a)



b)  c).

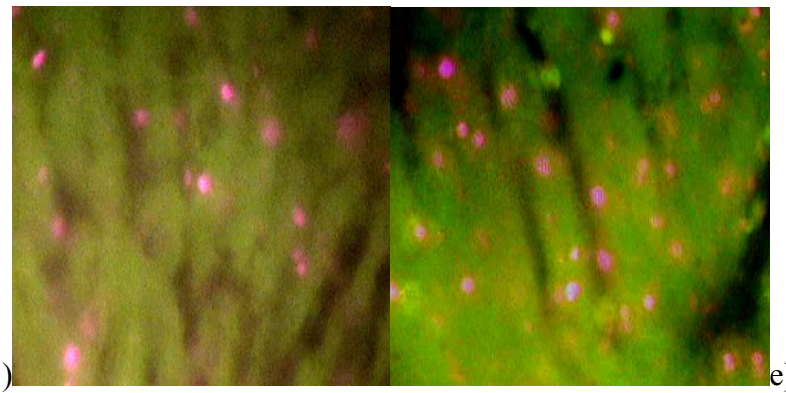
d)  e)

Figure 4.9

Figure a). Hepatocellular cell death in preconditioned group (RIPC+IRI) is significantly less compared to non preconditioned (IRI) group. HO inhibition significantly enhances hepatocellular death in preconditioned animals. Values expressed as mean $\pm$ sem. * = P<0.05 (IR/RIPC+IR), **= P< 0.05 (RIPC+IR/ ZNPP+RIPC+IR).

Fig b).Hepatocellular cell death in IRI 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 c).Hepatocellular cell death seen in RIPC+IR by propidium iodide staining (IVM).

Fig d). Hepatocellular cell death is also significantly less in PDTC+IR group.

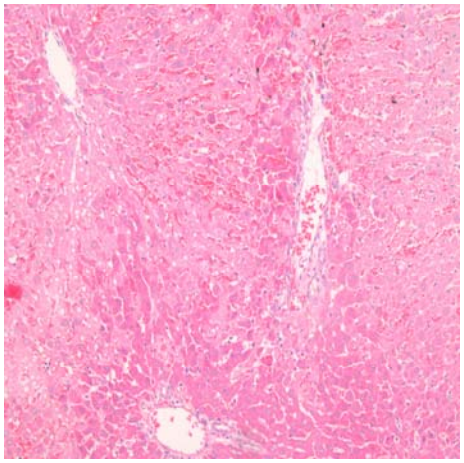
Fig e).HO inhibition by Zinc protoporphyrin significantly increases cell death in RIPC group.

Histology

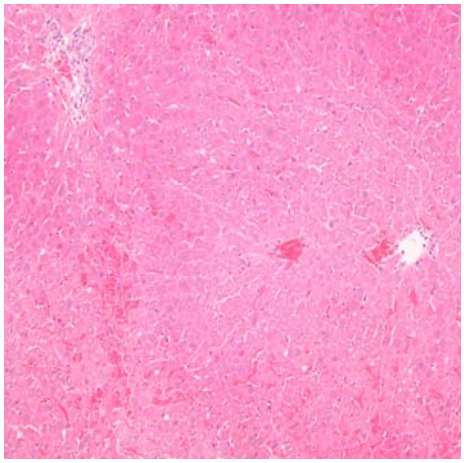
Group	IR	RIPC+IR	ZnPP+RIPC+IR	PDTC+IR	Sham	RIPC+Sham
Suzuki score	**8.83±0.7**	**6.2± 0.58**	**8.83 ± 0.6**	**4.5 ± 0.5**	**4±0.31**	**1.5 ± 0.34**

Table 5.1 (Suzuki criteria)

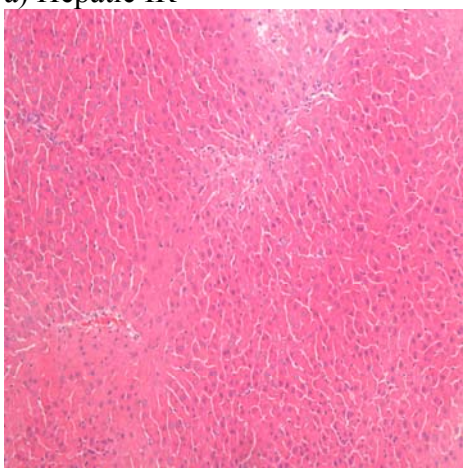
IR group showed significant periportal congestion in association with severe necrosis in zones 2 and 3 and also sub capsular necrosis. The changes are diffuse. RIPC+IR showed a significantly lower Suzuki score compared to the IR group. On inhibition of HO in RIPC+IR (ZNPP +RIPC +IR group) most of the sections showed diffuse and severe congestion and vacuolation. The necrosis is diffuse and focally associated with acute inflammatory cells. Changes are similar to IR group and significantly more compared to RIPC+IR. Only one animal showed exception to the necrosis with extensive congestion and vacuolation but minimal necrosis. On HO induction (PTDC+IR) most animals showed diffuse congestion and necrosis except for one which shows mild congestion with significant vacuolation and no well established infarction or necrosis. Sham animals revealed minimal changes. Congestion is present in both portal vein and central vein. Hepatocytes show vacuolation. RIPC+Sham animals show minimal changes, some vacuolation, but no necrosis, similar to sham.



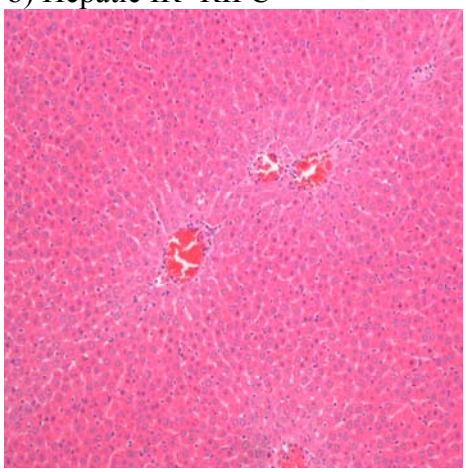
a) Hepatic IR



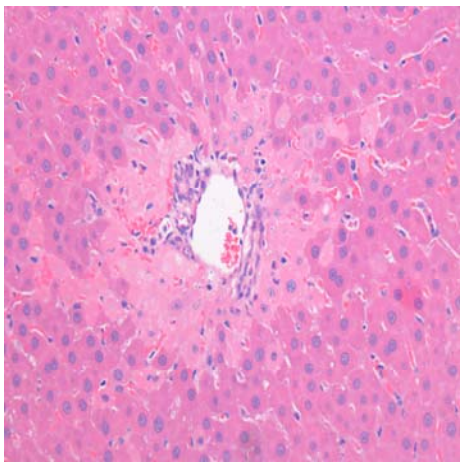
b) Hepatic IR+RIPC



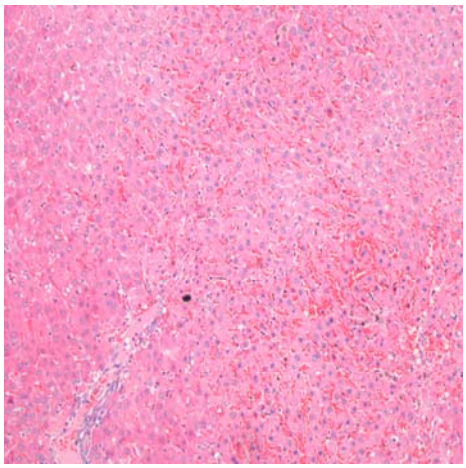
c) Sham



d) RIPC+Sham



e) PDTC+Hepatic IR



f) ZNPP+RIPC+IR

Figure 4.10

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

section reveals congested central vein but no other significant change.e) PDTC+IR- diffuse congestion and patchy necrosis. f) ZNPP+RIPC+IR- extensive necrosis seen

Immunohistochemistry and western blots for HO-1

Immunohistochemistry revealed increased HO-1 expression in kupffer cells in RIPC +IR animals in comparison to IR group. PDTC reproduced the effects of RIPC. ZnPP decreased HO expression in RIPC (results, chapter 6). Western blot analysis showed by densitometry significantly more HO expression in RIPC groups as compared to hepatic IR and PDTC reproduced the effects of RIPC. ZnPP inhibited HO expression in the RIPC group (results, chapter 7).

4.4 Discussion and conclusion.

New findings

This is the first study to demonstrate RIPC induced modulation of hepatic microcirculation through haemoxygenase by intravital microscopy.

Microcirculatory changes

Effect of IR. (Early phase)

In hepatic IR there was a significant decrease in RBC flow, sinusoidal flow and sinusoidal perfusion. There was no significant change in sinusoidal diameter as compared to sham group in the early phase of hepatic IR.

Effect of RIPC on hepatic IR (RIPC+IR) (Early phase)

In this experimental model RIPC+IR showed an increased velocity of RBC flow, sinusoidal perfusion compared to IR group at all time points of observation. No difference in sinusoidal diameter in the RIPC group as compared to IR injury group was seen. This observation was made in previous studies in ischaemic preconditioning which have demonstrated no significant variation in sinusoidal diameter between HO induction and controls (Hata et al).Moreover the HO produced in the initial phase is in response to stress (inducible HO-1) and is responsible for anti-inflammatory functions. The HO which modulates sinusoidal tone and diameter is constitutive HO (HO-2) and is not produced in kupffer cells in the early phase of hepatic IR (3hrs of reperfusion). Constitutive HO (HO-2) is produced in parenchmal cells after 6hrs of reperfusion as shown by Goda et and CO derived from an increase in HO-2 (Constitutive HO) in the parenchyma is responsible for modulation of sinusoidal tone and diameter (Goda N et al 1998).

Effect of HO induction on hepatic IR (PDTC+ IR)

This study shows increased RBC velocity and sinusoidal flow in the PDTC+IR group as compared to IR only. The perfusion index in the PDTC group is significantly higher than IRI at all time points suggesting that PDTC modulates sinusoidal perfusion. PDTC is known to induce HO-1, inhibit NF-KB and modulate IRI and increased HO-1 may be responsible for modulation of sinusoidal perfusion. This finding is supported by evidence from studies in animal models which have shown that HO-1 expression is associated with better sinusoidal perfusion {McCarter et al, 2004; Nie et al, 2002}. However there was no significant difference in sinusoidal perfusion between HO induction and HO inhibition (ZnPP group) upto 120 minutes of reperfusion. This observation suggests that HO pathways maybe responsible for modulation of perfusion after 120 min of reperfusion. This observation is supported by previous studies which have shown PDTC induced HO to increase sinusoidal perfusion at 120 minutes of reperfusion {Nie et al, 2002} in hepatic IR secondary to prolonged limb ischaemia. The initial modulation of sinusoidal perfusion in hepatic IR by PDTC maybe due to its anti-inflammatory action and inhibitory effect on NF-kβ.

A previous study by Hata demonstrated no significant difference between PDTC and ZnPP with regard to sinusoidal diameter however; recently Hata et al have demonstrated an increase in sinusoidal diameter due to increase in parenchymal HO-1 on administration of higher doses of PDTC (150 mg/kg) intramuscularly in a rat model of hepatic IRI. Hata has shown that CO derived from PDTC induced increase in parenchymal HO & modulated sinusoidal diameter in the early phase of hepatic IR. {Hata et al, 2003}.

Effect of HO inhibition on RIPC+IR (ZnPP+RIPC+IR)

Inhibition of HO in preconditioned groups showed a significant fall in RBC velocity and sinusoidal flow in the RIPC+IR+ZnPP group suggesting that RIPC induced HO modulates RBC flow and sinusoidal flow in hepatic IR. HO inhibition did not affect sinusoidal perfusion initially in preconditioned groups but a late fall in perfusion index was observed suggesting the role of RIPC induced HO in modulation of sinusoidal perfusion at 120 minutes of reperfusion. This finding is consistent with PDTC (HO inducer) induced modulation of sinusoidal perfusion which showed significantly increased sinusoidal perfusion as compared to ZnPP group at 120 minutes of reperfusion. The lack of any change in sinusoidal diameter on HO inhibition suggests that RIPC modulates IR injury by anti-inflammatory properties of HO rather than sinusoidal dilatation in the early phase of hepatic IR.

Correlation of histological changes in IR with microcirculatory flow, hepatocellular death, neutrophil adhesion, serum transaminases& HO expression.

The modified Suzuki score in IR injury was significantly higher (8.83 $\pm$ 0.7) than preconditioned group or PDTC group. This correlated with decreased flow and sinusoidal perfusion, increased hepatocellular death observed by propidium iodide staining under IVM in hepatic IR, increased sinusoidal and venular neutrophil adhesion and increased serum transminase levels suggestive of increased hepatocellular death. Increased HO expression was seen in kupffer cells in hepatic IR as compared to sham group. This is due to oxidative stress of IR and has been observed in hepatic IR in previous experiments by Katori et al and Hata et al.

Correlation of histological changes in RIPC+IR with microcirculatory flow, hepatocellular death, neutrophil adhesion, serum transaminases & HO expression.

The modified Suzuki score in preconditioned animals was significantly lower (6.2 ± 0.58) in preconditioned animals which correlated with improved blood flow, sinusoidal perfusion, decreased hepatocellular death observed by IVM , decreased sinusoidal and venular neutrophil adhesion and decreased serum transaminase levels suggestive of decreased hepatocellular death. Lai et al showed in an animal model decreased histological evidence of injury and serum transaminases supporting our findings in this study although they did not investigate microcirculatory flow. Increased HO expression was seen in RIPC as compared to IR only. This finding was observed by Lai et al (2006) and supports our finding.

Correlation of histological changes in PDTC+IR with microcirculatory flow, hepatocellular death, neutrophil adhesion, HO expression& serum transaminases.

PDTC+IR group showed a significantly lower Suzuki score (4.5± 0.5) suggestive of less congestion and necrosis. This correlated well with improved flow, sinusoidal perfusion, decreased neutrophil adhesion, hepatocellular death and serum transaminase levels suggestive of decreased hepatocellular injury. PDTC reproduced the effecs of RIPC. Increased HO expression was seen on both western blot analysis (Chapter 7) and Immunohistochemistry (chapter 6) as compared to IR.

Previous studies have shown by intravital microscopy improved flow and sinusoidal perfusion and decreased hepatocellular death following PDTC induced HO expression in the liver {McCarter *et al*, 2004} {Wunder *et al*, 2002}.

Correlation of histological changes seen on HO inhibition in RIPC with microcirculatory flow, hepatocellular death, neutrophil adhesion, serum transaminases and HO expression.

Inhibition of HO by ZnPP in the preconditioned group showed a significantly high Suzuki score (8.83 $\pm$ 0.6) suggestive of increased cell necrosis and congestion which correlated with decreased sinusoidal flow and perfusion, increased hepatocellular death on IVM , increased neutrophil adhesion and serum transaminase levels suggestive of significantly increased hepatocellular injury. Decreased HO expression was seen on immunohistochemistry and Western blot analysis in comparison to RIPC group. Previous studies by Busuttil et al have shown that HO inhibition of the donor with chromium mesoporphyrrin prior to harvest of the liver was associated with decreased recipient animal survival following subsequent implantation in recipient liver transplant models. Lai et al showed that inhibition of HO by ZnPP in RIPC was associated with significantly increased histological evidence of cell necrosis and congestion and increased serum transmanases.

ZnPP and sham and hepatotoxicity of ZnPP.

Previous experiments in sham animals have shown that ZnPP inhibits haemoxygenase if used in the darkness. If ZnPP is exposed to light its effect of HO inhibition is lost however, ZnPP inhibits hepatic artery dilatation when exposed to light and this potentially leads to hepatotoxicity (Greenbaum et al; 1991, zygmunt et al; 1994). Hepatoxicity can confound the experimental data and hence in order to avoid this confounding effect ZnPP was prepared in the dark and administered through a syringe covered with silver foil with minimal laboratory light. The dose used was 1.5 mg /kg (2.5μmol/kg) and this is much lower than the hepatotoxic toxic dose of ZnPP (5- 10μmol/kg) as shown by Greenbaum et al. Amersi et al (1999)

showed that ZnPP treatment in shams led to undetectable baseline HO and reduced portal flow and bile flow. Since previous experiments on shams have been undertaken we did not undertake experiments to investigate effect of ZnPP on sham group in our study. However toxic effects of ZnPP cannot be completely ruled out and future work to investigate the effects of ZnPP on Shams should be undertaken.

PDTC and sham and toxicity of PDTC.

Tsuchihashi et al (2003) demonstrated in an animal model that doses in excess of 600 mg /kg killed animals but animals did not die by injection of any dose upto 600mg/kg. Doses more than 200 mg/ kg offset the beneficial effects of PDTC as demonstrated by Liu et al (1999). Hence we chose a dose of 100 mg/ kg as used in experiments by Tsuchihashi. PDTC shows a dose dependent relation ship with regard to its beneficial effects. At doses of 100 mg/ kg PDTC induces HO in kupffer cells but does not influence sinusoidal dilatation. Hata et al showed that at doses of 150 mg/ kg {Hata et al, 2003} PDTC induced sinusoidal dilatation in sham animals increased HO-1 with peak mRNA levels detectable at 3 hrs after PDTC injection and then declined. HO Protein expression peaked at 24-48 hrs after injection and was dose dependent. Immunohistochemistry revealed that PDTC at higher doses induced HO-1 in both periportal kupffer cells and hepatocytes in pericentral areas.

Haemodynamics

Blood pressure

The model used was haemodynamically stable. However a fall in blood pressure on reperfusion was seen in hepatic IR, preconditioned animals, PDTC+IR and ZnPP group with recovery to baseline in 30-60 minutes. The blood pressure thereafter was stable throughout the time course of observation. Hence the potential effect of

hypotension leading to decreased parenchymal perfusion and confounding results was avoided.

Conclusion

This study has again demonstrated the hepatic haemodynamic changes which are associated with the early phase of liver warm IR injury. Sinusoidal perfusion and flow during this early period are improved with remote preconditioning. The effect of RIPC could be reproduced by HO-1 induction prior to the IR injury and inhibited by a HO-1 blocker. This data would suggest that HO-1 is key to these early haemodynamic changes. In this early period inducible HO-1 rather than constitutive HO-1 is likely to be responsible.

The next chapter focuses on investigation of microcirculatory changes in the late phase of hepatic IR and the effect of RIPC on the late phase of hepatic IR.

Chapter 5
New insights into the late phase of hepatic IR and the effect of RIPC on hepatocellular and microvascular changes after 24 hrs of reperfusion

5.1 Introduction

Why study the late phase of hepatic IR and the effect of RIPC on the late phase of hepatic IR?

The previous chapters have analysed the early phase of liver warm ischaemia reperfusion injury and demonstrated that HO-1 inhibition can abolish the protective hepatocellular and microvascular changes associated with RIPC in hepatic IR injury. In this chapter we have studied the late phase of hepatic IR and the effect of RIPC 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 preconditioning on protection of the liver in hepatic IR

Previous studies investigating the effect of RIPC in the late phase of IR (recovery models).

Eight studies have shown protection of the target organ 24-48hrs following RIPC.(Oxman et al. 1997; Pell et al. 1998; Schoemaker & van Heijningen 2000; Wang et al. 2001; Wolfrum et al. 2002) These studies showed delayed protection in all organ systems in animal models and in recent human studies (refer chapter one, page 47, 48). One study showed reduced myocardial infarct size following RIPC (Chen et al , 2005) induced HSP expression in the myocardium.The first clinical application of RIPC in children undergoing cardiac surgery on bypass showed that RIPC was most effective in myocardial protection if applied 24 hours prior to the coronary ischaemic insult.(Cheung et al. 2006).

There have been no studies which have investigated the effect of RIPC on the microvascular changes in late phase of hepatic IR and haemoxygenase expression in the late phase of hepatic IR following RIPC. This study has investigated the impact of RIPC on microvascular and hepatocellular injury in the late phase of hepatic IR. The study also investigates hepatic HO expression in the late phase of preconditioning.

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 in the early phase serves to prime macrophages and neutrophils for release of ROS(Jaeschke et al. 1993). Complement 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. 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 infiltratrion with these changes being significantly more in the late phase of hepatic IR.

Potential inducible mechanisms in the late phase of hepatic IR which may not be evident in the early phase of IR.

In the late phase of RIPC, synthesis or post translational modifications of Heat shock proteins, antioxidant enzymes and iNOS have been demonstrated. These mechanisms are triggered by adenosine, ROS, NO, Opioids and Catecholamines and subsequent pathways involve activation of kinases which lead to transcription of new proteins and culminate in formation of mediators such as inducible nitric oxide synthase (iNOS), HO-1, HSP which undergo postranslational modifications leading to the protective response (chapter 1 , page 52-80) . HO –mRNA protein is detectable as early as 2hrs after reperfusion and increased levels in the liver have been demonstrated at 18hrs of reperfusion following preconditioning (Terajima *et al,* 2000*)* and proteins such as iNOS are usually detectable after 24 hrs of reperfusion.

5.2Materials and methods

Animals and surgical procedures-refer chapter 2 (material and methods)**.**

5.3 Animal model

5.3a Hepatic I/R (24 hrs)

Refer chapter 2, material and methods, page 92.

5.3 b Limb preconditioning

Refer chapter 2, material and methods, page 92.

5.4 Experimental groups (n=6 in each group)

1. **Group one- Hepatic IR-24**- in which animals were subjected to ischaemia for 45 minutes and reperfusion for 24 hours. Ischaemia was induced by an atraumatic microvascular clamp.
2. **Group two- RIPC+IR-24** - were preconditioned immediately prior to ischaemia for 45 min and reperfusion for 24 hrs.Both groups were terminated at 24 hours after intravital microscopy. Standard protocols for preconditioning and inducing ischaemia were used.

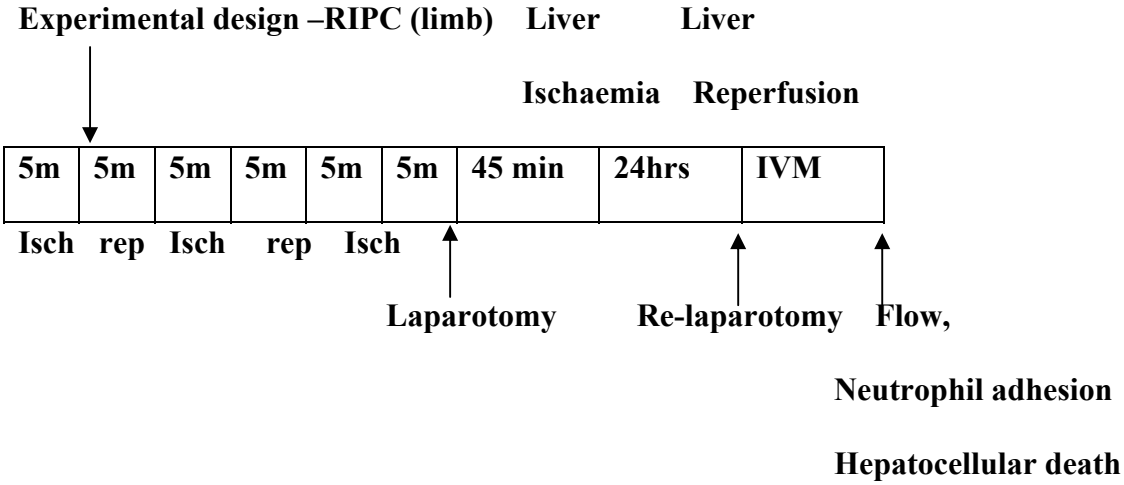


Figure- 5.1

5.5 Recovery after surgery and survival

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. Animals which appeared to do poorly were killed before the 24 hour reperfusion end point. **One animal died after 24 hours of reperfusion in the IR group only.** After 24 hours of reperfusion the animals were relaparotomised and cannulated in the carotid and jugular vessels of the opposite side of the neck. The liver was then subjected to intravital microscopy assessment.

5.6 Intravital videofluorescence microscopy

Refer chapter 2 - material and methods, pg 103.

5.7 Biochemical analysis

Liver transaminases were assessed by method described in material and methods chapter 2, page 105

5.8 Histology- refer chapter 2 material and methods, pg 105

5.9 Immunohistochemistry and western blots for HO – refer chapter 2 material and methods, pg 106- 110.

5.10 Statistical analysis

All data are presented as mean plus minus standard error of the mean (SEM).Differences were considered significant for $P<0.05$ .Comparisons between groups were performed by one way analysis (ANOVA). Bonferroni correction was applied for ANOVA.

5.11 Results

Velocity of flow

The velocity of blood flow is not significantly different in preconditioned animals in comparison to IR injury only at 24 hours of reperfusion. The mean velocity in RIPC+IR-24 was 415.75 ± 48.12 in comparison to 399.86± 48.09 in the IR-24 (**Figure 5.2).**

Sinusoidal flow

The sinusoidal flow is more in the RIPC+IR-24 group but not significantly different in comparison to IR injury at 24 hours of reperfusion. The mean sinusoidal flow in RIPC+IR-24 was 28.43± 2.99 in comparison to 21.88 ± 2.58 in IR-24 (**Figure 5.3).**

Sinusoidal diameter

The sinusoidal diameter is significantly more in the RIPC+IR group (9.33± 0.17) as compared to the IR group (8.37 ± 0.22) 24 hours of reperfusion injury. ($P<0.05$) (**Figure 5.4)**

Sinusoidal perfusion

The sinusoidal perfusion index which is the ratio of perfused (continuous and intermittent) to non perfused sinusoids is significantly higher in the RIPC+IR-24 group (0.83± 0 .016) in comparison to IR-24 (0.64 ± 0.05)(**Figure 5.5**)

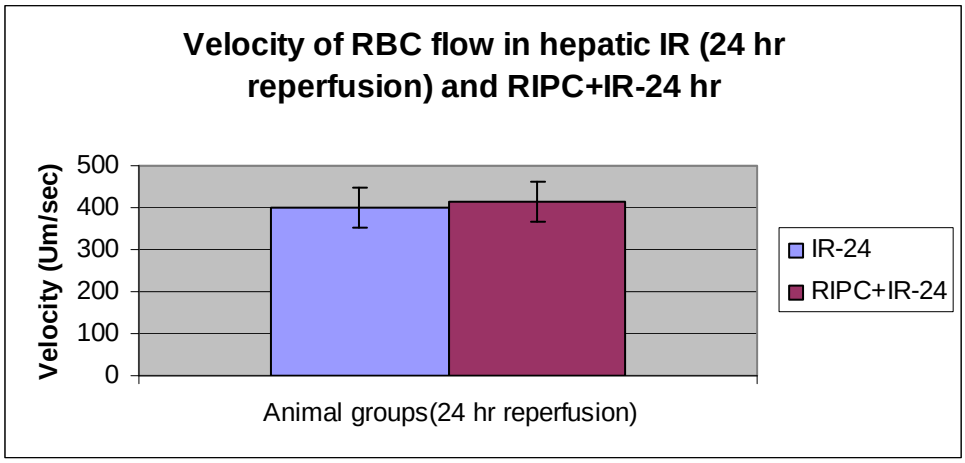


Figure 5.2

Figure - Velocity of RBC flow in RIPC+IR-24 as compared to IR-24. Values expressed as mean ± sem. P>0.05. (No significant difference seen between IR-24 and RIPC+IR-24)

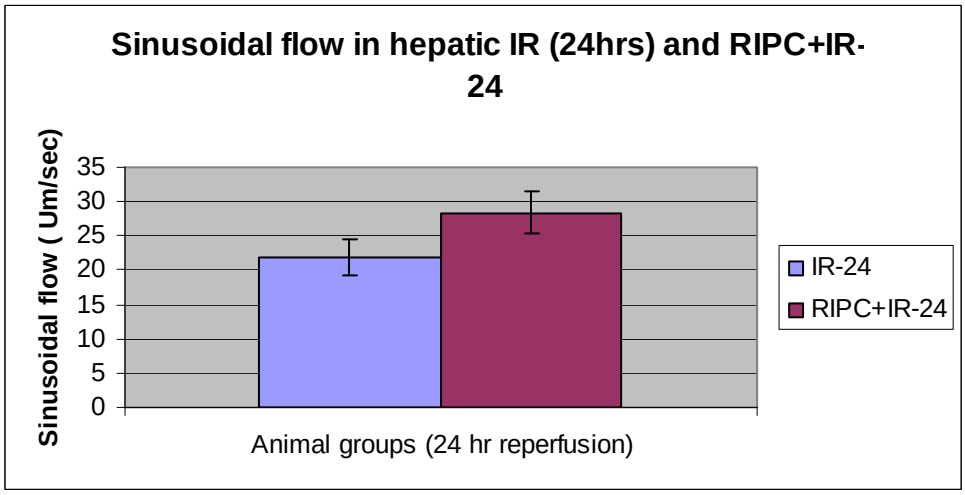


Figure 5.3

Sinusoidal flow - V× (D/2)2×л = V is velocity of RBC, D is sinusoidal diameter. Better flow in preconditioned animals (RIPC+IRI-24) as compared to non preconditioned (IR-24). Values expressed as mean ± sem. (P>0.05)

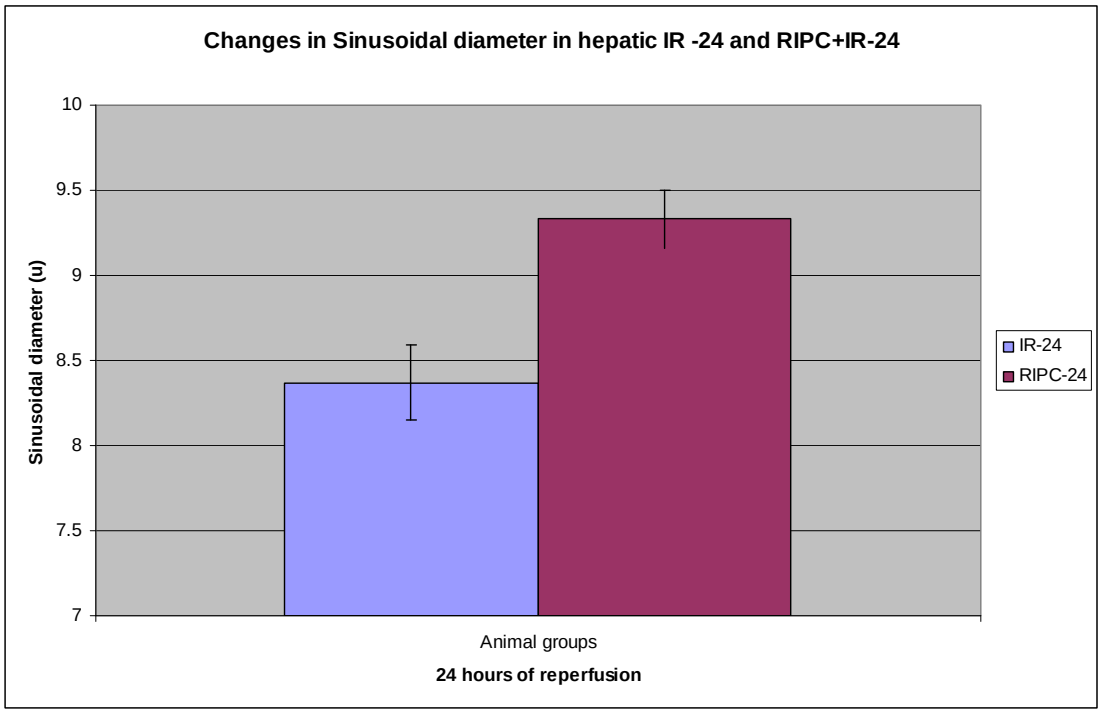


Figure 5.4

Figure - Sinusoidal diameter- Significantly increased diameter in the preconditioned group (RIPC+IR-24). Values expressed as mean ± sem. P<0.05. (Significantly increased sinusoidal diameter in the preconditioned group.)

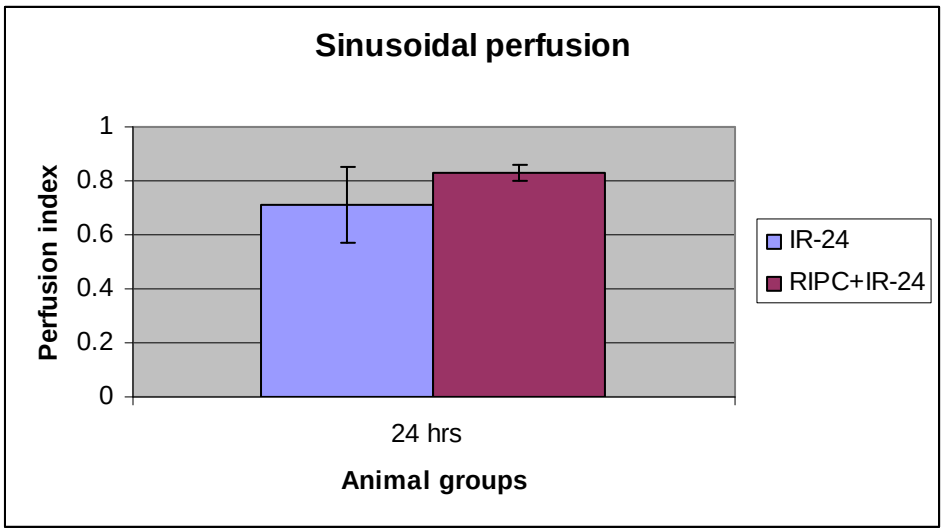
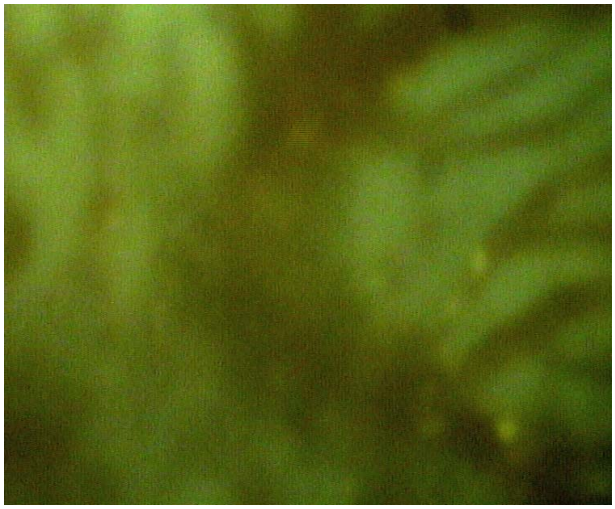
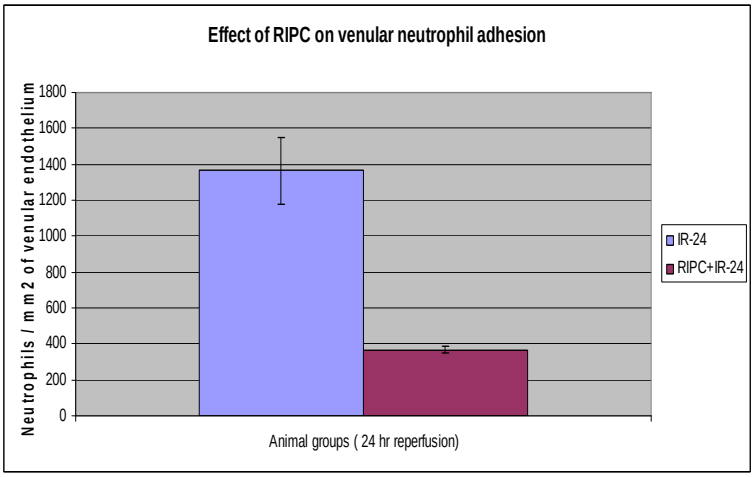
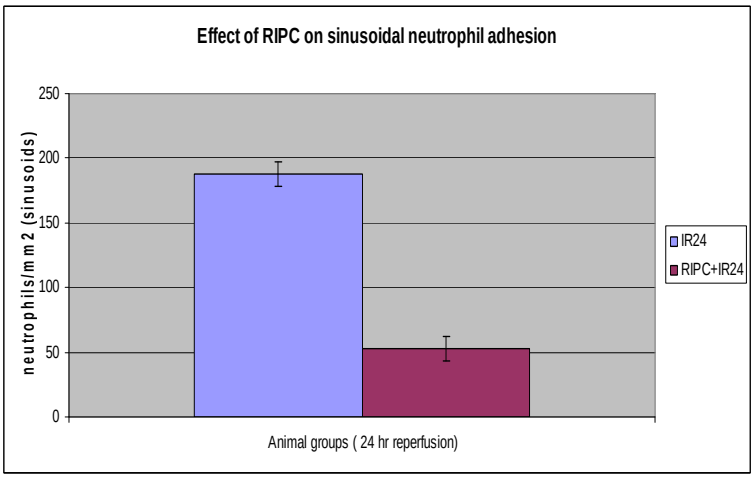


Figure 5.5

Sinusoidal perfusion index - The PI in remote preconditioned animals (RIPC+IR-24) is significantly higher than non preconditioned animals (IR-24). Values expressed as mean ± sem. * =P<0.05







Figures 5.6 a - 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 5.6 b - Significantly reduced venular neutrophil adhesion in preconditioned (RIPC+IR-24) group compared to non preconditioned group (IR-24). Values expressed as mean $\pm$ sem. * = P<0.05 (IR-24/RIPC+IR-24)

Figure 5.6 c- Significantly reduced sinusoidal neutrophil adhesion in preconditioned group (RIPC+IRI) compared to non preconditioned group (IRI). Values expressed as mean $\pm$ sem. * = P<0.05 (IR-24/RIPC+IR-24

Hepatic transaminases

The liver transaminases were higher in the IR-24 group in comparison to RIPC+IR-24 group. **(Figure 5.7)**

Hepatocellular death

The number of dead cells in the IR-24 group was significantly higher as compared to the RIPC+IR-24 group. The mean number of dead hepatocytes in IR group was 36± 2.9. The mean number of dead hepatocytes in RIPC+IR was 10± 1.92. **(Figure 5.8)**

Histology

Very severe injury with abundant ballooning degeneration and necrosis is seen in the IR-24 injury group. Very diffuse and significant neutrophil adhesion is seen in the IR group. Apoptosis is evident in the IR group.

RIPC +IR-24 group shows less injury with some ballooning and degeneration as well as neutrophilic infiltration. **(**Figure **5.9)**

Hepatic transaminases

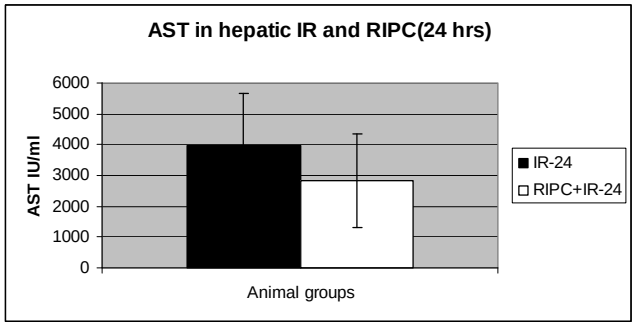
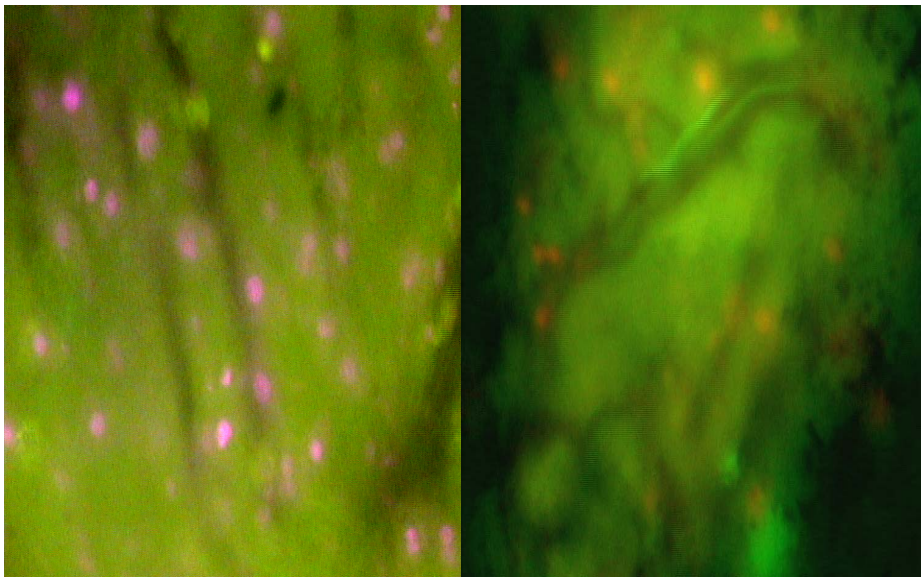


Figure 5.7
Transaminases show a lower rise in preconditioned animals.
Values expressed as mean ± sem. $P > 0.05$.

Hepatocellular cell death.



IR-24 RIPC+IR-24

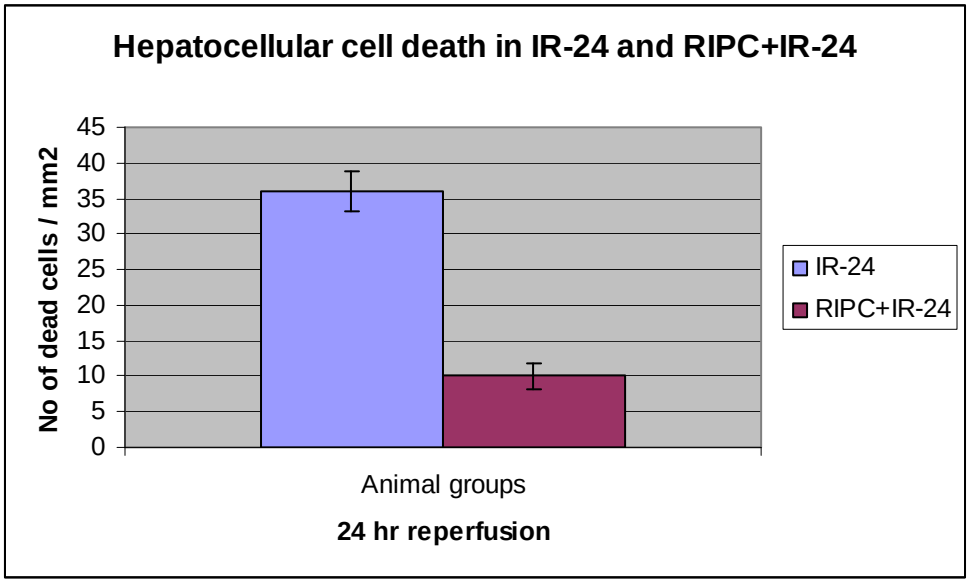
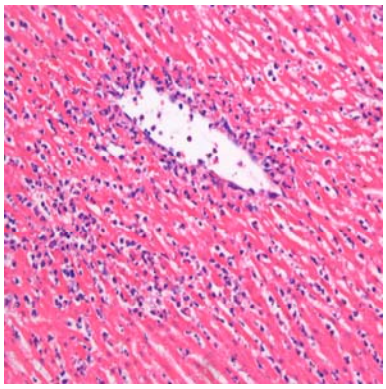
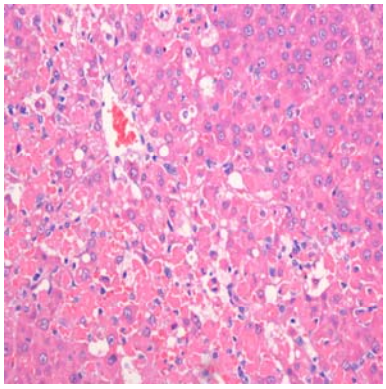


Figure 5.8a Hepatocellular cell death in IRI by propidium iodide staining (IVM). The of dead cells appear pink stained by propidium iodide. The number of cells divided by the surface area of the field above gives the number of cells/mm2.
Fig 5.8 b - Hepatocellular cell death seen in RIPC+IR-24 by propidium iodide staining (IVM).
Figure 5.8c- Hepatocellular cell death in preconditioned group (RIPC+IR-24) is significantly less compared to non preconditioned (IR-24) group. Values expressed as mean$\pm$ sem. * = $P<0.05$ (IR-24/RIPC+IR-24

Histology



a).IR-24



b). RIPC-24

Figure 5.9

Figure – a) IR-24
b) RIPC+ IR-24

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.

RIPC group shows less injury with some ballooning and degeneration as well as neutrophilic infiltration.

5.12 Discussion

Model stability, deaths and complications

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. Only one animal in the IR-24 group died after 24 hours of reperfusion. Post mortem examination revealed a completely necrotic liver in this animal. All RIPC+IR-24 animals were successfully recovered and all of them survived.

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 RIPC+IR 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 3) and late phase of hepatic IR were found to significantly low in the IR group.However the sinusoidal diameter is significantly less in the IR-24 group in comparison to preconditioned animals whereas in the early phase no difference was found in the sinusoidal diameter.

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 preconditioned animals. 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.

Effect of RIPC on microcirculatory changes in IR at 24 hours and comparison with early phase (3hrs)

As discussed above RIPC significantly enhanced sinusoidal perfusion and increased sinusoidal diameter in the late phase of hepatic IR. In the early phase RIPC improves sinusoidal perfusion but does not modulate sinusoidal diameter. The velocity of flow and sinusoidal flow were not significantly better in the late phase of hepatic IR in contrast to observations in preconditioned animals in the early phase of IR. These data suggest that RIPC modulates hepatic microcirculation and reduces hepatocellular injury primarily by improved RBC velocity and sinusoidal flow in the early phase whereas in the late phase RIPC primarily modulates sinusoidal diameter and perfusion to improve hepatic flow. The absence of any significant difference in velocity of flow between the two groups is partly explained by RBC velocity data from our study on the effect of RIPC in the early phase of hepatic IR (Chapter 3) which demonstrate that the velocity of RBC in the preconditioned group is significantly high at all time points of observation but the value of RBC velocity shows a downward trend from 30 min to 180 min of reperfusion and the difference between the IR injury and RIPC+IR group is less at 180 minutes of reperfusion in comparison to 30 minutes of reperfusion.

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

The data here clearly demonstrate that RIPC 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 preconditioned group. In the early phase of hepatic IR RIPC 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. The data in our study clearly demonstrate that modulation of neutrophil activation is a key mechanism responsible for preservation of sinusoidal perfusion in the RIPC+IR group in both phases.

Hepatocellular death in IR and RIPC+IR (comparison of early and late phases)

Hepatocellular death is significantly reduced in both the early and late phase of hepatic IR by RIPC as shown by intravital propidium iodide staining.

Since neutrophil activation is responsible for free radical release and parenchymal injury the reduced cell death seen maybe attributed to the preconditioning effect and modulation of both neutrophil activation and preservation of perfusion in the sinusoids as demonstrated by data in our study

Liver injury in IR and the effect of RIPC. (3hrs and 24 hrs)

Hepatic transaminases are significantly higher in the IR injury group at 24 hours as compared to the preconditioning group suggesting reduced hepatocellular injury in the RIPC group. Hepatocellular injury is also reduced by preconditioning in the early phase of hepatic IR although the difference was not statistically significant (chapter 3

Histological changes in IR -24 and the effect of RIPC. (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 RIPC group. In the early phase the modified Suzuki score was significantly more in IR injury in comparison to RIPC+IR. 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.

Conclusions

This is the first study to demonstrate the protective effects of RIPC on hepatic microcirculation in a recovery model of hepatic IR in rats. The study clearly demonstrates reduced hepatic injury in preconditioned animals.

In the next chapter HO expression and its spatial distribution in liver tissues in both the early and late phase of hepatic IR and RIPC shall be investigated by immunohistochemistry.

Chapter 6

Study of the spatiotemporal expression of HO-1 in the early and late phase of hepatic IR and RIPC by immunohistochemistry.

6.1 Introduction

Distribution and timing of HO-1 expression with hepatic IR and the experimental evidence for effect of RIPC on the degree and site of HO-1 expression.

HO-1 expression in hepatic IR

Upregulation of HO-1 is a critical cytoprotective mechanism against cellular stress induced by ischaemia, hypoxia or inflammation. HO-1 is produced in the hepatic macrophages (kupffer cells).HO-1 protein is expressed after 3hrs of reperfusion in kupffer cells. HO-1 expression peaks at 3hrs after reperfusion and then hepatic HO-1 expression decreases in 48 hrs. Increased expression in parenchymal cells has been shown and is usually constitutive HO i.e HO-2. Huawel et al have shown by bioluminescent activity detection of HO-1 signals at 3hrs of reperfusion.

Effect of RIPC on hepatic IR

Lai et al.(Lai et al. 2006) in an animal model of hepatic IR demonstrated that RIPC from the limb by four cycles of brief limb ischaemia prior to hepatic ischaemia increased HO-1 expression in the liver which was observed in kupffer cells in the early phase of hepatic IR.

Effect of haemin and HO-1 inducers in hepatic IR

Previous studies have shown the increased expression of HO-1 in kupffer cells suggesting that kupffer cells are the key source of HO-1.(Hirano et al. 2001)
HO-1 has been shown to enhance graft survival in experimental models.(Redaelli et al. 2002) Pretreatment with HO-1 inducer (Amersi et al. 1999) has shown increased survival in liver transplants in rat models and examination of liver specimens has demonstrated increased expression in macrophages(Amersi et al. 1999).

Why immunohistochemistry in this study?

The previous chapters in this study have shown the loss of protective effect of RIPC in hepatic IR after HO inhibition by zinc protoporphrin. The data demonstrate that HO-1 inducer (PDTC) protected hepatic microcirculation in hepatic IR. The aim of this study is to investigate the expression of HO-1 in hepatic IR and RIPC+IR in both the early and late phase of hepatic IR and the effect of RIPC on hepatic IR. The study also investigates the site and timing of HO expression by PDTC in hepatic IR.

Immunohistochemistry technique used in this study

The enzyme labelled antibody technique is a fast cheap and convenient method for light microscopy. It is a useful preliminary to ultrastructural methods.The enzyme labelled antibody technique is nearly as specific and sensitive as fluorescent techniques. It has a permanent staining unlike fluorescent techniques and does not require fluorescence or fluorescence microscopes. In addition auto fluorescence does not interfere with the visualisation of antigen (Nakane and Pierce, 1967). It has got good penetration into tissues.

6.2 Material and methods

a) Animals and surgical procedures

Refer chapter 2 – material and methods , pg 92

Six animals in each group as described previously in chapter 2 were studied. There were eight groups and hence the total no of samples evaluated were 48.

Sections from liver tissue from each animal were assessed.

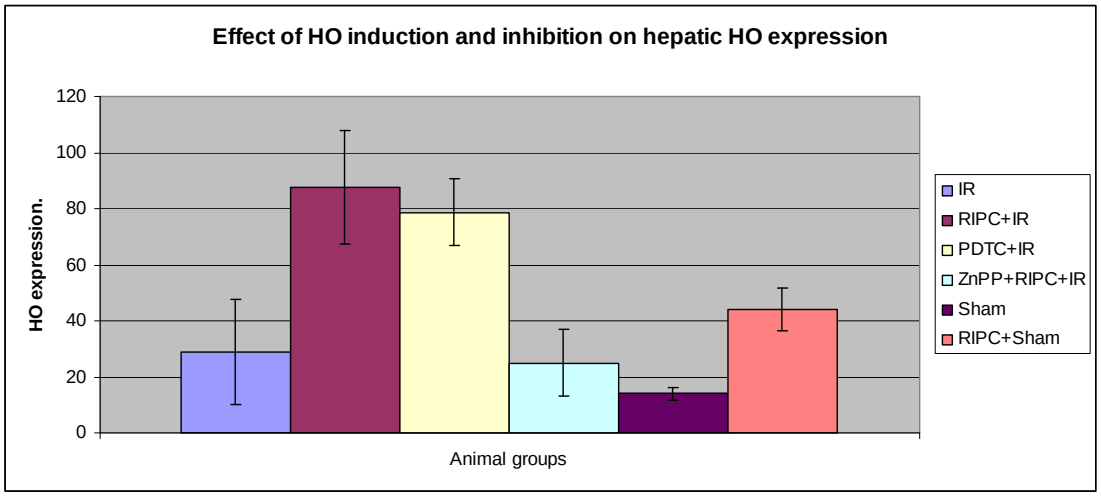
b) Method of tissue processing for immunohistochemistry.

Refer to chapter 2 - material and methods, pg 106

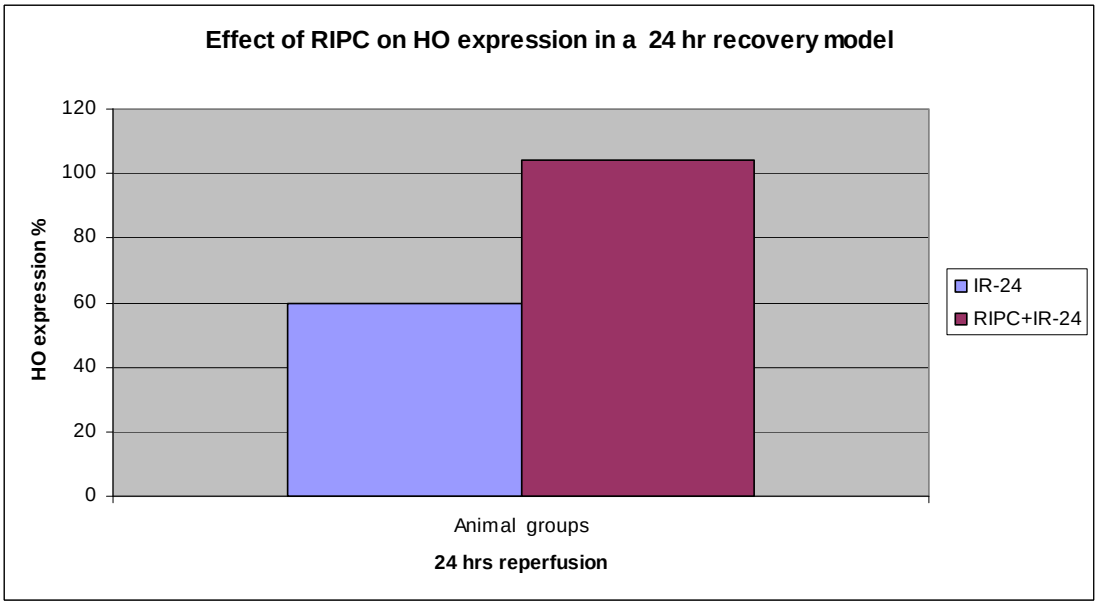
c) Immunohistochemistry criteria and assessment methodology.

Paraffin embedded tissue was stained with haematoxylin.An objective scoring method was devised which assessed the intensity of sinusoidal macrophage staining graded from 0 (no positive stain) to 4 (intense and crisp brown stain). A percentage positive score was obtained by multiplying the grade with the percentage of stained macrophages. The maximum score possible was 400 (4X100%) while the minimum was 0 (0X0%) {Harvey et al, 1999}.

6.3 Results

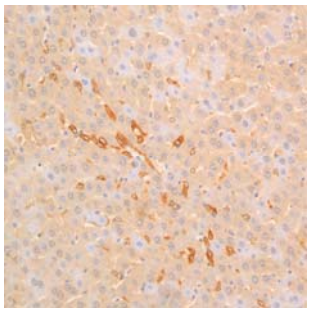


IR	RIPC+IR	PDTC+IR	ZnPP+RIP	Sham	RIPSham
28.66	87.5	78.7	25	14	44.1
18.77	20.27	8.75	11.83	2.44	7.57

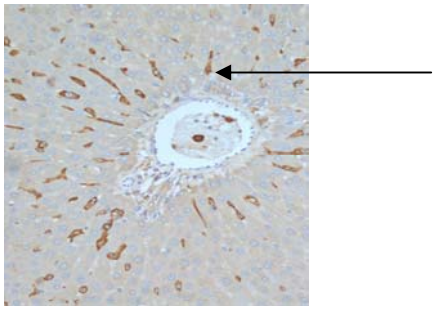


Immunohistochemistry scoring - table 6.1

Immunohistochemistry for HO-1



6.1 IR



6.2 RIPC+IR (Increased HO-1 expression in kupffer cells)

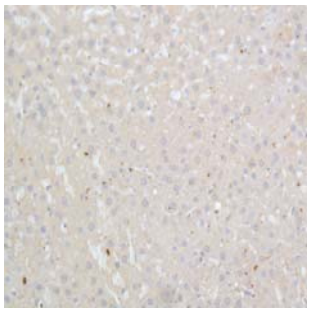


Fig 6.3 PDTC+IR (HO inducer)

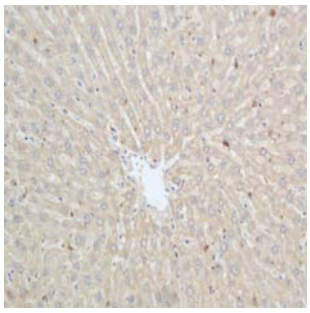


Fig 6.4 ZnPP+RIPC+IR (HO inhibition)

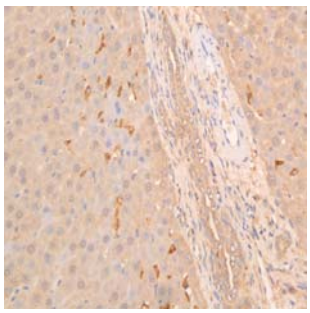


Fig 6.5 Sham

Fig 6.6 RIPC+Shams

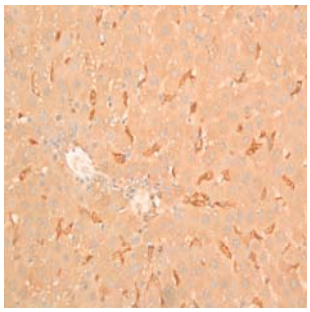


Fig 6.7 IR-24

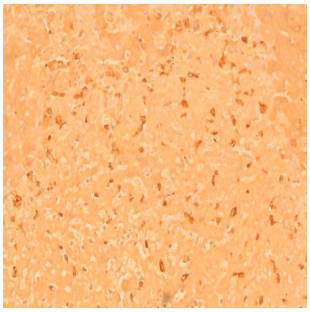


Fig 6.8 RIPC+IR-24

Fig 6.1- There is some HO expression seen in macrophages (kupffer cells) in IR injury in response to oxidative stress. Fig 6.2- The expression of HO-1 was significantly more in kupffer cells in the RIPC+IR group. Fig 6.3-PDTC (HO-1

inducer) showed increased HO-1 expression in macrophages and reproduced effects of RIPC+IR group. Fig 6.4- Prior administration of ZnPP significantly reduced the expression of HO-1 in preconditioned animals ZNPP+RIPC+IR. Fig 6.5- Sham animals showed baseline HO-1 expression in sinusoidal kupffer cells. Fig 6.6- RIPC in sham induced HO-1 above baseline levels in response to oxidative stress.

6.4 Discussion

Site of HO-1 expression

Immunohistochemistry showed HO-1 expression in macrophages at the end of three hours of reperfusion. Lai et al.(Lai et al. 2006) demonstrated that RIPC from the limb increased HO-1 expression in the liver and increased HO-1 was seen in kupffer cells. Previous studies have shown the increased expression of HO-1 in kupffer cells suggesting that kupffer cells are the key source of HO-1.(Hirano et al. 2001)

HO-1 has been shown to enhance graft survival in experimental models.(Redaelli et al. 2002) Pretreatment with HO-1 inducer (Amersi et al. 1999) has shown increased survival in liver transplants in rat models and examination of liver specimens has demonstrated increased expression in macrophages .(Amersi et al. 1999)

Reproducibility of scoring system, validity & references for new scoring system

The scoring on immunohistochemistry for HO-1 macrophage marker is based on the system routinely used in the laboratories to assess Oestrogen receptor status of Invasive ductal carcinoma cells of the breast in paraffin embedded sections (Harvey *et al* 1999, leake R *et al* 2000, Rhodes *et a*l 2001). The cells which are positive on immunohistochemical staining for the HO-1 macrophage marker (name of the stain) is graded into 4 grades depending on the intensity of staining.

0= No staining

1= Weak staining

2= Moderate staining

3= Strong staining

This is subsequently multiplied with the percentage of cells which are positive. The percentage is arrived at by counting the positive cells in a total of 100 cells with in the particular high power field.Hence the minimum score possible is 0 and the maximum is 300 This system is valid and has been used for oestrogen receptor status and is reproducible.,,

Variability in methodogy and assessment of immunohistochemistry

Interobserver variability is due to differences in methodology of dilution and pipetting of samples.

HO expression in shams

Baseline HO expression is seen in kupffer cells in sinusoids of sham group.

HO distribution in hepatic IR (3hrs of reperfusion and 24 hrs of reperfusion).

Haemoxygenase expression in IR (3hrs of reperfusion) was more compared to sham.This is due to increased oxidative stress from which acts a direct stimulus to HO-1 expression. The increased HO expression was observed in kupffer cells which are the primary source of HO production and degradation of haem to biliverdin and CO. At 24 hrs of reperfusion more HO was observed in Kupffer cells due to a longer period of oxidative stress in comparison to 3 hrs of reperfusion.

HO expression in RIPC+IR (early and late phase)

Haemoxygenase expression is seen to be significantly more in RIPC group as compared to IR group at 3hrs of reperfusion suggesting that the remote preconditioning stimulus induced increased HO-1 expression in the liver. RIPC induces a low grade oxidative stress which maybe responsible for increased HO in the liver. The increased HO expression was observed in kupffer cells at 3hrs of reperfusion. HO-1 pathways are initiated after oxidative stress and previous studies (Potter, Bussutil) have shown expression of mRNA as early as 2 hours after the initial oxidative stress. The increase in velocity and flow seen after 30 minutes of

reperfusion corresponds to 2 hours after the first cycle of preconditioning and the time point of earliest HO-mRNA expression suggesting that HO-1 pathways maybe responsible for modulation of hepatic microcirculation in this study.

Lai et al {Lai et al, 2006} demonstrated increased hepatic HO-1 expression in both kupffer cells and parenchymal cells. Hirano et al demonstrated increased HO-1 in kupffer cells in response to injected senescent RBCs and increased bilirubin in the bile suggesting that kupffer cells are the principal source of HO-1 in the liver.{Hirano et al, 2001 } Previous studies have demonstrated increased free radicals in the blood following RIPC.{Chen et al , 2005} Increased ROS may induce a low grade oxidative stress in the liver and since kupffer cells are the key cells in the initial inflammatory response this may explain the increased HO-1 in the kupffer cells.

In the late phase of hepatic IR (24 hours) there was increased HO expression in both kupffer cells and hepatocytes (parenchymal cells) in preconditioned animals in comparison to IR only. This may explain the increased sinusoidal diameter observed in RIPC group at 24 hrs on intravital microscopy.Increased HO in parenchymal cells modulates sinusoidal tone and diameter as shown by Goda et al. Goda et al have shown that CO derived from an increase in HO-2 (Constitutive HO) in the parenchyma is responsible for modulation of sinusoidal tone and diameter in the late phase of hepatic IR (Goda N et al 1998). Increased HO-1 in kupffer cells in the early phase of hepatic IR was seen in preconditioned animals but there was no evidence of parenchymal expression of HO explaining the absence of sinusoidal dilatation in the early phase of hepatic IR.

HO expression with inducible (PDTC) and inhibitory agents (ZNPP)

.PDTC induced HO-1 in macrophages in the early phase of hepatic IR. Inhibition of haemoxygenase by ZnPP (ZnPP inhibits both HO-1 and HO-2) showed decreased HO

expression in macrophages in the early phase of hepatic IR. This explains the intravital findings of increased neutrophil adhesion and hepatocellular death in comparison to preconditioned animals.

PDTC (100mg/kg) did not cause any significant change in sinusoidal diameter. This observation is supported by previous studies in ischaemic preconditioning which have demonstrated no significant variation in sinusoidal diameter after HO induction with PDTC at doses of 100mg/kg s. c. Recently Hata has shown that CO derived from PDTC induced increase in parenchymal HO-1 modulated sinusoidal diameter in the early phase of hepatic IR {Hata et al, 2003}. Hata used higher doses of PDTC i.e 150mg/kg s.c. and the effects were dose related.

Animal model and in vivo assessment (chapter 4 and 5)

Early phase of hepatic IR (3 hrs of reperfusion) and effects of RIPC (Refer Chapter 4)

Intravital microscopy demonstrated in real time manner significantly better RBC flow, sinusoidal flow, sinusoidal perfusion and decreased endothelial neutrophil interaction as well as hepatocellular cell death in RIPC+IR as compared to IR only. The improved flow was observed as early as 30 minutes after reperfusion.

There was no difference in sinusoidal diameter between RIPC+IR and IR only. Zinc protorporhyrin abolished all protective effects in preconditioned animals suggesting the role of haemoxygenase in modulation of hepatic microcirculation.

Late phase of hepatic IR (24hrs) and effect of RIPC. (Refer Chapter 5)

Intravital microscopy showed significantly better sinusoidal perfusion, increased sinusoidal diameter and decreased neutrophil adhesion as well as hepatocellular cell death in RIPC+IR-24 in comparison to IR-24 only. There was no difference in velocity of flow and sinusoidal flow.

Lack of change in sinusoidal diameter on HO inhibition suggests that RIPC modulates IR injury by anti-inflammatory properties of HO rather than sinusoidal dilatation in the early phase of hepatic IR.

Conclusions and future assessment.

Immunohistochemistry strongly suggests increased haemoxygenase expression in the liver of preconditioned animals in hepatic IR following preconditioning. Increased HO is seen in kupffer cells in the early phase of hepatic IR and in both kupffer cells as well as parenchymal cells in the late phase of hepatic IR following preconditioning. Although the cellular distribution of haemoxygenase has been identified it is necessary to quantify haemoxygenase expression.

The next chapter focuses on quantification of HO expression by western blot analysis in all animal groups.

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Chapter 7

Quantification of HO-1 protein expression in the early and late phase of hepatic IR and RIPC by Western Blot analysis

7.1 Why western blotting for HO-1?

Immunohistochemistry has shown increased HO expression primarily in macrophages in the early phase of hepatic IR following RIPC and in both macrophages and hepatocytes in the late phase of hepatic IR following RIPC. Immunohistochemistry is useful for showing distribution of HO expression. However it is not specific for HO-1 as the antibody cross reacts with HO-2 and also the antibody may cross react with other proteins. Moreover immunohistochemistry could not be used to quantify HO expression.

Western blotting is a technique which involves extraction of HO protein using specific antibodies and is a semiquantitative method which helps quantify HO expression. In order to quantify HO-1 expression in RIPC, western blots for HO-1 expression were carried on snap frozen liver tissues.

7. 2 Material and methods

Animals and surgical procedures

As described in chapter 2 (Pg 92). Liver tissues were snap frozen in – 80 degrees celsius for HO western blot analysis.

Experimental groups (n=6 in each group)

Eight groups of animals were studied as described in material and methods pg 92.

Method of western blots and densitometry for HO-1 expression.

Refer material and methods, chapter 2, pg 110.

Number of samples

Liver tissues from six animals in each group (eight groups) were processed. The total number of samples analysed for HO expression were 48.

Controls

The positive control used was recombinant HSP-32 (protein lysate) from Stressgen laboratories.

Quantitation

This was done using densitometry analysis and software for measurement of band densities as described in chapter 2 material and methods.

Statistical analysis

All data are presented as mean plus minus standard error of the mean (SEM).Differences were considered significant for $P<0.05$.Comparisons between groups were performed by one way analysis (ANOVA). Bonferroni correction was applied for ANOVA.

7.3 Results (early and late phase of IR)

The results show a significantly increased HO-1 expression in RIPC groups as compared to IR groups and administration of HO-1 blocker ZnPP significantly reduced the haemoxygenase expression in the preconditioned groups. Also PDTC is a known HO-1 inducer and the expression of HO-1 in PDTC reproduced the effects of RIPC in the early phase (Fig 7.1, 7.2). Sham showed baseline HO expression and preconditioning in sham induced HO expression over baseline levels. In the late phase of IR signififcantly more HO-1 expression is seen in RIPC-24 as compared to IR-24 only (Fig 7.1, 7.3).

HO-1

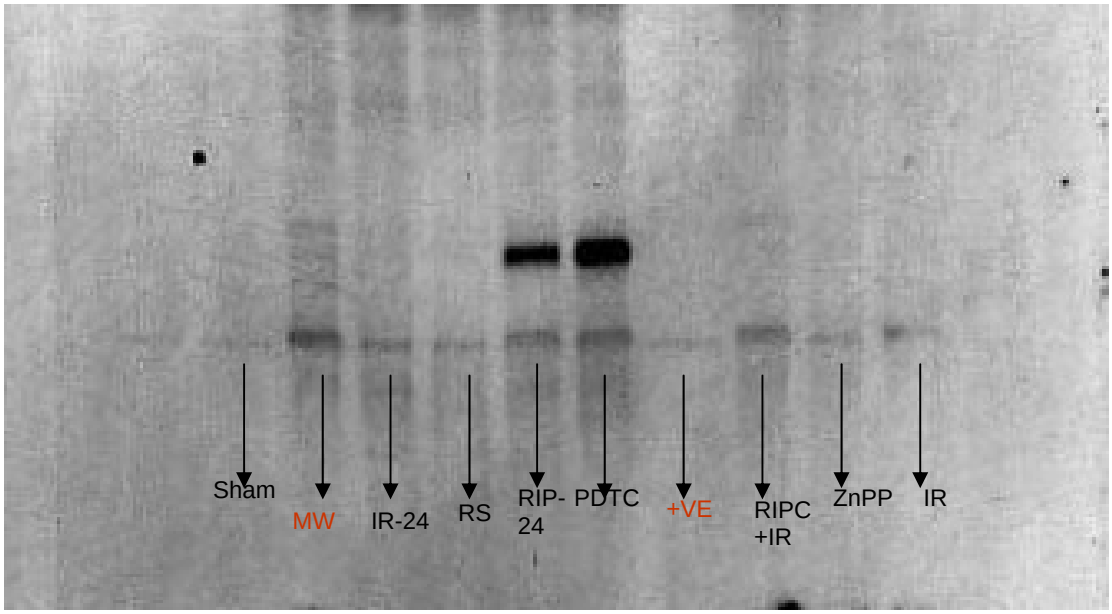


Fig 7.1 Representative example of western blots done for all groups.

Lane 1- Sham

Lane 2- MW- 32 Kda for HO protein.

Lane 3- IR-24 (HO expression in late phase of hepatic IR)

Lane 4-RS

Lane 5- RIPC+ IR-24 (effect of RIPC on HO expression in late phase of IR)

Lane 6- PDTC- HO inducer

Lane 7- Positive control

Lane 8- ZnPP- HO inhibitor

Lane 9- Ischaemia reperfusion inury (early phase)

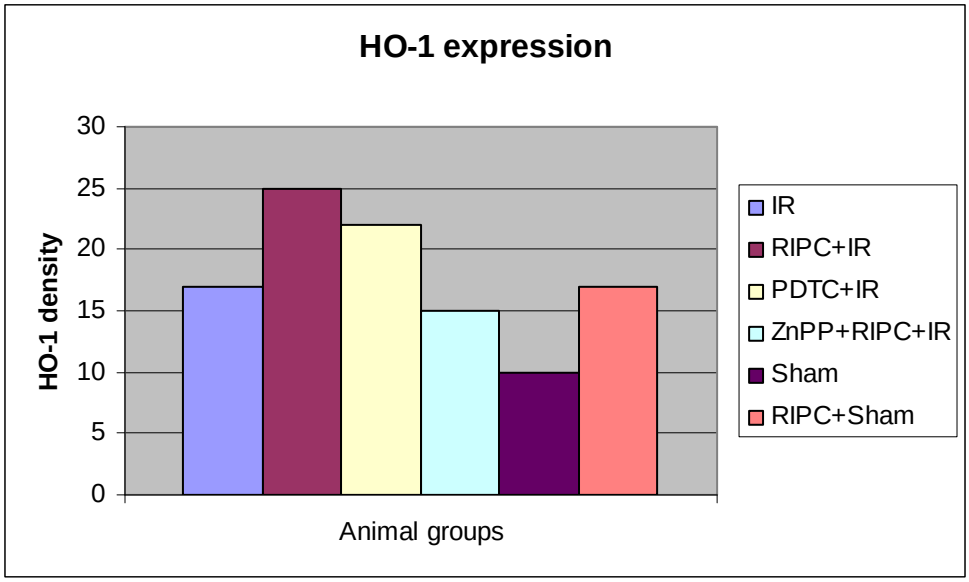


Fig 7.2 - Early phase of hepatic IR (above). Densitometry for western blots showing increased HO-1 expression in RIPC+IR and PDTC+IR which was abolished by ZnPP +RIPC+IR.

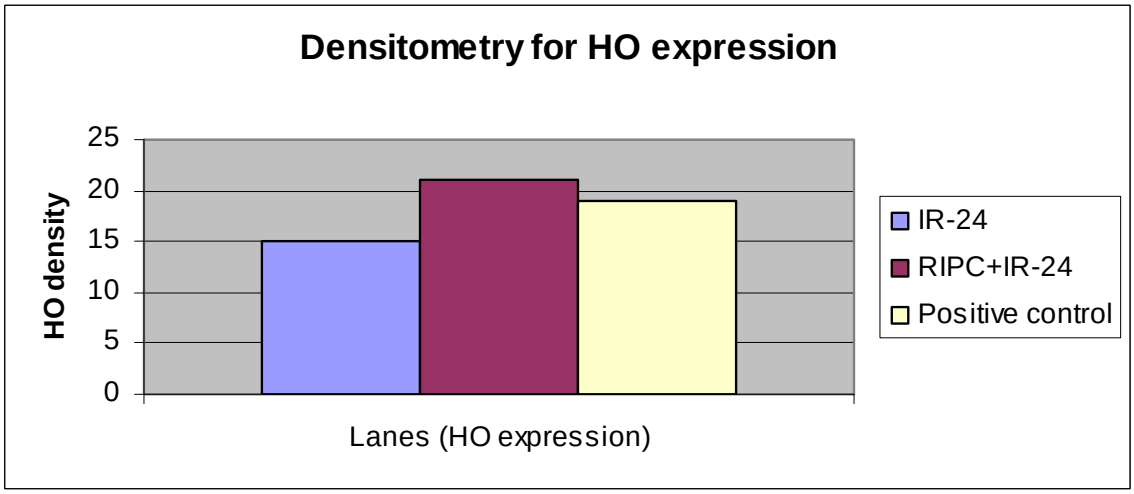


Fig7.3 – Densitometry for late phase of hepatic IR and RIPC western blot showing increased HO-1 expression in the RIPC group at 24 hours.

7.4 Discussion and conclusion

Method of assessing HO-1

Reliability, reproducibility of technique and assay variability

The technique used is a well described semiquantitative technique used for detecting HO protein. It has been used in rats, rabbits and mice. Interobserver variability is possible due to differences in pipetting and dilution. This was minimised by the same person doing all the samples. All the samples from different groups were compared to a standard control. The technique is reproducible and has been validated for use in other species i.e rabbits, humans, mouse etc (stressgen).

Positive Control used was recombinant HSP-32 (protein lysate).

Validation and antibodies

The primary and secondary antibodies chosen were commercially standardised anitobodies from Stressgen laboratories. These antibodies have been validated for detection of HO-1 by Stressgen. The method used and dilution of antibodies was according to manufacturer's guidelines.

Extraction of protein and incubation with antibody makes the technique more sensitive and specific than immunohistochemistry and helps quantification.

We chose polyclonal rabbit IgG (StressGen, Canada) as it was easily available commercially as against monoclonal antibodies and it was diluted 1; 1000. The rat HO-1 antibody does not cross react with rabbit HO-1 **because** it is raised in rabbits.

Biotinylated secondary antibody helps develop a peroxidase based reaction. HRP conjugated secondary antibody (1:2000) was used and HRP conjugated antibiotin antibody (1:1000).

HO-1 in sham (Early phase)

Studies by Tsuchihashi (2003), Hata et al (2003) have shown that baseline HO is essential for RIPC or other stressful stimuli to induce HO expression as HO is not inducible in HO knockout mice as shown by Tsuchihashi et al. These observations suggest that genes expressing HO are required for protection of the liver from oxidative stress.

HO-1 in IR (Early and late phase of hepatic IR)

Increased HO expression in IR group was seen as compared to shams in the early phase of hepatic IR. This is due to oxidative stress in hepatic IR and has been shown in previous studies by Lai et al in the early phase and Katori et al in liver transplant models respectively.

HO-1 in RIPC+IR (Early and late phase of hepatic IR)

Significantly more hepatic HO protein expression was observed in our study in both the early and the late phase of RIPC in hepatic IR. This is the first study to demonstrate increased HO expression findings in a recovery model of hepatic IR and RIPC. Lai et al (2006) have demonstrated increased hepatic HO expression in the early phase of RIPC+IR by western blot analysis.

Effects of HO induction and inhibition (Early phase of hepatic IR)

RIPC+IR and PDTC+IR induced increased HO protein in the liver which was significantly more compared to IR and shams. HO inhibition with ZnPP led to significantly reduced HO protein expression in RIPC group. Our findings in RIPC+IR are supported by Lai et al (2006) who observed increased HO after 4 hrs of reperfusion in the liver following brief limb RIPC. Hata et al have demonstrated PDTC induced increased hepatic HO expression supporting our observation. ZnPP

inhibits hepatic HO protein expression as seen in our study and this observation is supported by Lai et al (2006).

Correlation with Immunohistochemistry (for HO-1) in the early and late phase of hepatic IR.

Immunohistochemistry supports our findings from western blots for HO-1. Baseline HO-1 expression was observed in sinusoids in sham animals on immunohistochemistry and western blot analysis demonstrates baseline levels of HO in sham animals. Increased HO expression was seen in IR (early phase) in kupffer cells as compared to sham in the early phase of IR and this is confirmed by western blot analysis. In RIPC +IR significantly more HO expression is seen in kupffer cells in the early phase on immunohistochemistry and this finding is supported by significantly increased HO protein expression found on western blot analysis. PDTC induced HO expression is seen both by immunohistochemistry and western blot analysis. Immunohistochemistry in our study showed primarily increased HO in sinusoidal kupffer cells. PDTC induced HO expression on both western blots and immunohistochemistry has previously been demonstrated by Hata et al (2003). Hata showed a dose dependent relationship between PDTC and HO expression with HO expression in Kupffer cells at doses of 100 mg/ kg as observed in our study and in both parenchymal cells and kupffer cells at doses of 150 mg/kg. Hata et al showed that ZnPP abolished the effects of PDTC. ZnPP significantly reduced HO expression in RIPC+IR in our study as observed by both immunhistochemistry and western blots. This finding is supported by Lai et al (2006).

Relation of HO protein data to hepatic microcirculatory flow in the early and late phase of hepatic IR and RIPC.

In the early phase of RIPC+ hepatic IR increased HO protein is associated with significantly better RBC flow, sinusoidal flow and sinusoidal perfusion. There was no change in sinusoidal diameter on HO induction or inhibition. Sinusoidal dilatation on HO induction by PDTC was not observed in our study since the dose we used was 100mg/kg as against 150 mg/kg which has been shown to induce sinusoidal dilatation recently by Hata et al. Moreover the HO-1 induced by RIPC or PDTC in the early phase of IR was observed in kupffer cells and HO-1 induced in kupffer cells is in response to oxidative stress as shown by previous studies. Inducible HO-1 in kupffer cells is responsible for anti-inflammatory functions (Katori et al). HO-1 induced in parenchymal cells is responsible for generating CO and modulation of sinusoidal tone as shown by Hata et al using higher doses of PDTC (150mg/kg) { Hata et al 2003}.

In the late phase there was a significant increase in HO seen on western blot analysis in RIPC +IR -24 as compared to IR-24 only . Immunohistochemistry demonstrated increased HO both in sinusoids and parenchymal cells in RIPC+IR-24. Increased HO in the sinusoids has anti-inflammatory functions as shown previously by Katori et al. Goda et al have shown parenchymal HO-2 (constitutive) expression in the late phase of ischaemic preconditioning to be responsible for CO generation and sinusoidal dilatation. This suggests that the sinusoidal dilatation seen in our study in the late phase of RIPC maybe due to HO produced in the hepatic parenchymal cells.

Relation of HO protein to leukocyte adhesion and hepatocellular death in the early and late phase of hepatic IR and RIPC.

Increased HO protein correlated with decreased sinusoidal and venular neutrophil adhesion in RIPC in both the early and late phases, correlated with decreased histological necrosis and congestion in both the early and late phases of RIPC, decreased hepatocellular death as observed by propidium iodide staining in both the phases of RIPC+IR and in PDTC induced HO expression and decreased serum transaminase levels in both phases of RIPC+IR. This suggests that HO protein is responsible for protection of liver parenchyma, hepatic microcirculation and reduced hepatocellular injury.

Conclusions and future assessment

Western blots have demonstrated that RIPC induced hepatic haemoxygenase expression in both the early and late phase of hepatic IR has a significant role in modulation of hepatic microcirculation and reduction of hepatocellular injury. Further investigation in HO knockout models would be necessary to specifically investigate the effects of haemoxygenase.

Since intravital microscopy has shown decreased neutrophil adhesion suggesting reduced neutrophil activation the focus of the next chapter was to investigate if hepatic HO expression in this study modulated CINC-1 levels in order to reduce neutrophil activation.

Chapter 8

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

8.1 Introduction

Relationship between IR, HO-1 and neutrophil adhesion.

IR injury is associated with increased venular and sinusoidal neutrophil adhesion seen by intravital microscopy and histologically in both the phases of hepatic IR. RIPC reduced neutrophil adhesion in both phases of hepatic IR and this was associated with increased HO-1 protein as observed by immunhistochemistry and western blot analysis in our study. These data suggest that HO-1 is associated with modulation of neutrophil activation and adhesion. Previous studies have shown HO to reduce neutrophil activation. This study was carried out to study the effect of RIPC on serum CINC-1 levels and its correlation with hepatic HO expression.

What is CINC-1?

Functionally, CINC-1 is described as a major neutrophil chemoattractant and activator. CINC-1, induced by IL-1, TNF- and bacterial products, promotes both neutrophil rolling and adhesion, likely through the upregulation of surface integrins. Thus, it directs neutrophils to sites of bacterially-induced inflammation. It is also reported to stimulate neutrophil activity by promoting cathepsin G release from azurophilic granules. It does not, however, seem to impact nitrite production. Thus it makes a partial contribution to bacterial killing. 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 (16). The significance of this is unclear.

. CINC (Cytokine-induced neutrophil chemoattractant), a peptide in the IL-8 superfamily has been reported to be a potent neutrophil chemoattractant in rats

{Watanabe, 1989; Watanabe, 1989}. Kupffer cells, which comprise the largest fixed macrophage population in the liver, produce cytokines when activated and represent an important source of chemoattractants.

What is the role of CINC-1 in hepatic IR?

Neutrophils play an important role in hepatic IR. The infiltration of neutrophils intoinflammatory lesions is brought about chemotactic substances. CINC, a peptide in the IL-8 superfamily has been reported to be a potent neutrophil chemoattractant in rats {Watanabe, 1989; Watanabe, 1989}. Kupffer cells, which comprise the largest fixed macrophage population in the liver, produce cytokines when activated and represent an important source of chemoattractants. Hisama et al {Hisama et al, 1996} demonstrated that serum CINC levels peaked 6 hours after reperfusion and then gradually decreased. The expression of CINC mRNA transcripts in the liver peaked at 3 hrs in untreated animals as shown by northern blot analysis. Treatment of animals wih heparin or gadolinium chloride inhibited serum CINC levels and CINC transcript expression in liver and both together had an additive inhibitory effect on CINC m RNA expression, serum CINC levels. The number of neutrophils which infiltrated the liver 24 hours following reperfusion significantly decreased following CINC inhibition.

Studies showing effect of RIPC on CINC-1.

There have been no studies demonstrating the effect of RIPC on CINC-1. Pharmacological preconditioning by Glycine and heat shock preconditioning have been shown to reduce serum CINC-1 levels in hepatic reperfusion injury. Since data from previous chapters have shown a significant effect of RIPC on neutrophil adhesion in both the early and late phase of hepatic IR we investigated the effects of RIPC on CINC levels in hepatic IR (early and late phase).

Aim of this study

The aim of this study was to investigate the effects of RIPC on CINC levels in hepatic IR and correlate with microcirculatory data and HO data from the previous chapters.

8.2 Material and methods

Animals and surgical procedures

As detailed in the chapter on methodology, pg 92.

Experimental groups (n=6 in each group)

Eight (8) groups of animals were studied as described in chapter 2.

CINC Elisa

Elisa for quantification of CINC was done as described in chapter 2, pg 112.

Intravital microscopy

As described in chapter 2, pg 9103.

Western blot analysis for HO

As described in chapter 2, pg 110**.**

Statistical analysis

All data are presented as mean plus minus standard error of the mean (SEM).Differences were considered significant for $P<0.05$.Comparisons between groups were performed by one way analysis (ANOVA). Bonferroni correction was applied for ANOVA.

8.3 Results

The CINC data shows significant difference between IR injury, RIPC+IR and ZnPP+RIPC+IR in the early phase of hepatic IR. In the late phase of IR there is a significant difference seen between IRI and RIPC+IRI suggesting that preconditioning modulates release of CINC-1 from kupffer cells.

CINC -1 levels in Sham and RIPC+Sham

The CINC cytokine levels in sham were significantly low (101.32± 6.42). RIPC in sham led to relatively high CINC levels (412.18 ± 65.24) as compared to sham. (Fig 8.1)

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

Hepatic IR injury produced a significantly high serum CINC level in comparison to sham animals in the early phase of hepatic IR (644.08 ± 181.24). The serum CINC levels were significantly high in the late phase of hepatic IR (15306± 1222.04). (Fig 8.1, 8.2)

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

RIPC significantly reduced CINC -1 levels in both the early (401.62 ± 78.56) and late phase (467.46 ± 26.06) of hepatic IR in comparison to IR only.

Effect of HO induction (PDTC) and inhibition (ZNPP) on CINC-1 levels (early phase).

PDTC (HO inducer) significantly reduced CINC-1 levels in serum in hepatic IR (413.36 ± 63.06)

HO inhibition in preconditioned animals with Zinc protoporphyrin increased serum CINC levels (521.81± 74.9).

Correlation of CINC-1 with intravital findings on neutrophil adhesion

Intravital microscopy has shown increased neutrophil adhesion in IR in the early and late phase and after HO inhibition in preconditioned animals. PDTC+IR (HO induction) significantly reduces Serum CINC levels. Neutrophil adhesion is significantly less in RIPC+IR in both the early phase of IR and late phase of IR.

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 preconditioned animals. Also increased hepatocellular death was observed after HO inhibition in RIPC+IR (early phase)

Correlation of CINC-1 with HO western blots.

Increased HO expression is seen in RIPC+IR animals in both the early and late phase of hepatic IR and after HO induction with PDTC prior to hepatic IR. In these groups reduced serum CINC levels are observed. Reduced HO in IR injury in both phases and after HO inhibition with ZNPP prior to RIPC are observed (Chapter 7). In these groups increased CINC levels are observed.

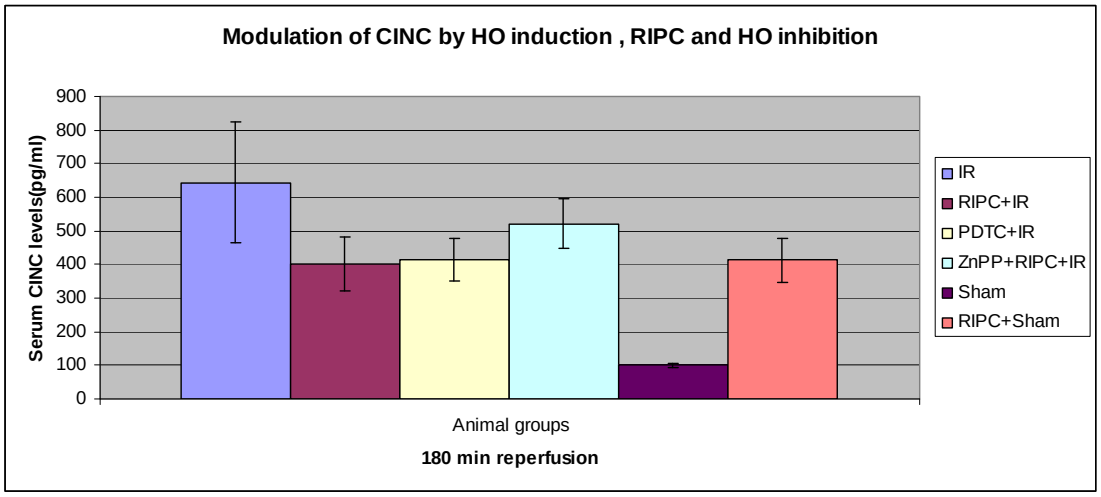


Figure 8.1 Modulation of CINC in early phase of hepatic IR

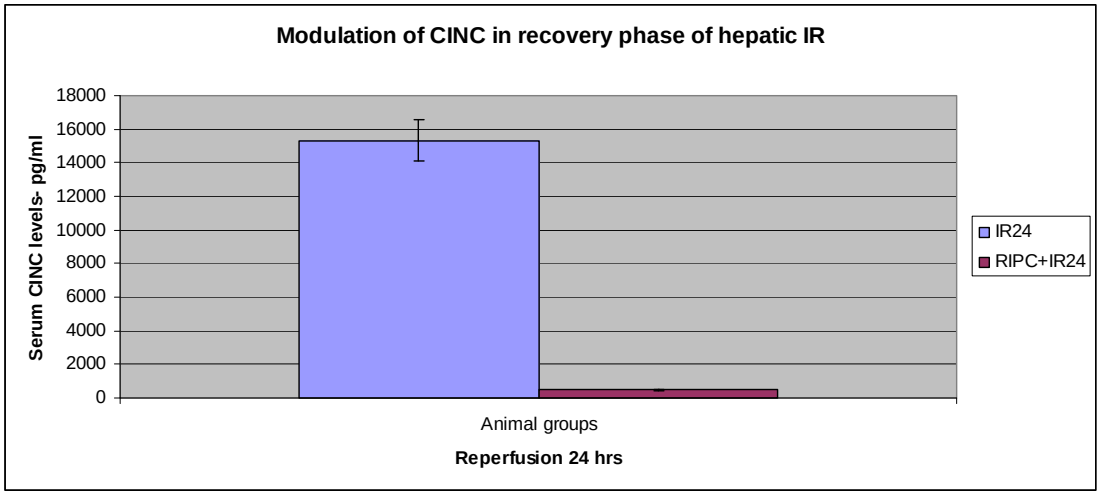


Figure 8.2 Modulation of CINC in late phase of hepatic IR

8.4 Discussion

Assay, controls and variability

If samples generate values higher than the highest standard, further dilute the samples with the Calibrator Diluent and repeat the assay.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. Until all factors have been tested, however, the possibility of interference cannot be excluded.

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 levels in sham in comparison with RIPC+Sham and correlation with intravital findings of neutrophil adhesion and hepatocellular death and HO western blots.

The serum levels of CINC are more in RIPC+Sham. These may be due to low grade oxidative stress induced by RIPC in sham animals. This correlates with more neutrophil adhesion & hepatocellular death in RIPC+sham as compared to sham animals. Due to oxidative stress HO expression is more in RIPC+Sham.

Serum CINC levels in hepatic IRI and correlation with intravital findings of neutrophil adhesion and hepatocellular death and HO western blots.

The serum CINC levels in the early phase of hepatic IR were significantly higher compared to sham animals. Liang et al, 2000 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. Oxidative stress due to ROS results in increased NF-κB production and NF-κB stimulates CINC production by kupffer cells. Increased CINC levels correlate with increased neutrophil adhesion and hepatocellular death in the early and late phase of hepatic IR. HO expression is less in IR compared to RIPC+IR.

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

RIPC reduced CINC in the early phase of hepatic IR and in the late phase of hepatic IR (24hrs) CINC levels are significantly inhibited following preconditioning. The serum CINC levels correlate with reduced neutrophil adhesion and hepatocellular death as well suggesting modulation of CINC and neutrophil activation by RIPC in hepatic IR. This needs to be clarified in future studies.

Effect of HO induction in IR and HO inhibition in RIPC+IR on CINC-1 levels and correlation with intravital findings of neutrophil adhesion and hepatocellular death and HO western blots.

RIPC reduced CINC levels and PDTC (HO inducer) reproduced the effects of RIPC in hepatic IR. PDTC induced HO correlated with decreased neutrophil adhesion and hepatocellular death and increased HO expression in liver tissue. Inhibition of HO by ZnPP in RIPC reduced hepatic HO expression, increased serum

CINC levels, increased neutrophil adhesion and hepatocellular death suggesting the role of HO in modulation of CINC.

Does HO inhibit CINC?

Our study demonstrates that HO induction is associated with decreased neutrophil adhesion and serum CINC levels.Kubulus et al have shown that hemin arginate caused a profound increase in HO expression in the liver after 24 hours, decreased TNF-α, improved hepatic sinusoidal perfusion and protected hepatic microcirculation. H.P. Yu et al have shown that HO upregulation in proestrus rats significantly attenuates shock lung following trauma haemorrhage and reduces ICAM, CINC and MPO activity in lungs{Yu et al , 2006 } {Uchinami et al 2002}.

Conclusion

These data suggest that RIPC reduces serum CINC levels and decreases neutrophil activation in hepatic IR and also the potential role of RIPC induced HO in modulating CINC & neutrophil activation.

The next chapter focuses on investigating the role of HO in modulation of mitochondrial cytochrome c and apoptosis and whether or not HO inhibits release of mitochondrial cytochrome c.

Chapter 9

Mitochondrial function in the early and late phase of hepatic IR, modulation by RIPC and correlation with biochemical, histological and hepatocellular injury markers

9.1 Introduction

What is the role of mitochondria in hepatic IRI?

Recent studies have provided evidence for the role of mitochondria in both apoptosis and cellular necrosis (A elimadi, J. pharmacol.exp.ther. 286(1998). Mitochondria are the main source of intracellular ROS production which are produced excessively following reperfusion and damage mitochondrial membranes and proteins through lipid peroxidation. This peroxidation damages and increases the permeability of mitochondrial membrane leading to loss of mitochondrial integrity resulting in release of cytochrome c into cytoplasm and subsequent activation of caspase activity which initiates apoptotic cell death.

Ischaemia leads to ATP depletion, loss of mitochondrial transmembrane potential which is associated with Ca^{2+} overload and opening of mitochondrial membrane pores. The mitochondria then undergo rapid swelling and rupture due to osmotic effects eventually leading to cytochrome c release and caspase activation causing DNA fragmentation and apoptosis.

Thus mitochondria are involved in both apoptotic and oncolytic necrosis pathways of IR injury. Neutrophils are the main source of extracellular ROS which are also responsible for cellular necrosis.

Does RIPC induced HO -1 reduce mitochondrial membrane disruption and release of cytochrome C?

Wang et al have demonstrated blood borne transferred preconditioning in an animal model of explanted hearts from rabbits which were perfused prior to non infarct cardioplegic arrest with blood from rabbits whose hearts were preconditioned by RIPC. They showed rapid recovery of perfused hearts following non infarct cardioplegic arrest and reperfusion. Mitochondrial cytochrome c assessment showed

decreased release of cytochrome c into cytosol in preconditioned animals suggesting the role of RIPC induced blood borne factors which induce preconditioning and modulate cytochrome c release.

Does reperfusion cause hepatocellular death through apoptosis or necrosis?

Reperfusion causes release of reactive oxygen species which directly attack cellular molecules and indirectly promoting synthesis of pro-inflammatory mediators. These effects lead to hepatocellular cell death which typically follows one of the two patterns: oncolytic necrosis and apoptosis. Oncolytic necrosis is caused by the direct effect of ROS and is characterised by loss of membrane integrity leading to swelling and rupture of cells which release their denatured cytoplasmic contents into plasma. These lead to a secondary inflammatory response and apoptotic cell death of surrounding cells. In addition to necrosis it is possible that cells sublethally damaged undergo programmed cell death i.e apoptosis. Eum et al demonstrated that hepatic IR caused periportal cell death by necrosis and pericentral cell death by apoptosis {Eum et al, 2007}.

Aim

The aim of this study was to investigate whether RIPC induced HO-1 inhibits mitochondrial cytochrome c release following ischaemia reperfusion injury (early phase).

9.2 Material and methods

Animals and surgical procedures

As described in chapter 2, material and methods, pg 92.

Tissue and Blood collection.

As described in chapter two, material and methods, pg 105**.**

Number of animals sampled

Liver tissues were taken from all animals in the early phase of hepatic IR and late phase of IR.

48 samples were processed for mitochondrial cytochrome c western blot.

Experimental groups (n=6 in each group)

Sham

RIPC+SHAM

Hepatic IR

RIPC+IR

PDTC+IR

ZnPP+RIPC+IR

IR-24

RIPC+IR-24

Western blot analysis for cytochrome C (early and late phase)

This was carried out as described in chapter 2, material and methods, pg 114.

Western blot for HO (early and late phase)as described in chapter 2, pg 108

9.3 Results

Effect of RIPC in Sham on cytochrome c levels in comparison to Sham only animals

There is no significant difference in mitochondrial cytochrome c levels in sham animals and preconditioned shams. (Fig 9.1)

Effect of hepatic IR (early phase) on mitochondrial cytochrome c release in comparison to sham.

In the early phase of hepatic IR the mitochondrial cytochrome c levels are lower compared to sham however the difference is not significant.(Fig 9.1)

Effect of RIPC+IR (early phase) on mitochondrial cytochrome c release.

RIPC did not significantly inhibit release of mitochondrial cytochrome c in IR injury. (Fig 9.1)

Effect of HO induction in hepatic IR (early phase) on mitochondrial cytochrome c release

PDTC induced HO did not significantly inhibit release of mitochondrial cytochrome c in hepatic IR. (Fig 9.1)

Effect of HO inhibition in RIPC+IR on mitochondrial cytochrome c release.

Inhibition of HO did not significantly decrease in mitochondrial cytochrome c. (Fig 9.1)

Sinusoidal perfusion in the early phase of hepatic IR, RIPC+IR, PDTC+IR, ZNPP+RIPC+IR, Sham and RIPC+Sham (Chapter 4).

Sinusoidal perfusion was observed to be significantly low in hepatic IR and after HO inhibition in comparison to sham. After preconditioning and HO induction there was significant increase in sinusoidal perfusion.

Haemoxygenase western blots in the early phase of hepatic IR, RIPC+IR, PDTC+IR, ZNPP+RIPC+IR, Sham and RIPC+Sham (Chapter 4).

(Chapter 4).

HO expression in the liver was significantly more in RIPC+IR compared to IR. After HO inhibition there was a significant decrease in HO liver expression.

Effect of IR and RIPC on mitochondrial cytochrome c in the late phase of IR

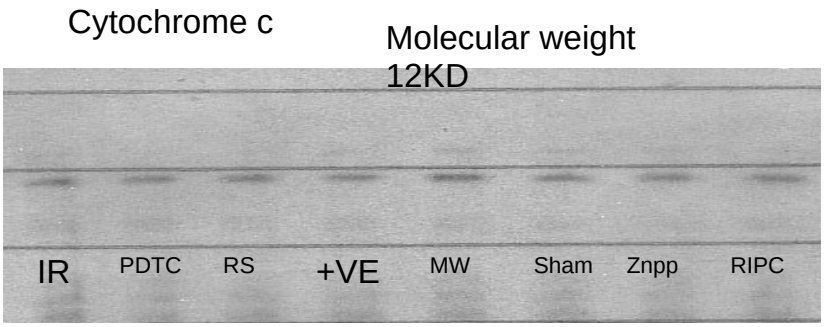
In the late phase RIPC mean cytochrome c levels were higher in the mitochondria in IR injury compared to RIPC. This suggests that RIPC doe not modulate cytochrome c levels in the late phase of IR as well.

Sinusoidal perfusion in the late phase of IR and RIPC+IR

The sinusoidal perfusion is more in RIPC+IR as compared to IR only at 24 hrs of reperfusion (chapter 5). Hepatocellular death and neutrophil adhesion are significantly less in RIPC+IR.

Haemoxygenase western blots in the late phase of IR and RIPC+IR

HO-1 expression is significantly more in RIPC+IR-24 as compared to IR-24 only **(chapter 7).**



+ve control RatPC12 cell lysate

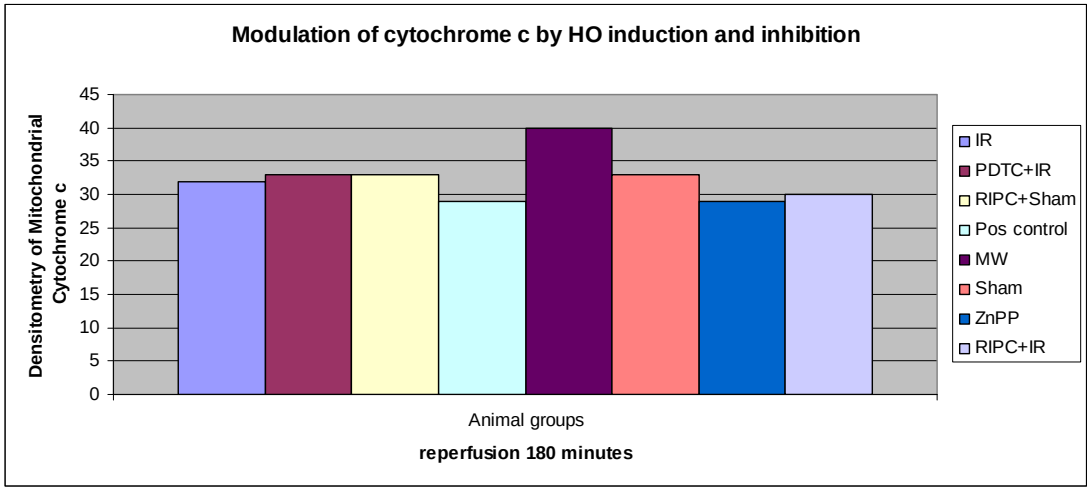


Figure 9.2 - No significant difference in the mitochondrial cytochrome c levels between hepatic IR/RIPC+IR/ ZnPP+RIPC+IR.

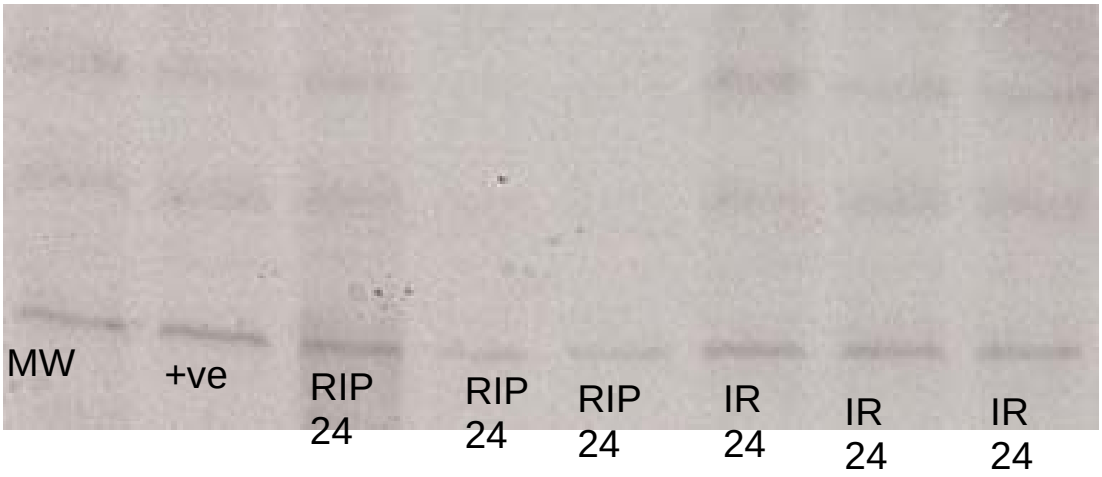


Fig 9.3 Western blots for cytochrome c – late phase of IR and RIPC+IR (representative picture)

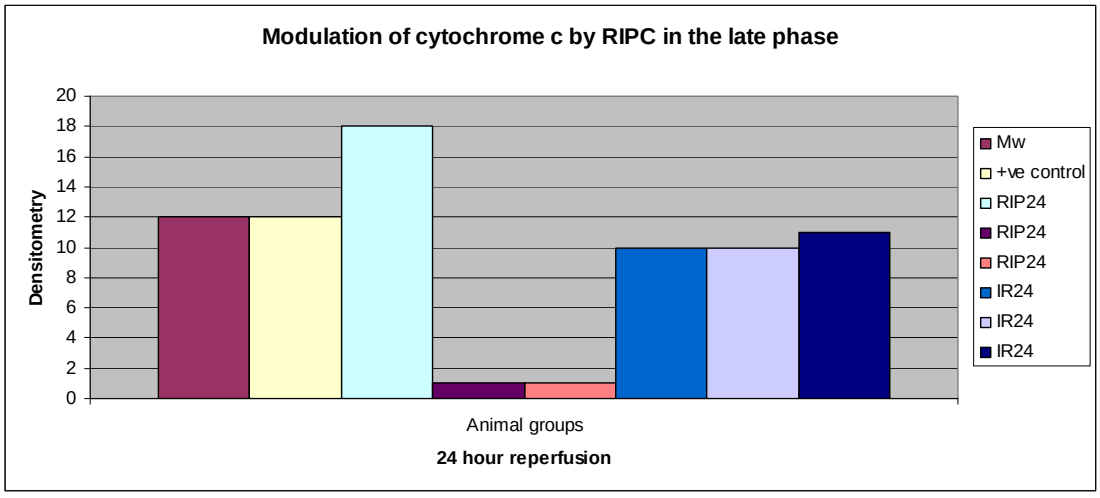


Fig 9.4 Densitometry for cytochrome c late phase.

Fig 9.4- Mitochondrial cytochrome c in IR injury at 24 hours was more than the RIPC- 24 samples except for one animal as shown above. This suggests that RIPC does not prevent release of mitochondrial cytochrome c following hepatic reperfusion for 24 hrs.

9.4 Discussion and conclusion

The assay and variability.

Variability in the assay can arise due to different methods of pipetting, dilution.These were minimised by using standard techniques of dilution, assays being done by the same person.

Controls

Rat lysate was used as positive control**.**

Cytochrome c release in Sham, comparison with RIPC+Sham and correlation with hepatocellular death, Sinusoidal, neutrophil adhesion on IVM

There was no significant difference between mitochondrial cytochrome c levels between sham and RIPC+Sham. This correlates well with no significant difference seen in hepatocellular death and neutrophil adhesion observed by IVM between Sham and RIPC+Sham. .

Effect of IR on cytochrome c release, correlation with hepatocellular death by IVM, sinusoidal perfusion, neutrophil adhesion (early phase)

This study has shown decreased mitochondrial cytochrome c in comparison to shams suggesting the release of cytochrome c into cytosol after IR. Reperfusion injury causes mitochondrial ROS generation, disruption of mitochondrial permeability and efflux of cytochrome c. IVM showed increased hepatocellular death by propidium iodide staining. This cell death maybe secondary to apoptosis induced by cytochrome c release or due to oncolytic necrosis induced by ROS from neutrophils. Increased neutrophil adhesion and decreased sinusoidal perfusion on intravital microcopy suggest a prominent role for neutrophil induced endothelial injury. Decreased hepatic HO by western blot analysis in IR in correlation with increased neutrophil adhesion and increased cell death would suggest a role for HO in protecting liver parenchyma

by modulation of neutrophil function rather than modulation of cytochrome c release in the early phase of IR.

Effect of RIPC on cytochrome c release in the early phase of IR and correlation with haemodynamics, neutrophil adhesion, apoptosis.

RIPC does not significantly decrease cytochrome c release from mitochondria in the early phase of IR. This does not correlate with decreased hepatocellular death seen on propidium iodide staining by intravital microscopy. There is a significant decrease in neutrophil adhesion in RIPC and RIPC significantly improved sinusoidal flow and perfusion. In the early phase of hepatic IR oxidative stress leads to free radical release and cell injury. Since RIPC reduces CINC hence it acts to reduce neutrophil activation and decrease release of ROS. Thus the main mechanism of cytotoxicity in the early phase is ROS generation and cell damage rather than mitochondrial release of cytochrome c and activation of apoptosis.

Hai et al showed that IR injury led to a significant increase in cytosol mitochondrial cytochrome c and activation of caspase 3 and increased translocation of Bax (proapoptotic proten) into the mitochondrial membrane promotes efflux of cytochrome c into the cytosol. Hirakawa (JSR, 2003) demonstrated the role of mitochondrial permeability pore resulting in increased cytosolic caspase 3 following reperfusion injury (Hirakawa, JSR). Hirakawa showed that cytosol cytochrome c appeared as early as 30 min after ischaemia and caspase 3 activity began to increase after 90 min. Soeda et al demonstrated the role of ROS after reperfusion in disruption of mitochondrial integrity and cytochrome c release early into the cytosol {Soeda et al, 2001}.

Effect of HO inhibition and HO induction on cytochrome c release and correlation with HO expression on western blots (early phase of IR).

HO induction or inhibition did not significantly influence cytochrome c release from mitochondria. Increased HO was observed after HO induction with PDTC and RIPC induced HO in the liver by western blot analysis. This does not correlate with decreased cytochrome c release in our study suggesting that HO may not modulate Cytochrome c release from mitochondria in the early phase of hepatic IR. HO expression in the early phase of hepatic IR modulates CINC and neutrophil adhesion and hence influences release of ROS.

Effect of IR on cytochrome c release, correlation with hepatocellular death by IVM, sinusoidal perfusion, neutrophil adhesion.

IR injury the mitochondrial c levels were high. This did not correlate with the increased hepatocellular death, increased neutrophil adhesion and decreased perfusion observed by IVM. This suggests that cytochrome c pathway is unlikely to be of significance in late phase of hepatic IR and that free radicals released from neutrophils are the key factors responsible for hepatocellular death.

Effect of RIPC on cytochrome c release in the late phase of IR and correlation with haemodynamics, neutrophil adhesion, apoptosis, HO expression

RIPC did not significantly modulate mitochondrial cytochrome c levels although there was a significant decrease in neutrophil adhesion, hepatocellular death and significant increase in hepatic HO expression suggesting that RIPC and HO modulate IR injury by modulation of neutrophil activation rather than the cytochrome c pathway.

Conclusion

RIPC in the early and late phase of hepatic IR reduces cell death due to oncolytic necrosis secondary to free radicals. RIPC does not modulate apoptosis (programmed cell death) in the early phase and the late phase of IR.

Chapter 10

Discussion of thesis

10.1 Chapter 1- Background literature

This chapter has discussed the pathophysiology of hepatic IR, evidence for remote ischaemic preconditioning and potential mechanisms of preconditioning. Review of experimental studies has clearly demonstrated that brief ischaemia of the heart, mesentery, kidney and limb conferred protection on remote organs. RIPC prior to IR of limbs, heart, liver and kidney conferred protection on these organs as demonstrated by these studies. Only one study by Kanoria et al demonstrated improved hepatic haemodynamics following brief RIPC of the hind limb and Lai et al showed improved liver function. The studies reviewed have shown release of biochemical messengers such as cytokines, free radicals, opioids, NO which act either through the neurogenic or humoral pathway on the target organ.

In RIPC of the liver Kanoria et al suggested the role of NO however there was no clear evidence of the mechanism in RIPC. Lai et al showed haemoxygenase expression in the liver following RIPC and reduced hepatic IR. This formed the basis of further investigation in this study.

10.2 Chapter 2- Methodological considerations

Adequacy of model

The experimental model used is a previously well described hepatic lobar ischaemia model of warm IR. In the model 45 minutes of partial ischaemia (70% of the liver) was induced followed by three hours of reperfusion (Koti et al). Intravital microscopy has shown that IRI significantly reduced velocity of flow and perfusion as compared to sham animals (Menger et al). Therefore the model was considered suitable to study the effects of RIPC.

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 RIPC 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 however this was not done in our study.

Technique of preconditioning and its adequacy.

RIPC was produced using a tourniquet around the Hind limb for four cycles of five minute ischaemia followed by reperfusion. This technique of RIPC has previously been used in the modulation of cardiac IR injury following transplantation (Kristiansen et al). Three or more cycles of preconditioning have been shown to be more effective than a single cycle in previous experimental studies (Addison et al, Kristiansen et al) and this formed the basis for four cycles of RIPC used in the current study. Preliminary experiments demonstrated a significant effect of four cycles of RIPC on hepatic IR and hence the number of preconditioning cycles was considered optimal.

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 outbred 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 insignificant alteration of systemic haemodynamics.

In vivo fluorescent 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 exteriorized with a minimum of trauma. This method of exteriorisation of the liver also eliminated the respiratory movements in the tissue. 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.

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.

10.3 Effect of RIPC on hepatic IR in a rat model of warm hepatic IR.(Chapter 3)

Remote ischaemic preconditioning modulated hepatic microcirculation leading to increased red blood cell velocity (i.e. blood flow), sinusoidal flow, sinusoidal perfusion and decreased leukocyte- endothelial interaction as well cell death. There have been no previous studies which have demonstrated these findings.

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 histologic markers, realising 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.

10.4 Effect of HO-1 inhibition on RIPC in hepatic IR and HO-1 induction in hepatic IR. (Chapter 4)

RIPC was associated with an increase in hepatic haemoxygenase production and protection of liver function.The administration of HO-1 inducer Pyrrolidine

dithiocarbamate produced results similar to RIPC, i.e. improvement in sinusoidal perfusion, flow, improved red blood cell velocity and attenuated leukocyte-endothelial interactions. Pyrrolidine dithiocarbamate administration was associated with increased expression of HO-1.The administration of Zinc Protoporphyrin (HO-1 inhibitor) abolished the protective effect of remote ischaemic preconditioning strongly suggesting the role of haemoxygenase in modulation of hepatic microcirculation.

10.5 New insights into the late phase of hepatic IR (Chapter 5)

Intravital microscopy in a real time manner has provided new insights into the modulation of the late phase of hepatic IR by remote ischaemic preconditioning. The data clearly demonstrates that RIPC modulates sinusoidal perfusion, neutrophil adhesion and hepatocyte death in hepatic IR after 24 hours of reperfusion. There is evidence of sinusoidal dilatation seen in the late phase of RIPC in contrast to the early phase. These effects are associated with better liver function and significantly increased haemoxygenase expression in the liver in preconditioned animals.

10.6 Haem Oxygenase studies (Chapter 6&7) Immunohistochemistry and W blots.

In this study HO estimations were used as marker of HO production and to affirm the response to pharmacological manipulation with Pyrrolidine dithiocarbamate and Zinc Protoporphyrin. The expression of haemoxygenase and its cellular distribution was assessed by immunohistochemistry using polyclonal primary and monoclonal seconday antibodies. Immunohistochemistry demonstrated HO-1 expression in Kupffer cells in the early phase of IR following RIPC and in parenchymal cells as well as kupffer cells in the the late phase of hepatic IR. The limitation of immunohistochemistry is lack of quantification.

Western blot technique is a semiquantitative technique used to quantify HO expression in this study and western blotting showed increased expression of HO-1 in preconditioned animals and PDTC induced HO-1 groups in the early phase and in the late phase of hepatic IR. Inhibition of HO showed decreased HO expression in the ZnPP group.

Polyclonal antibodies against the specific HO isoforms are commercially available and can identify the isoforms on tissues sections and these were used for western blotting in this study. A limitation of molecular biology techniques is that they only indicate the presence of protein and as such may indicate synthesis of new protein but these techniques do not give an indication of protein phosphorylation and therefore activity. The other option is to use enzyme phosphorylation assays, but these are indirect measurements and again do not help in establishing a direct link between protein and enzymatic HO. In theory, a combination of specific HO isoform genetic knockout model and molecular biology techniques would give a better understanding of HO-1 activity in a given setting.

10.7 Cytokine studies (Chapter 8)

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 enxyme-linked immunosorbent assay detects CINC at concentrations of 3 pg/ml to 30ng/ml.The significantly decreased CINC following RIPC and decreased neutrophil endothelial interaction observed in intravital microscopy suggests modulation of neutrophil activation by RIPC. RIPC modulated neutrophil activation and neutrophil endothelial interaction through inhibition of CINC and

haemoxygenase pathways may have a role in modulation of CINC as suggested by the data in this study.

10.8. Cytochrome c mitochondrial apoptosis (chapter 9)

Western blot analysis for mitochondrial cytochrome c in hepatic IR and RIPC+IR did not show any significant difference between the two groups following reperfusion injury. Haemoxygenase inhibition or induction did not demonstrate any effect on mitochondrial cytochrome c levels in the early phase of hepatic IR. These data suggest that RIPC does not modulate release of mitochondrial cytochrome c and activation of the mitochondrial apoptotic pathway in the early and late phase of hepatic IR. Haemoxygenase induction or inhibition does not appear to have an effect on mitochondrial cytochrome c activation in early IR. This can be explained by the fact that in the early phase necrosis is predominantly responsible for cell death as against apoptosis.

Observations on Sinusoidal dilatation

Ischaemia reperfusion injury is known to cause sinusoidal constriction due to endothelial injury and effects of endothelin. Our observations did not show any change in sinusoidal diameter compared to shams which was an unexpected finding. The extent of endothelial neutrophil adhesion and hepatocellular death was significantly more in IR injury in the early and late phase suggesting that the model was appropriate for the study. RIPC did not induce any change in sinusoidal diameter since HO-1 expression was mainly seen in the Kupffer cells in response to oxidative stress. PDTC did not cause any sinusoidal dilatation. The dose chosen for PDTC in our study was 100mg/kg. Hata et al have shown that PDTC at doses of 150mg/kg or higher induces sinusoidal dilatation and the dilatation is proportional to PDTC dosage. They explained that PDTC at higher doses induced parenchymal HO-1 expression which led to release of CO into the space of disse and CO induced sinusoidal

dilatation. ZnPP did not induce any change in sinusoidal dilatation in preconditioned animals suggesting that RIPC induced HO in the early phase of hepatic IR primarily has anti-inflammatory effects. In our study there was increased parenchymal HO expression in the late phase of RIPC and there was significant sinusoidal dilatation in comparison to IR injury. Goda et al have also shown that expression of constitutive HO (HO-2) in parenchymal cells is responsible for inducing sinusoidal dilatation. Future studies would need to investigate parenchymal HO expression and the subtypes of HO expressed in parenchymal cells in the early and late phase of hepatic IR following RIPC.

Overall conclusions arising from the thesis .

The results of the study have been discussed in detail in each chapter detailing the experiments (Chapters 3 to 9).The above conclusions support the hypothesis presented in this study and strongly suggest a role for HO-1 in the protection conferred by RIPC in early and late phase of ischaemia reperfusion induced hepatic injury. Although the technique of RIPC is simple and easily applicable there are concerns about increased operative time (due to period of time involving brief ischaemia and reperfusion) which may not be tolerated well in many operating theatres. However this limitation can be overcome by commencing preconditioning from the time the patient is anaesthetized to the time a laparotomy incision is made. Finally, although current research on the mechanisms of preconditioning seems to diverge more and more, it is possible that all these mechanisms converge into an unidentified final common pathway. This conclusion is based on the belief that a powerful adaptive phenomenon that is induced so easily and reproducibly in different models, laboratories, and species is almost certain to be mediated by a universal mechanism. The data from this study suggests that haeme oxygenase is the candidate

molecule in remote ischaemic preconditioning of the liver and is likely to be central to the universal mechanism.

Clinical application of RIPC/ IPC and the wider implications of this work.

IPC trials in liver IR.

Rela et al (transplantation 2006) showed that 10 min of clamping of the hepatic pedicle prior to retrieval led to significantly lower hepatic injury in donor livers , decreased serum lactate, neutrophilic infiltration in preconditioned livers and also decreased ITU stay. This trial showed a beneficial effect for ischaemic preconditioning. Recent Cochrane review by Kurinchi et al showed no evidence to support or refute the use of ischaemic preconditioning in hepatic transplantation. This review showed no difference in mortality, primary graft function or delayed graft function {Kurinchi et al, Cochrane 2007). Pharmacological preconditioning particularly with agents inducing NO has shown some promise recently. During liver transplantation patients were exposed to inhale a high but non toxic dose of nitric oxide (80 ppm). Inhaled NO improved serum transaminase and coagulation times and reduced hepatocyte apoptosis and hospital stay {Lang *et al*, J Clin Inv 2007}.

Potential clinical applications of RIPC .

There have been no published clinical trials investigating modulation of ischaemia reperfusion injury of the liver by remote ischaemic preconditioning. The first clinical trial evaluating effects of RIPC has been in children undergoing cardiac surgery and this study demonstrated that RIPC significantly reduced trop I levels, peak airway resistance and inotrope requirements in patiens compared to those who were not preconditioned. The patients were preconditioned by four cycles of 5 min ischaemia and 5 min of reperfusion {Cheung *et al*, 2007}. In another recent study RIPC reduced

myocardial injury in CABG patients receiving cold –blood cardioplegia {Venogopal *et al,* Heart 2009}.

This study has demonstrated that preconditioning of livers prior to hepatic IR conferred protection of hepatic microvasculature and flow. However preconditioning shams induced a low grade oxidative stress and reduced flow transiently in normal livers. These data suggest that preconditioning recipients may lead to free radicals damaging normal organs in the recipient and causing organ failure and eventually death. In contrast preconditioning donors would protect the donor organ from IR without causing injury to normal organs in the recipient.

Amersi et al showed that inducing HO-1 through adenovirus HO-1 gene transfer in genetically fat zucker rats significantly reduced hepatic IR after explantation and reimplantation. Also Koito et al have shown that cold preservation injury due to sinusoidal endothelial ell injury is reduced after pre-treatment with HO-1 inducers. These data suggest that preconditioning prior to retrieval may reduce hepatic ischaemia reperfusion injury significantly and enhance organ usage.

Future plans

Knock out models

Further studies need to be taken up in HO-1 knockout mice models to pinpoint the definitive role of HO-1 in RIPC in hepatic IR. The use of small interfering RNA sRNA which specifically inhibit HO-1 pathways would also be useful in investigating the role of HO-1 in hepatic IR. ZnPP is both toxic and a non-specific inhibitior of HO as it inhibits both HO-1 and HO-2. This limits its role in investigating HO-1 pathways in preconditioning.

Effect of HO on endothelial function

The effect of HO on endothelial function would need to be demonstrated in experimental models. This study has shown RIPC induced increased parenchymal expression of HO in the late phase of hepatic IR which led to increased sinusoidal dilatation. It has been suggested by Goda et al that this is due to increased CO production by HO-1 or HO-2 expression in parenchmal cells. In the early phase HO-1 was primarily expressed in kupffer cells in response to oxidative stress. Hence it did not cause sinusoidal dilatation . Hata et al have shown increased doses of PDTC to induce HO-1 and sinusoidal dilatation in the early phase of hepatic IR. The effect of HO-1 on endothelial adhesion molecules and endothelin which is a sinusoidal constrictor and has been shown to cause sinusoidal constriction in hepatic IR will need to be investigated in future experimental studies.

Future therapeutic regimes.

PDTC and its limitations in humans.

There have been no satisfactory drugs which have been developed to induce HO-1 in the human liver and modulate ischaemia reperfusion injury. PDTC is extremely toxic and cant be used in humans.

Clinical trials

RIPC would need to be taken up in clinical trials in patients undergoing liver resection and liver transplant. Preconditioning of heart beating donors would lead to an increased donor pool of organs and also reduce ischaemia reperfusion injury in the grafts thus reducing patient morbidity , mortality and costs in patient care.

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Appendix – abstracts and publications arising from the thesis

Abstracts

1. Title-Remote ischemic preconditioning modulates hepatic microcirculation to ameliorate hepatic ischaemia reperfusion injury in a rat model.

N.Tapuria, S.P.Junnarkar, Wenxuan Yang, A. Seifalian, B.R.Davidson.

Introduction- Liver resection and transplantation surgery involve a period of ischaemia reperfusion injury (IRI) which initiates an inflammatory cascade resulting in hepatic and remote organ injury. It has been established that remote ischemic preconditioning (RIPC) protects the liver.RIPC entails brief periods of limb ischaemia followed by reperfusion. The effect of RIPC on the hepatic microcirculation has not been studied previously.

Hypothesis-RIPC would protect the liver from ischaemia reperfusion injury by modulation of hepatic microcirculation.

Aim-To use a well described model of ischaemia reperfusion injury to study the effect of RIPC on hepatic microcirculation under intravital microscopy.

Material and methods-The effect of RIPC was studied in a rat model of ischaemia reperfusion injury with 45 minutes of partial liver ischaemia(70%) followed by 3 hours of reperfusion. Three groups of animals namely Sham, IRI and RIPC +IRI were studied with six animals in each group .Hepatic microcirculation was assessed by studying velocity of blood flow and sinusoidal perfusion and apoptosis was assessed by propidium iodide staining under intravital microscopy.

Results-. The velocity of blood flow was significantly better in the RIPC group at the end of three hours of reperfusion compared to IRI group ($P<0.05$) but no difference was found between RIPC and Sham. Sinusoidal perfusion was better in the RIPC group. The number of apoptotic cells in the RIPC group was significantly less as compared to IRI only ($P<0.05$).

Conclusions-RIPC protects the liver from IRI by modulation of hepatic microcirculation.

Abstract published in the HPB sep 2006 supplement.

2. Title- Remote ischemic preconditioning modulates hepatic microcirculation through the hemeoxygenase pathway in a rat model of warm ischaemia reperfusion injury of the liver.

N.Tapuria, S.P.Junnarkar, Neelanjana Dutt, Barry Fuller, A. Seifalian, B.R.Davidson.

Introduction- Liver resection and transplantation surgery involve a period of ischaemia reperfusion injury (IRI) which initiates an inflammatory cascade resulting in hepatic and remote organ injury. IRI leads to nonfunction or dysfunction of liver grafts in clinical transplant settings. Davidson's group at the Royal Free have shown that remote ischemic preconditioning (RIPC) protects liver function. However the mechanism of protection has not been studied. This is the first study under intravital microscopy to investigate the effect of remote ischemic preconditioning upon hepatic microcirculation and the role of hemeoxygenase pathways as a candidate mechanism.

Aim-To study the role of hemeoxygenase pathways in RIPC as the candidate mechanism in the modulation of hepatic microcirculation in a rat model of hepatic IRI.

Material and methods-The effect of RIPC was studied in a rat model of ischaemia reperfusion injury with 45 minutes of partial liver ischaemia(70%) followed by 3 hours of reperfusion. Five groups of animals namely Sham, IRI and RIPC +IRI , RIPC+Sham, PDTC (HO donor)+IRI, ZNPP(HO blocker)+RIPC+IRI were studied with six animals in each group .Hepatic microcirculation was assessed by studying velocity of blood flow , sinusoidal perfusion , sinusoidal flow, sinusoidal diameter and neutrophil adhesion . Apoptosis was assessed by propidium iodide staining under intravital microscopy. Liver functions were assessed in all groups.

Results- The velocity of blood flow was significantly better in the RIPC group at the end of three hours of reperfusion compared to IRI group ($P<0.05$) but no difference was found between RIPC and Sham. Sinusoidal perfusion and flow was better in the RIPC group. The number of apoptotic cells in the RIPC group was significantly less as compared to IRI only ($P<0.05$). The PDTC group (HO Donor) showed significantly improved liver functions compared of IRI and ZNPP groups. ZNPP significantly blocked the effects of RIPC on the microcirculation at about two hours of reperfusion strongly suggesting the role of HO-1 pathways in RIPC mediated amelioration of hepatic IR injury.

Conclusions- This is the first study to demonstrate that RIPC modulates hepatic microcirculation in order to ameliorate IRI and hemeoxygenase pathways are one of the key pathways in the mechanism of RIPC.

Abstract published in the IHPBA supplement of the American chapter of HPB April 2007.

3. NEW INSIGHTS INTO PROTECTION OF HEPATIC FUNCTION BY REMOTE ISCHEMIC LIMB PRECONDITIONING IN THE LATE PHASE OF HEPATIC ISCHEMIA REPERFUSION INJURY.

Tapuria, Niteen; Junnarkar, Sameer; Fuller, Barry; Seifalian, Alexander M; Davidson,Brian
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BACKGROUND: Ischemia reperfusion injury (IRI) may lead to nonfunction or dysfunction of transplanted liver grafts. At the Royal Free (UK) experimental studies by intravital microscopy have demonstrated that remote ischemic limb preconditioning (RIPC)modulates hepatic microcirculation by increasing RBC velocity, sinusoidal flow, sinusoidal perfusion and decreasing neutrophil adhesion as well as apoptosis to protect liver function in the early phase of hepatic reperfusion injury. This is the first study to focus on the role of RIPC in protection of hepatic function in the late phase of reperfusion injury. **AIM**: To investigate the effect of RIPC on late phase of reperfusion injury in an animal model of 24 hour hepatic ischemia reperfusion injury. **MATERIAL AND METHODS**:A rat model of IRI with 45 minutes of partial liver ischemia (70%) followed by 24 hours of reperfusion was used for this study. Twoanimal groups studied included IRI-24 and RIPC+IR-24. The animals were preconditioned by 4 cycles of right hind limb ischemia(5minutes), reperfusion (5 minutes) prior to ischemia. Hepatic microcirculation was assessed by studying velocity of RBC flow,sinusoidal perfusion, sinusoidal flow and sinusoidal diameter under intravital microscopy. The number of neutrophils adherent toendothelium/mm2 and the numbers of stained apoptotic cells/HPF were assessed by intravital microscopy. Liver functions wereassessed. **RESULTS:** There was no significant difference in the RBC velocity, sinusoidal flow or sinusoidal diameter between the IR-24 and RIPC+IR-24 groups. Sinusoidal perfusion was significantly better in the RIPC+IR-24 group. Neutrophil adhesion and apoptosis were significantly reduced and liver functions were significantly better in the RIPC group. **CONCLUSION**: Modulation of hepatic microcirculation and neutrophil activation by RIPC in the late phase of hepatic IR is a key mechanism in protection of liverfunction. The role of modulation of neutrophil activation to prolong graft survival needs further investigation

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RESEARCH REVIEW

Remote Ischemic Preconditioning: A Novel Protective Method From Ischemia Reperfusion Injury—A Review

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Background. **Restoration of blood supply to an organ after a critical period of ischemia results in parenchymal injury and dysfunction of the organ referred to as reperfusion injury. Ischemia reperfusion injury is often seen in organ transplants, major organ resections and in shock. Ischemic preconditioning (IPC) is an adaptational response of briefly ischemic tissues which serves to protect against subsequent prolonged ischemic insults and reperfusion injury. Ischemic preconditioning can be mechanical or pharmacological. Direct mechanical preconditioning in which the target organ is exposed to brief ischemia prior to prolonged ischemia has the benefit of reducing ischemia-reperfusion injury (IRI) but its main disadvantage is trauma to major vessels and stress to the target organ. Remote (inter organ) preconditioning is a recent observation in which brief ischemia of one organ has been shown to confer protection on distant organs without direct stress to the organ. Aim: To discuss the evidence for remote IPC (RIPC), underlying mechanisms and possible clinical applications of RIPC.**

Methods of search. **A Pubmed search with the keywords "ischemic preconditioning," "remote preconditioning," "remote ischemic preconditioning," and "ischemia reperfusion" was done. All articles on remote preconditioning up to September 2006 have been reviewed. Relevant reference articles from within these have been selected for further discussion.**

Results. **Experimental studies have demonstrated that the heart, liver, lung, intestine, brain, kidney and limbs are capable of producing remote preconditioning when subjected to brief IR. Remote intra-organ preconditioning was first described in the heart where brief ischemia in one territory led to protection in other areas. Translation of RIPC to clinical application has been demonstrated by the use of brief forearm ischemia in preconditioning the heart prior to coronary bypass and in reducing endothelial dysfunction of the contra lateral limb. Recently protection of the heart has been demonstrated by remote hind limb preconditioning in children who underwent surgery on cardiopulmonary bypass for congenital heart disease. The RIPC stimulus presumably induces release of biochemical messengers which act either by the bloodstream or by the neurogenic pathway resulting in reduced oxidative stress and preservation of mitochondrial function. Studies have demonstrated endothelial NO, Free radicals, Kinases, Opioids, Catecholamines and K_{ATP} channels as the candidate mechanism in remote preconditioning. Experiments have shown suppression of proinflammatory genes, expression of antioxidant genes and modulation of gene expression by RIPC as a novel method of IRI injury prevention.**

Conclusion. **There is strong evidence to support RIPC. The underlying mechanisms and pathways need further clarification. The effective use of RIPC needs to be investigated in clinical settings.** **© 2008 Elsevier Inc. All rights reserved.**

Key words: **Ischemic preconditioning; remote preconditioning; remote ischemic preconditioning; ischemia reperfusion.**

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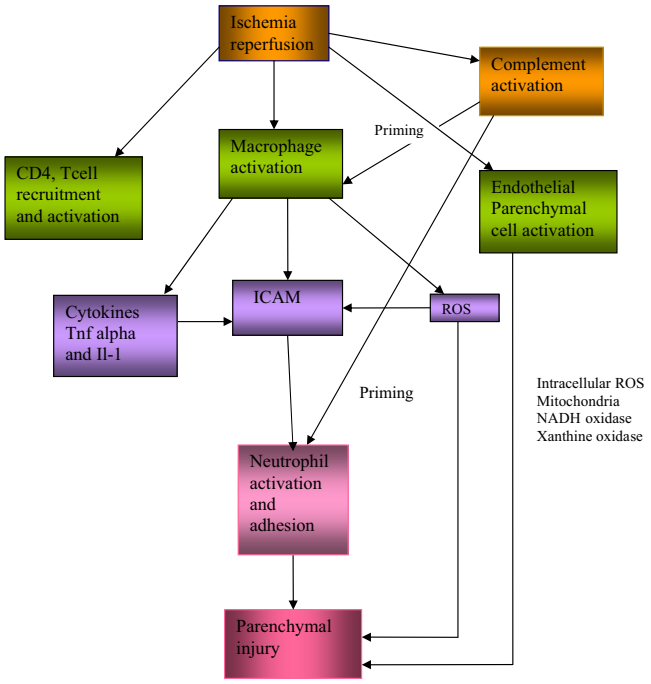

FIG. 1. Diagramatic representation of ischemia reperfusion injury. (Color version of figure is available online.)

INTRODUCTION

The restoration of blood supply to organs after a certain period of no flow ischemia results in parenchymal damage referred to as ischemia-reperfusion injury (IRI). The critical ischemia period is dependent on the organ and is 15–20 min [1] in the liver and kidney, 2.5 h in skeletal muscle [2–4], whereas in the brain ischemia for more than 5 min, leads to considerable neuronal death and infarction. Reperfusion following periods exceeding the critical ischemia period results in endothelial and parenchymal injury. The liver is resilient to hypoxic injury. Low-flow ischemia found in hemorrhagic shock (mean arterial pressure [MAP]-40 mmHg for 120 min) followed by restoration of normal flow does not lead to activation of Kupffer cells, generation of free radicals and associated IRI in the initial resuscitation period [1]. This is because most Kupffer cells are located in the periportal region and hemorrhagic shock is characterized by ischemia in the pericentral regions with sinusoidal perfusion failure in the periportal region as a result of which Kupffer cells are not affected by pericentral hypoxia.

Pathophysiology of IRI (Fig. 1)

Following a period of ischemia, tissues adapt to anaerobic metabolism [5]. Restoration of blood supply results in oxygen supply in excess of the requirements that lead to activation of macrophages in the vasculature and consequently generation of super oxide radicals, also referred to as reactive oxygen species (ROS), causing oxidative stress. The key event in the initial phase of reperfusion injury is activation of macrophages that are the primary source of extracellular ROS. ROS are the key initiators of reperfusion injury, which leads to endothelial injury and further release of pro-inflammatory cytokines. Thus neutrophils are the key cells in the late phase of IRI.

The Clinical Relevance of IRI

IRI often happens following transplantation of organs, major organ resections, and trauma. IRI following transplantation can lead to primary nonfunction of the implanted organ (<5%), primary dysfunction (10–30%), and also multiple organ dysfunction syndrome, resulting in morbidity and mortality in adult and pediatric transplants [6–8]. In hypervolemic shock resuscitation leads to IRI in brain, gut, and pancreas [9], which results in more extensive tissue infarction. In the liver shock resuscitation reduces its tolerance to subsequent warm ischemia [10]. In major organ resections inflow occlusion [11] and subsequent restoration of blood supply causes reperfusion injury. This can lead to postoperative organ insufficiency. Ischemia reperfusion injury is also associated with chronic rejection due to arteriosclerosis [12] caused by IRI. IRI results in adipocutaneous and musculoskeletal flap necrosis as well as nonfunctioning microvascular flaps.

IPC

Protective strategies have been developed for protection of organs from ischemia reperfusion injury, which are referred to as organ preconditioning. IPC is only a method by which the target organ is conditioned prior to the ischemic insult to reduce the extent of injury. IPC could be mechanical or pharmacological. Mechanical preconditioning is either direct or remote (indirect). In IPC a brief direct ischemic insult to the target organ followed by reperfusion results in tolerance to subsequent insults of ischemia. Ischemic tolerance is induced by regulation of endothelial function, blood flow, and decreased macrophage as well as neutrophilic activity. This results in decreased endothelial injury and eventually decreased parenchymal injury. Direct IPC has been investigated as a surgical tool for many years [13]. Although direct IPC does reduce reperfusion injury [14–16] as well as its systemic consequences [17, 18], its main disadvantage is direct stress to the target organ and mechanical trauma to major vascular structures, which have limited its clinical application.

Remote ischemic preconditioning (RIPC) is a novel method where ischemia followed by reperfusion of one organ is believed to protect remote organs either due to release of biochemical messengers in the circulation or activation of nerve pathways, resulting in release of messengers that have a protective effect. This protects

TABLE 1

Studies on Intraorgan Regional Preconditioning

Author/reference	Site of preconditioning stimulus	Site of index ischemia	Model	Endpoint	Organ protection	Proposed mechanism
Intracardiac preconditioning						
Przyklenk *et al.* [21]	Circumflex occlusion	Lad occlusion	Dog	Infarct size	Reduced infarct size	Not identified
Nakano *et al.* [22]	Cardiac ischemia	Total LV:global ischemia	Rabbit	Infarct size	Reduced infarct size	Not applicable
Tanaka *et al.* [131]	Cardiac ischemia	Remote myocardial preconditioning	Rabbit	Infarct size	Reduced infarct size	Heat shock proteins
Gho *et al.* [26]	Cardiac ischemia	Cardiac ischemia	Rat	Infarct size	Reduced infarct size	?Neurogenic pathway, ?humoral

target tissue without direct stress. RIPC was first demonstrated in myocardium [19] by McClanahan in 1993. He found that ischemia in the kidney followed by reperfusion protected myocardium from ischemia and reduced infarct size. In animal models brief ischemia reperfusion of the limb, gut, mesenteric, or kidney reduces myocardial infarct size. In humans skeletal preconditioning has been used for myocardial protection with the beneficial effect being attributed to regulation of endothelial protection [20].

Aim

The aim of this article was to discuss the evidence for existence of remote preconditioning, the underlying mechanisms and pathways in different methods of remote preconditioning, the mediators of preconditioning, and the possible clinical applications of remote preconditioning.

Methods of Search

A PubMed search with the keywords "ischemic preconditioning," "remote preconditioning," and "remote ischemic preconditioning" and "ischemia reperfusion" was done. All articles on remote preconditioning up to September 2006 have been reviewed. Relevant reference articles from within these have been selected for further discussion.

Evidence for RIPC

Intracardiac Regional Preconditioning (Table 1)

The very first evidence of RIPC can be traced to 1993 when Przyklenk conducted regional myocardial preconditioning in an experimental dog model [21]. In this model the circumflex coronary artery was occluded four times for 5 min followed by 5 min reperfusion prior to 1 h of sustained left anterior descending coronary artery occlusion. A significant reduction in myocardial infarct size was seen as compared to non-preconditioned groups. Nakano *et al.* questioned the existence of RIPC as in an experimental Langendorff model they showed two cycles of 5 min occlusion of a branch of coronary artery followed by reperfusion (5 min) prior to global cardiac ischemia protected the myocardium supplied by the arterial branch but not the rest of the myocardium against sustained ischemia [21, 22]. However, their experiments were in a rabbit model and they used a different preconditioning protocol. Moreover, their argument is not supported by any other studies. Hence evidence for nonexistence of RIPC in other species cannot be concluded from their study.

TABLE 2

Studies on RIPC by Brief Cerebral Ischemia

Author/reference	Site of preconditioning stimulus	Site of index ischemia	Model	Endpoint	Organ protection	Proposed mechanism
Preconditioning by cerebral ischemia						
Tokuno *et al.* [23]	Cerebral ischemia	Cardiac ischemia	Mouse isolated heart	Infarct size	Reduced infarct size	iNOS and NO
de Zeeuw *et al.* [119]	Cerebral ischemia	Cardiac ischemia	Pig	Infarct size	No change in infarct size	Norepinephrine

TABLE 3

Studies on RIPC by Brief Renal Ischemia

Author/reference	Site of preconditioning stimulus	Site of index ischemia	Model	Endpoint	Organ protection	Proposed mechanism
Preconditioning by renal ischemia						
McClanahan *et al.* [19]	Renal	Cardiac ischemia	Rabbit	Infarct size	Reduced infarct size	Not identified
Pell *et al.* [27]	Renal	Cardiac ischemia	Rabbit	Infarct size	Reduced infarct size	Adenosine receptors and sarcolemmal KATP channels
Takaoka *et al.* [25]	Renal	Cardiac ischemia	Rabbit	Infarct size	Reduced infarct size	Adenosine
Singh *et al.* [28]	Renal	Cardiac ischemia	Rat	Infarct size	Reduced infarct size	Angiotensin receptors
Lang *et al.* [138]	Renal	Cardiac ischemia	Rat	Infarct size	Reduced infarct size	Humoral factor (MW <8 kDa), ?Neurogenic pathway

Interorgan Preconditioning

RIPC is not confined to within an organ and can be transferred from one organ to another.

The Effect of Cerebral Ischemia on the Heart (Table 2)

Spontaneous ischemic events in the brain have been shown to induce adaptation of the heart to ischemia [23] and Tokuno *et al.* were the first to demonstrate in a mouse model decreased myocardial infarction and improved myocardial function in mice by prior brain ischemia (bilateral internal carotid ligation) with no evidence of brain damage except for transient neurological patterns [23]. Importantly, this study demonstrated that ischemia rather than reperfusion stimulated signaling pathways, which led to remote protection.

The Effect of Renal Ischemia on Remote Organs (Table 3)

Five studies showed that brief renal ischemia reduced the size of infarct resulting from myocardial ischemia [24–28]. The first evidence of RIPC dates back to 1993 when McClanahan carried out experiments on a rabbit model and showed that a 10 min period of renal ischemia was as effective as a 5 min period of coronary artery occlusion in preconditioning the heart [19]. In 1996, Gho *et al.* [26] showed that brief periods of mesenteric artery occlusion (MAO) (15 min) and reperfusion prior to 60 min coronary artery occlusion (CAO) was as effective as direct CAO and 15 min renal artery occlusion (RAO) prior to 60 min CAO reduced infarct size under hypothermic conditions but not normothermic conditions, demonstrating the unmasking of effects of RAO by hypothermia. The onset of myocyte injury is associated with adenosine triphosphate (ATP) depletion and breaks in sarcolemmas, which are linked to intracellular acidosis and raised phosphocreatinine levels. Takoaka *et al.* showed by nuclear magnetic resonance spectroscopy that RAO (10 min) followed by reperfusion (20 min) in rabbits led to a decrease in myocardial infarct size on histology, attenuated depletion of ATP, preserved myocyte pH, and improved recovery of myocardial phosphocreatinine as well as ATP levels during subsequent ischemia-reperfusion (IR) [25]. Two studies, one in a rabbit model [27] and the other in a rat model [28], showed RIPC by RAO reduced myocardial infarct size prior to CAO (30 min) followed by 2 h reperfusion. One study showed that RIPC from kidney was as effective as direct myocardial IPC in reducing infarct size [25]. These studies collectively demonstrate that brief periods of RAO and reperfusion are necessary for RIPC. RIPC was not seen in permanent arterial occlusion, suggesting that infarct size limitation was due to washout of a protective substance during reperfusion. Both rat and rabbit models demonstrated the benefit of RIPC. The extent of myocardial protection by RAO was more in Takoaka's study as compared to McClanahan's, although both used rabbit models, possibly due to differences in core temperatures of the animal models. This remained unclear as McClanahan did not report core body temperature in his study. Further studies in different animal models and in clinical settings are needed to test the efficacy of RIPC by RAO and to define the indications for its application.

Role of Mesenteric IPC (Table 4)

Ten studies demonstrated that RIPC from mesentery reduced myocardial infarct size on histology in a rat model. Seven studies [26, 29–33] showed that RIPC by single cycle of MAO (15 min) and subsequent reperfusion preconditioned the intestine and reduced myocardial infarct size. In contrast, two studies showed that RIPC, by multiple cycles of MAO [34, 35], reperfusion preconditioned the intestine and reduced myocardial infarct size, while one study demonstrated myocardial protection even after induction of myocardial ischemia 24 h later [36]. These studies collectively

TABLE 4

Studies on RIPC by Brief Mesenteric Ischemia

Author/reference	Site of preconditioning stimulus	Site of index ischemia	Model	Endpoint	Organ protection	Proposed mechanism
Preconditioning by mesenteric ischemia						
Gho *et al.* [26]	Mesenteric and renal	Cardiac ischemia	Rat	Infarct size	Reduced infarct size	Neurogenic, humoral
Schoemaker *et al.* [29]	Mesenteric ischemia	Cardiac ischemia	Rat	Infarct size	Reduced infarct size	Bradykinin mediated and neuronal pathway
Liem *et al.* [32]	Mesenteric ischemia	Cardiac ischemia	Rat	Infarct size	Reduced infarct size	Increased interstitial adenosine levels; afferent autonomic nerve stimulation, activation of myocardial adenosine receptors
Wang *et al.* [36]	Mesenteric	Cardiac ischemia	Rat	Infarct size	Reduced infarct size	Role for iNOS
Wolfrum *et al.* [30]	Mesenteric	Cardiac ischemia	Rat	Infarct size	Reduced infarct size	Myocardial PKC activation through both neuronal and bradykinin DEP humoral pathway
Wolfrum *et al.* [31]	Mesenteric	Cardiac ischemia	Rat	Infarct size	Reduced infarct size	Calcitonin gene related peptide
Patel *et al.* [33]	Mesenteric	Cardiac ischemia	Rat	Infarct size	Reduced infarct size	Endogenous opioids
Tang *et al.* [35]	Mesenteric	Cardiac ischemia	Rat	Infarct size	Reduced infarct size	Capsaicin-sensitive sensory nerves
Xiao *et al.* [34]	Mesenteric	Cardiac ischemia	Rat	Infarct size	Reduced infarct size	Capsaicin-sensitive sensory nerve and NOS
Petrishcev *et al.* [43]	Direct cardiac and remote mesenteric ischemia	Cardiac ischemia	Rats	Infarct size	Reduced infarct size	NO unlikely mechanism
Verdouw *et al.* [139]	Mesenteric ischemia, renal ischemia and ventricular pacing	Cardiac ischemia	Pig	Infarct size	Reduced infarct size	Ventricular pacing by activation of KATP channels Mesenteric ischemia effective at low and high core temp. 31 and 36°C Renal ischemia only at high temp.−36°C
Vlasov *et al.* [37]	Intestinal ischemia	Heart and intestine	Rat	Infarct size	No myocardial protection. Intestinal adaptation	NO in direct preconditioning but not in remote preconditioning
Liem *et al.* [62]	Mesenteric ischemia	Cardiac ischemia	Rat	Infarct size	Reduced infarct size	Mesenteric ischemia Adenosine dependent pathway
Huda *et al.* [140]	Mesenteric ischemia	Cardiac ischemia	Rat	Infarct size	Reduced infarct size	Inflammatory gene suppression

support the evidence for RIPC by MAO and reperfusion. All studies demonstrated an effective preconditioning protocol with brief MAO and only one study [33] demonstrated that a single cycle of RIPC was more effective than multiple cycles. Only one study (Vlasov [37]) showed that direct IPC is more effective than RIPC and one study (Wang *et al*. [36]) demonstrated the existence of late phase mesenteric RIPC. Histology is a common endpoint in all studies but myeloperoxidase activity (MPO) activity or myocardial creatinine kinase levels were not evaluated in all studies. One study showed that RIPC reduced myocardial MPO, which is a marker of systemic inflammatory response [36]. Two studies found significant hemodynamic alterations [30, 36] and showed that MAP increased on mesenteric occlusion and decreased on reperfusion with a gradual return to baseline. None of the studies measured ventricular function. Future studies aimed at clarifying the functional importance of mesenteric RIPC, comparisons of preconditioning protocols to establish the ideal protocol, and clinical applications are needed.

The Protective Effects of Prior Limb Ischemia on Remote Organs (Table 5)

Twelve studies have shown the beneficial effects of brief limb ischemia in preconditioning the heart. Oxman *et al.* showed reduced cardiac tachyarrythmias following brief limb ischemia [38], while Birnbaum *et al.* demonstrated that gastrocnemius electrical stimulation coupled with brief limb ischemia reduced myocardial infarct size [39]. Konstantinov *et al.* [40] and Kharbanda *et al.* [20] by multiple cycles of limb preconditioning and Weinbrenner *et al.* by a single cycle of preconditioning [41] demonstrated that RIPC reduced myocardial infarct size in animal models. Kharbanda showed that limb preconditioning reduced endothelial injury in humans [20]. IR of the upper limb (ischemia 20 min, 200 mm Hg cuff) blunted its vasodilatory response to acetylcholine; however, RIPC of the contralateral arm (3 × 5 min ischemia) attenuated neutrophil activation following ipsilateral limb IR. Gunaydin *et al.* showed that RIPC by two cycles of 3 min upper limb ischemia (tourniquet pressure 300 mm Hg) separated by 2 min of reperfusion in patients undergoing coronary bypass showed an equal rise in creatinine phosphokinase-MB in both preconditioned and control groups; however, there was a significant increase in lactate dehydrogenase (LDH) in the preconditioned group with levels of myocardial lactate and lactate efflux twice as high compared to the control group, providing biochemical evidence for maintenance of myocardial anaerobic glycolysis in the preconditioned group [42]. From a clinical perspective RIPC (Kharbanda model, [20]) may be useful in patients undergoing coronary angioplasty for reducing myocyte and endothelial injury and in patients undergoing cardiopulmonary bypass (Gunaydin model, [42]) where RIPC by transient upper limb ischemia can be used to protect the myocardium against subsequent IR when the aorta is clamped. Patients with intermittent claudication may have a better tolerance to myocardial ischemia due to preconditioning by brief limb ischemia and consequently have a longer window period for thrombolytic therapy to salvage ischemic myocardium. Vlasov [43] showed that both brief limb ischemia and cardiac ischemia reduced myocardial infarct size. A significant reduction in nicotine amide dehydrogenase (NADH) diaphorase and LDH activity was seen in the ischemic zone in the IR group, which was attenuated by both RIPC and direct IPC. In addition an increase in NADH diaphorase activity was seen in intact cardiomyocytes of the preconditioned groups as compared to IR group animals. This provided evidence that both RIPC and direct IPC induced some form of metabolic activity and ischemic adaptation in both ischemic and intact cardiac myocytes. This is supported by Gunaydin *et al.* [42], who showed attenuated LDH activity due to anaerobic glycolysis in preconditioned myocardium. Li *et al.* [44] in a mouse model demonstrated that RIPC significantly reduced myocardial infarct size. Expression of nuclear factor-kappa B (NF-κB) proteins from both limb skeletal muscle after RIPC and myocardium suggested the induction of protective signals in the limb being transferred to the heart and leading to ischemic adaptation. Two studies demonstrated the role of RIPC in a rat cardiac transplant model. Kristiansen *et al.* preconditioned the donor (rat model, 4 × 5 min IR cycles, hindlimb ischemia), which significantly reduced coronary IR in donor hearts on implantation in the recipient [45]. In contrast, Konstantinov *et al.* preconditioned the recipient (rat model, 4 × 5 min IR cycles, hindlimb), which reduced myocardial infarct size in the donor heart on implantation [46].

Eight studies have demonstrated the beneficial effect of skeletal preconditioning on adipocutaneous, muscle, and cremasteric muscle flaps as well as remote skeletal tissue [47–54]. Moses *et al.* [47] demonstrated reduced LD muscle flap infarct size in a pig model following RIPC (3 × 10 min). Contralateral hindlimb RIPC [48, 50–53] prior to ipsilateral cremasteric flap ischemia (2 h) and reperfusion in a rat model improved RBC flow and reduced neutrophil adhesion. Similarly Liauw *et al.* in a rat model showed that RIPC (ipsilateral Gracilis) reduced muscle necrosis of contralateral muscle by 60% as compared to non-preconditioned [54].

RIPC reduced flap necrosis, which is of particular benefit in patients with irradiated tissues, smokers, and obese patients. This protective effect is more pronounced in the late phase of preconditioning. These studies showed remote ischemic preconditioning to be associated with better microcirculation, decreased leukocyte endothelial sticking, and endothelial dysfunction as well as better capillary blood flow with terminal arteriolar dilation [55].

The following four studies have shown hindlimb ischemic preconditioning to be beneficial in protection of the lung and the brain [56–58]. (1) RIPC by hindlimb ischemia (3 × 5 min), 5 min reperfusion prior to 2 h bilateral hindlimb ischemia, and 2.5 h reperfusion in a porcine model [58] protected against lung dysfunction secondary to limb IRI. RIPC reduced plasma cytokine levels, ameliorated impaired gas exchange and oxygen transport, reduced the elevation in pulmonary arterial pressure and vascular resistance, reduced pulmonary edema, and decreased lung tissue myeloperoxidase activity. During surgery of the lower limbs, there is release of cytokines after prolonged limb ischemia leading to acute respiratory distress syndrome and limb preconditioning could protect against lung dysfunction found with acute respiratory distress syndrome. (2) RIPC by left femoral artery occlusion (3 × 5 min) prior to lung IRI (90 min ischemia, 5 h reperfusion) [59] in a porcine model reversed the detrimental effects of lung IRI on lung function and attenuated pulmonary hypertension and impaired gas exchange. Thus, both models of RIPC

TABLE 5

Studies on RIPC by Brief Limb Ischemia

Author/reference	Site of preconditioning stimulus	Site of index ischemia	Model	Endpoint	Organ protection	Proposed mechanism
RIPC by limb ischemia						
Oxman *et al.* [38]	Hindlimb	Cardiac ischemia	Rat	Reperfusion arrhythmias	Decreased arrythmias	Norepinephrine
Birnbaum *et al.* [39]	Gastroenemius and reduced flow	Cardiac ischemia	Rabbit	Infarct size	Reduced infarct size	Not identified
Liauw *et al.* [54]	Gracilis of ipsilateral limb	Gracilis of contralateral limb	Rat	Muscle necrosis	Reduced by 60%	Not investigated
Kharbanda *et al.* [20]	Hindlimb	Cardiac ischemia	Pig	Infarct size	Reduced infarct size	Suggested involvement of adrenergic pathway and blockade by reserpine.
Gunaydin *et al.* [42]	Upper limb	Cardiac ischemia (Cardiopulmonary bypass)	Human	LDH, CK release	Not conclusive	Enhanced anaerobic glycolysis
Xia *et al.* [57]	Iliac artery	Cardiac ischemia	Sheep	Pulmonary function and pulmonary vascular resistance	Lung protection and decreased pulmonary vascular resistance	KATP channel–pulmonary vascular dilatation and myocardial protection
Kharbanda *et al.*[20]	Upper limb	Upper extremity (contralateral arm)	Human	Endothelial dysfunction	Skeletal protection	Not identified
Addison *et al.* [49]	Hindlimb	Global skeletal protection	Pig	Ischemic necrosis	Skeletal protection	Opioid delta-1 receptors
Kuntscher *et al.* [48]	Hindlimb	Adipocutaneous flaps	Rat	Flap necrosis	Protection	NO
Kuntscher *et al.* [52]	Hindlimb	Rat cremasteric muscle flaps	Rat	Flap necrosis	Protection	NO
Kuntscher *et al.* [50]	Hindlimb	Adipocutaneous flaps	Rat	Flap necrosis	Flap protection	NO
Kuntscher *et al.* [53]	Hindlimb	Flap ischemia	Rat	Flap necrosis	Flap protection	?
Liem *et al.* [141]	Hindlimb		Rat		Skeletal muscle protection	?Mechanism
Moses *et al.* [47]	Hindlimb		Rat	Flap necrosis	Skeletal muscle protection	KATP channels (early phase)
Wang *et al.* [55]	Hindlimb, femoral A	Cremaster flap	Rat	Flap necrosis	Reduced flap necrosis	?Mechanism
Weinbrenner *et al.* [78]	Infrarenal	Cardiac ischemia	Rat	Infarct size	Reduced infarct size	Opioid receptors and PKC; humoral pathway.
Harkin *et al.* [58]	Hindlimb	Hindlimb reperfusion injury	Porcine	Pulmonary artery pressure. PO_2(alv)-PO_2(art) Lung MPO activity and weight	Lung protection	Decreased circulating IL-6, circulating primed phagocytes, and pulmonary neutrophil infiltration
Li *et al.* [44]	Hindlimb	Cardiac ischemia	Mice	Infarct size	Reduced infarct size Delayed protection	NFkB and iNOS
Konstantinov *et al.* [46]	Hindlimb	Cardiac ischemia in transplanted heart	Rat	Infarct size	Reduced infarct size	KATP channels modulation in recipient
Konstantinov *et al.* [40]	Hindlimb	Cardiac ischemia	Mouse	Infarct size	Reduced infarct size	Inflammatory gene suppression

TABLE 5

Continued

Author/reference	Site of preconditioning stimulus	Site of index ischemia	Model	Endpoint	Organ protection	Proposed mechanism
Chen *et al.* [75]	Hindlimb	Cardiac ischemia	Rat	Infarct size	Reduced infarct size	NO
Moses *et al.* [142]	Hindlimb	Skeletal muscle	Rat	Infarction	Reduced skeletal infarction	KATP, late phase
Loukogeorgakis *et al.* [74]	Hindlimb	Endothelial limb injury	Humans	Flow mediated dilatation	Endothelial function Protected	Autonomic nervous system
Chen *et al.* [76]	Hindlimb	Cardiac ischemia	Rat	Infarct size	Reduced infarct size	Free radicals
Waldow *et al.* [59]	Hindlimb	Lung IRI	Pigs	Lung functions	Protection of lung functions	Cytokine IL-6
Kristiansen *et al.* [45]	Hindlimb	Cardiac ischemia in explanted heart	Rat	Infarct size	Reduced infarct size	KATP channels
Zhang *et al.* [122]	Hindlimb	Cardiac ischemia	Rat	Infarct size	Reduced infarct size	Opioid receptors and KATP channels
Dave (2006)	Hindlimb	Brain ischemia	Rat	Neuronal damage	Increased neuroprotection	Not investigated
Kanoria *et al.* [77]	Hindlimb	Hepatic ischemia	Rat	Liver function hepatic blood flow	Reduced IRI Improved liver function	?Adenoisne, NO
Lai *et al.* [136]	Hindlimb	Hepatic ischemia	Rat	Liver function	Reduced IRI, improved liver function	Hemoxygenase (HO-1)
Cheung *et al.* [73]	Hindlimb	Cardiac ischemia	Human	Trop I Lung function Cytokines	Reduced Trop I Improved lung function	Reduced cytokine levels

were effective in protecting the lung. Harkin *et al.* demonstrated that IPC of the hindlimb prior to limb IR injury reduced cytokines (IL-1 and IL-1β), and activated neutrophils, systemic inflammatory response syndrome, and lung dysfunction [58]. In contrast, Waldow's experimental model demonstrated that RIPC (hindlimb ischemia) protects the lung from acute IR injury but does not modulate all indices of systemic inflammation (IL-6, ROS, and activated granulocytes were not modulated by RIPC) [59]. (3) Xia *et al.* [57] showed that RIPC by three episodes of 5 min occlusion and 5 min reperfusion of the iliac artery preserved lung function and prevented a rise in pulmonary vascular resistance as well as arterial pressure following myocardial reperfusion injury. This has important clinical applications in cardiac surgery as coronary IR has deleterious effects on the lung, which is a major cause of mortality in these patients. In beating heart surgery it is not possible to directly clamp the aorta and therefore direct cardiac preconditioning is not possible. Under such circumstances preconditioning from a remote organ such as the limb may be useful as shown by Xia *et al.* [57]. (4) RIPC by femoral artery occlusion (30 min), reperfusion (15 min) and (30 min) occlusion, 48 h (reperfusion) [56] prior to IR (30 min brain ischemia by carotid artery occlusion and 48 h reperfusion) reduced brain edema and circulating endotheliocytes and improved blood flow, demonstrating ischemic adaptation in the early and late phase of IRI.

Most models of limb preconditioning supported the effectiveness of multiple brief cycles of limb ischemia followed by reperfusion; conversely, Weinbrenner *et al.* showed a single cycle to be more effective than multiple cycles and prolongation of length of the single cycle led to more effective preconditioning [41].

The Protective Effect of Brief Hepatic Ischemia and Myocardial Ischemia on Remote Organs (Table 6)

Four studies showed the protective effects of brief myocardial and hepatic ischemia on remote organs [17, 24, 60, 61]. Both brief hepatic and myocardial ischemia have protective effects on the stomach [60, 61]. In an experimental rat model, Brzozowski *et al.* showed that two 5 min episodes of hepatic/myocardial ischemia followed by 10 min of reperfusion each was as effective as direct gastric preconditioning in reducing gastric erosions as well as increasing gastric blood flow following sustained gastric IR. Ates *et al.* demonstrated in a rat model that brief hepatic ischemia (10 min) prior to 45 min of ischemia in the left kidney was associated with better creatinine clearance as well as improved sodium fractional excretion 24 h after preconditioning. They showed reduced mitochondrial swelling, basement membrane detachment on electron microscopy, reduced renal tubular swelling, necrosis, tumor necrosis factor-alpha (TNF-α) levels, decreased lipid peroxidation (TBARS levels) [24], and a relatively rapid decline in LDH levels in the remote preconditioned group as compared to IRI group. This study provides evidence of preservation of ultrastructural,

TABLE 6

Studies on RIPC by Brief Hepatic Ischemia

Preconditioning by hepatic ischemia						
Ates *et al.* [24]	Brief hepatic ischemia	Renal ischemia	Rat	Renal blood flow and creatinine clearance	Decreased renal ischemia	Adenosine, Bradykinin, KATP, possible neuronal reflex
Peralta *et al.* (2003)	Hepatic ischemia	Kidneys, lungs, and other organs	Rat	Organ failure	Protection of remote organs	Inhibition of TNF-alpha-induced selectin up-regulation
Brzozowski *et al.* [53]	Hepatic/ cardiac	Gastric ischemia reperfusion injury	Rat	Gastric lesions and blood flow	Protection of the stomach	Cyclo-oxygenase 1 and cyclo-oxygenase-2, sensory nerve activation and release of CGRP, No, inhibition of IL-1 and TnF alpha
Brzozowski *et al.* [54]	Hepatic/ cardiac	Gastric ischemia	Rat	Gastric lesions	Protection	Brain gut axis, sensory and vagal activation, CGRP release.

histopathological, and biochemical renal function in the RIPC group and supports data for beneficial effects of brief hepatic ischemia on remote tissues.

Does Ischemic Preconditioning Tolerance Induce Cross Tolerance to RIPC?

In an experimental rat model Liem *et al.* demonstrated that two cycles of CAO of 15 min ischemia followed by 15 min reperfusion preconditioned the myocardium prior to 60 min CAO and reduced infarct size, which was abrogated by 8-sulfophenyl theophylline (adenosine receptor blockade) prior to IPC, suggesting the role of adenosine in IPC. However, four cycles of 15 min CAO followed by 15 min reperfusion rendered the myocardium tolerant to IPC and subsequent preconditioning was ineffective in reducing myocardial infarct size [62] due to depletion of cardiac interstitial adenosine. However, RIPC with two cycles of MAO 15 min in tolerant myocardium was effective in reducing myocardial infarct size, suggesting alternate signaling pathways. This data suggests that repetitive brief ischemia of same duration may render tissue tolerant to preconditioning; however, in the clinical setting patients with unstable angina are unlikely to develop tolerance to adenosine since angina is of varying severity and duration. However, exogenous adenosine is effective in ameliorating IRI even in those who develop tolerance, as shown by Liem *et al.,* and RIPC may be useful in situations where direct preconditioning is ineffective due to adenosine tolerance.

Remote Ischemic Preconditioning of Trauma (Fig. 2 and Table 7)

The role of remote trauma in preconditioning has been addressed in a recent study by Ren *et al.* [63]. They demonstrated that carotid artery catheterization (remote nonischemic vascular surgical trauma) aggravates myocardial ischemia; however, abdominal incision (remote nonischemic nonvascular surgical trauma) reduces myocardial infarct size following cardiac IR. This effect is more in the early phase of remote preconditioning (80% reduction in infarct size) and less in the late phase of preconditioning (40% reduction in infarct size). Remote preconditioning of trauma (RPCT), unlike ischemic preconditioning or remote ischemic preconditioning, does not involve ischemic insults to initiate preconditioning in remote organs. There is both an early and a late preconditioning phase in RPCT. The underlying mechanism of RPCT is unclear. RPCT further reduces myocardial infarct size in TNF-α knockouts, supporting the argument

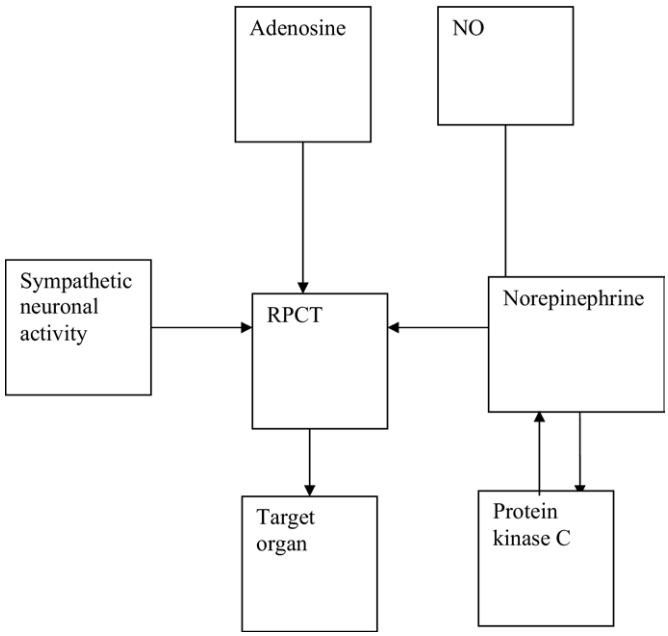

FIG. 2. Remote preconditioning of trauma.

TABLE 7

Studies on Remote Preconditioning of Trauma

Author/reference	Site of preconditioning stimulus	Site of index ischemia	Model	Endpoint	Organ protection	Proposed mechanism
Remote preconditioning of trauma						
Ren *et al.* [63]	1. RPCT (Vascular, ischemia) 2. RPCT (Nonvascular, non-ischemic) 3. Direct IPC	Cardiac ischemia	Mouse	Infarct size	1. Increased infarct 2. Reduced infarct 3. Reduced infarct	1. TNF-α 2. ?Adenosine, NO, catechol 3. TNF-α

that TNF-α does not mediate remote preconditioning of trauma [63]. The role of adenosine in RIPC of the heart and noradrenaline in IPC has been shown. Therefore adenosine activity, sympathetic neuronal activity, and catecholamines are potential mediators of RPCT. Since norepinephrine is involved in cross-signaling with protein kinase C (PKC) [64], nitric oxide (NO) [65] modulates release of norepinephrine from skeletal muscle in ischemia and both PKC and NO play a role in remote preconditioning. One may speculate the role of catecholamines, PKC, and NO pathways in RPCT. Further studies in animal models using sympathetic blockade, knockouts of NO and PKC, and ganglion blockade prior to RPCT are needed to pinpoint the candidate mechanism and elucidate the pathway of signal transmission.

Transferred Preconditioning (Table 8)

Ischemic preconditioning of the heart followed by transfer of coronary effluent from the preconditioned heart to the recipient heart proved beneficial in protecting the recipient heart from ischemia reperfusion injury [66–69]. The mechanism of transferred preconditioning was via opioids such as metencephalins and not due to epinephrine or adenosine release into the effluent. Naloxone blocked opioid receptors and abrogated the beneficial effect of transfer of coronary effluent from the preconditioned heart.

Coronary effluent from preconditioned hearts is also effective in preconditioning segments of jejunum via opioid receptors and K_{ATP} channels [66]. Mesenteric ischemic tolerance induced by pretreatment of small bowel segments with coronary effluent leads to quicker recovery of contractile function of small bowel following reoxygenation and reperfusion.

Remote Microvascular Preconditioning (Table 9)

Mechanisms and the Role of Candidate Compounds in the Process of Remote Ischemic Preconditioning

Adenosine, NO, TNF-α, opioids, bradykinins, PKC, calcitonin gene related peptide (CGRP), cyclo-oxygenase, K_{ATP} channels, capsaicin, heat shock proteins, and norepinephrine are all involved in the mechanism of remote ischemic preconditioning. These substances are released as a response to stress and act via the neuronal or humoral pathway to produce organ protection. The path-

TABLE 8

Studies on Transferred Preconditioning

Author/reference	Site of preconditioning stimulus	Site of index ischemia	Model	Endpoint	Organ protection	Proposed mechanism
Transferred preconditioning						
Dickson *et al.* [67]	Coronary effluent of donor following cardiac ischemia	Cardiac ischemia	Rabbit	Infarct size		Opioid receptors
Dickson *et al.* [69]	Whole blood transfusion following cardiac and renal ischemia	Cardiac ischemia	Rabbit	Infarct size	Reduced infarct	Opioid receptors and KATP channel
Dickson *et al.* [68]	Coronary effluent following cardiac ischemia	Cardiac ischemia	Rabbit	Infarct size	Reduced infarct	Metenkephalins
Dickson *et al.* [66]	Coronary effluent following cardiac ischemia	Mesenteric ischemic tolerance	Rabbit	Jejunal peristalsis	Protection of intestine Yes	Metenkephalins

TABLE 9

Studies on Remote Microvascular Preconditioning

Author/reference	Site of preconditioning stimulus	Site of response	Model	Endpoint	Organ protection	Proposed mechanism
Remote microvascular preconditioning Mabanta *et al.* [129]	Proximal microvascular network	Distal microvascular network	Microvascular network	Local response	Microvascular	KATP channels

ways involved are different in response to different ischemic stimuli and often overlap. The evidence for the role of each of these compounds and the mechanisms by which they act and the pathways involved are reviewed.

Direct Preconditioning and Evidence for Different Phases of the Adaptive Response (Fig. 3)

In direct IPC there is evidence for different phases of ischemic adaptation and protection [70]. These phases were first identified in the heart and subsequently in other organs. In the heart the early phase protects against infarction but not against myocardial dyskinesia [70], whereas the late phase protects against both. The early phase begins soon after reperfusion and lasts for up to 3 h in ischemic preconditioning, whereas the late phase starts 12–24 h later [71]. The early phase is independent of protein synthesis and is due to release of endogenous substances, which stimulate posttranslational modifications in proteins, whereas the late phase is stimulated by release of endogenous substances, which lead to synthesis of new proteins and altered gene expression. This is referred to as the second window of protection [72]. The effects of the acute phase are short lived, lasting for 3–4 h, whereas the effects of the delayed phase are longer, lasting for 48–96 h or sometimes for weeks [25, 27, 28].

The protective effect of the delayed phase of preconditioning is less compared to the early phase and mechanistically different [70].

Does RIPC Confer Early and Late Protection?

Eight studies have shown two phases of protection (ischemic adaptation) in RIPC [27, 29, 30, 36, 38]. These studies showed the existence of both phases in all organ systems in animal models and in recent human studies. (1) Two studies [25, 27, 28] showed brief MAO-induced early and delayed preconditioning in the heart. (2) Li *et al.* showed a pronounced reduction in myocardial infarct size 24 h after hindlimb RIPC [44]. (3) Four studies demonstrated late RIPC in remote skeletal muscle following skeletal muscle preconditioning [51–53]. (4) One study showed ischemic adaptation of the brain to hindlimb ischemia after 24 h of preconditioning [56]. (5) One study showed late phase of RIPC in the heart and liver due to heat shock protein (HSP) expression. (6) The first clinical application of RIPC in children undergoing cardiac surgery on bypass showed that RIPC was most effective in myocardial protection if applied 24 h prior to the coronary ischemic insult [73].

Evidence for the Pathway of Transduction of Remote Protection

Four studies [25–28] in renal preconditioning and four studies [30, 32, 33, 55] in mesenteric precondition-

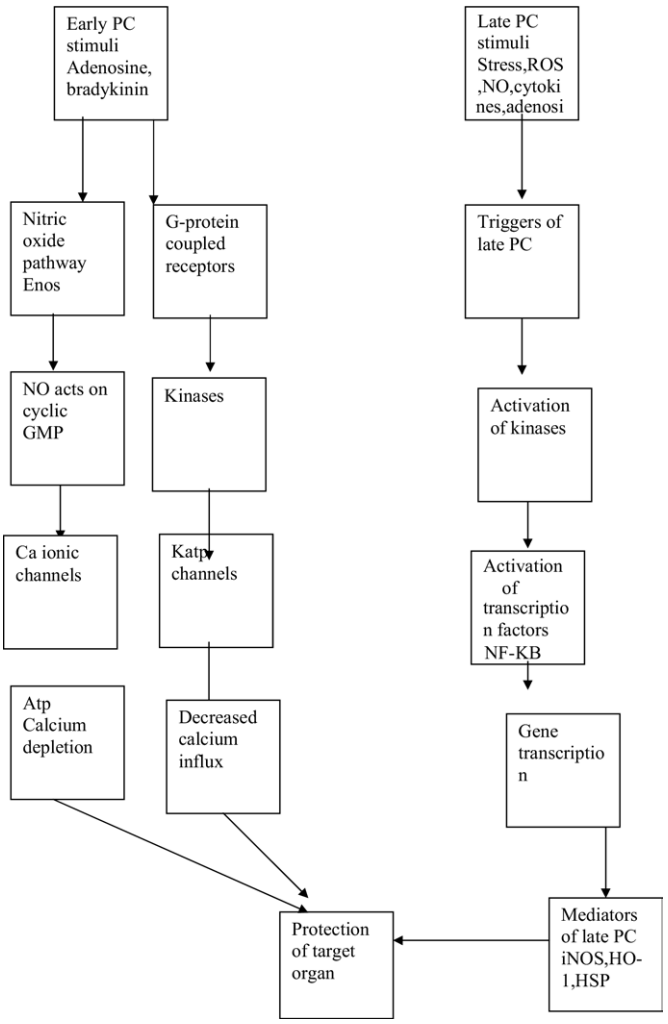

FIG. 3. Phases of preconditioning.

ing showed that remote organ protection was associated with brief periods of reperfusion of the remote organ; however, persistent ischemia without reperfusion did not protect remote organs. Liem *et al.* [32] showed that brief MAO and reperfusion conferred myocardial protection demonstrating that reperfusion of virgin intestine was necessary for activation of the neurogenic pathway. Subsequent permanent MAO reduced infarct size significantly more than brief MAO alone, suggesting that brief periods of MAO are cardioprotective but not optimal as their effect was enhanced by further permanent occlusion. Four studies demonstrated the role of the neurogenic pathway in transduction of RIPC from the limb. In an animal model, Oxman *et al.* [38] showed rise in plasma catecholamine levels on preconditioning, which was abolished by autonomic nerve blockade with reserpine. Loukogeorgakis *et al.* showed the role of the autonomic nervous system in transduction of the protective signal. However, they were unable to define the specific component of the autonomic system [74]. Birnbaum *et al.* demonstrated RIPC by electrical stimulation of gastroenemius muscle in conjunction with brief limb ischemia in a rabbit model [39] and Kharbanda showed RIPC from the limb was abolished by sympathetic nerve blockade with reserpine [20]. In contrast six studies demonstrated rises in plasma levels of nitrates, opioids, free radicals, and catecholamines [36, 38, 41, 49, 75–77] following RIPC, providing evidence in support of a humoral pathway. In Weinbrenner's study [41], the protective effect was only seen in groups who had a period of reperfusion after ischemia in comparison to those who had no reperfusion after ischemia [25, 78] and simultaneous aortic occlusion along with coronary occlusion did not confer protection, indicating that preconditioning had to be prior to allow for the substance released to reach the heart. These findings demonstrate the release of protective substances into the circulation. Autonomic nerve blockade with hexamethonium did not abolish RIPC [75]. Two studies showed cardioprotection in a rat cardiac transplant model (denervated heart), suggesting the role of bloodborne factors in preconditioning [45, 46]. Whether the neurogenic pathway was activated locally in the mesenteric, renal, or skeletal beds or by release of mediators into the circulation (humoral pathway) and subsequent stimulation of sensory afferent fibers is unclear from these studies. The need for reperfusion to confer remote protection and rise in plasma levels of catecholamines, adenosine, neuropeptides, cytokines, or free radicals suggests that these substances may activate neuronal pathways after release into circulation. It seems that both the neurogenic pathway and the humoral pathway have some element of overlap and are not mutually exclusive. Measurement of interstitial levels of mediators and sensory afferent nerve activity prior to and after preconditioning and specific blockade of synthesis and release of the mediators would help clarify their role in activation of neuronal pathways. Blockade of neuronal pathways and end receptors would help define the predominant pathway for transduction of preconditioning in different organ systems.

Role of Nitric Oxide

Studies in direct IPC have demonstrated the protective effect of NO on microcirculation [14, 79–90]. NO is a free radical produced from L-arginine by the enzyme NO synthase, which has three forms: endogenous NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS) [82, 83]. Of these, eNOS and nNOS are constitutively expressed, while iNOS is produced in response to cytokines and NF-κB. eNOS has a protective effect on microcirculation and always produces NO in small amounts, which predominates in the circulation, producing a protective effect on the microcirculation.

Evidence in Remote Ischemic Preconditioning (Table 10, Studies 1–10)

Six studies have demonstrated the role of NO [48, 50–53, 56] in remote preconditioning of skeletal muscle, intestine, brain, and heart. These studies showed hindlimb RIPC induced protection of muscle flaps in the early and late phase of RIPC [36, 51, 52], induction of myocardial iNOS following mesenteric RIPC, and reduction in myocardial infarct size as well as MPO activity (marker of neutrophilic activation). Blockade of NO activity abrogated the decrease in myocardial infarct size and MPO activity, suggesting that NO inhibits neutrophil infiltration. Tokuno *et al.* showed that spontaneous brain ischemic events 24–48 h prior to cardiac IR injury reduced myocardial infarct size [23, 49] with loss of RIPC in iNOS knock out mice. Vlasov *et al.* [56] showed recovery of brain blood flow at 120 min after reperfusion and reduced cerebral edema in the late phase of RIPC (hindlimb) due to iNOS-induced cerebral ischemic adaptation. A recent study has shown expression of NF-κB due to brief limb ischemia and NF-κB-induced iNOS 24 h later, which reduced myocardial infarct size [44] with loss of protection in iNOS knockout mice. This observation is supported from studies of direct IPC that have demonstrated that cytokines induce iNOS production. Conversely Petrischev showed that NO is not involved in RIPC [43] since the nonspecific NO blocker L-NNA did not abolish increase in nicotinamide adenine dinucleotide phosphate activity in intact cardiomyocytes following limb/mesenteric remote ischemia [43] or block attenuation of decrease in nicotinamide adenine dinucleotide phosphate activity in ischemic tissue by preconditioning. These studies demonstrate the role of NO in acute RIPC of the heart and muscle but its role in

TABLE 10

Studies on Mechanisms in RIPC

	Proposed mechanism	Site of index ischemia	Site of preconditioning stimulus	Author/reference	Model	Endpoint	Organ protection
1	iNOS expression (humoral mechanism)	Hindlimb (right) cremaster flap	Hindlimb (femoral artery)	Wang *et al.* [55]	Rat	Flap necrosis	Flap protection
2	iNOS and NO	Cardiac ischemia	Cerebral ischemia	Tokuno *et al.* [23]	Mouse isolated heart	Infarct size	Reduced infarct size
3	NFkB and iNOS	Cardiac ischemia	Hindlimb	Li *et al.* [44]	Mice	Infarct size	Reduced infarct size Delayed protection
4	NO	Adipocutaneous flaps	Hindlimb	Kuntscher *et al.* [48]	Rat	Flap necrosis	Protection
5	NO	Adipocutaneous flaps	Hindlimb	Kuntscher *et al.* [50]	Rat	Flap necrosis	Flap protection
6	NO	Cardiac ischemia	Hindlimb	Chen *et al.* [75]	Rat	Infarct size	Reduced infarct size
7	NO	Rat cremasteric muscle flaps	Hindlimb	Kuntscher *et al.* [52]	Rat	Flap necrosis	Protection
8	NO in direct preconditioning but not in RIPC	Heart and intestine	Intestinal ischemia	Vlasov *et al.* [56]	Rat	Infarct size	No myocardial protection Intestinal adaptation
9	Role for iNOS	Cardiac ischemia	Mesenteric	Wang *et al.* [36]	Rat	Infarct size	Reduced infarct size
10	NO unlikely pathway	Cardiac ischemia	Direct cardiac and remote mesenteric ischemia	Petrishcev *et al.* [43]	Rats	Infarct size	Reduced infarct size
11	Increased interstitial adenosine levels; afferent autonomic nerve stimulation, activation of myocardial adenosine receptors	Cardiac ischemia	Mesenteric ischemia	Liem *et al.* [32]	Rat	Infarct size	Reduced infarct size
12	Adenosine receptors and sarcolemmal KATP channels	Cardiac ischemia	Renal	Pell *et al.* [27]	Rabbit	Infarct size	Reduced infarct size
13	Adenosine	Cardiac ischemia	Renal	Takaoka *et al.* [25]	Rabbit	Infarct size	Reduced infarct size
14	Adenosine	Cardiac ischemia	RPCT	Ren *et al.* [63]	Mouse	Infarct size	Reduced infarct size
15	Angiotensin receptors	Cardiac ischemia	Renal	Singh *et al.* [28]	Rat	Infarct size	Reduced infarct size
16	Autonomic nervous system	Endothelial limb injury	Hindlimb	Loukogeorgakis *et al.* [74]	Humans	Flow-mediated dilatation	Endothelial function protected
17	Neurogenic, humoral	Cardiac ischemia	Mesenteric and renal	Gho *et al.* [26]	Rat	Infarct size	Reduced infarct size
18	Bradykinin-mediated and neuronal pathway	Cardiac ischemia	Mesenteric ischemia	Schoemaker *et al.* [29]	Rat	Infarct size	Reduced infarct size
19	Brain gut axis, sensory and vagal activation, CGRP release	Gastric ischemia	Hepatic/cardiac	Brzozowski *et al.* [54]	Rat	Gastric lesions	Protection
20	Calcitonin gene-related peptide	Cardiac ischemia	Mesenteric	Wolfrum *et al.* [31]	Rat	Infarct size	Reduced infarct size
21	Capsaicin sensitive sensory nerve and NOS	Cardiac ischemia	Mesenteric	Xiao *et al.* [34]	Rat	Infarct size	Reduced infarct size
22	Capsaicin-sensitive sensory nerves	Cardiac ischemia	Mesenteric	Tang *et al.* [35]	Rat	Infarct size	Reduced infarct size
23	Cyclo-oxygenase 1 and cyclo-oxygenase-2, sensory nerve activation and release of CGRP, NO, inhibition of IL-1 and TNF-α	Gastric ischemia reperfusion injury	Hepatic/cardiac	Brzozowski *et al.* [53]	Rat	Gastric lesions and blood flow	Protection of the stomach

TABLE 10

Continued

	Proposed mechanism	Site of index ischemia	Site of preconditioning stimulus	Author/reference	Model	Endpoint	Organ protection
24	Cytokine IL-6	Lung IRI	Hindlimb	Waldow *et al.* [59]	Pigs	Lung functions	Protection of lung functions
25	Inhibition of TnF-α induced selectin up regulation	Kidneys, lungs, and other organs	Hepatic ischemia	Peralta *et al.* (2003)	Rat	Organ failure	Protection of remote organs
26	Enhanced anaerobic glycolysis	Cardiopulmonary bypass	Upper limb	Gunaydin *et al.* [42]	Human	LDH, CK release	Not conclusive
27	Free radicals	Cardiac ischemia	Hindlimb	Chen *et al.* [76]	Rat	Infarct size	Reduced infarct size
28	Heat shock proteins	Remote myocardial preconditioning	Cardiac ischemia	Tanaka *et al.* [131]	Rabbit	Infarct size	Reduced infarct size
29	Inflammatory gene suppression	Cardiac ischemia	Hindlimb	Konstantinov [40]	Mouse	Infarct size	Reduced infarct size
30	Inflammatory gene suppression	Cardiac ischemia	Mesenteric ischemia	Huda *et al.* [140]	Rat	Infarct size	Reduced infarct size
31	K ATP channels	Cardiac ischemia in explanted heart	Hindlimb	Kristiansen *et al.* [45]	Rat	Infarct size	Reduced infarct size
32	K ATP, Late phase	Skeletal muscle	Hindlimb	Moses (2005)	Rat	Infarction	Reduced
33	KATP channel–pulmonary vascular dilatation and myocardial protection	Cardiac ischemia	Iliac artery	Xia *et al.* [57]	Sheep	Pulmonary function and pulmonary vascular resistance	Lung protection and decreased pulmonary vascular resistance
34	KATP channels modulation in recipient	Cardiac ischemia in transplanted heart	Hindlimb	Konstantinov [46]	Rat	Infarct size	Reduced infarct size
35	KATP channels (early phase)		Hindlimb	Moses *et al.* [47]	Rat	Flap necrosis	Skeletal muscle protection
36	Ventricular pacing by activation of KATP channels	Cardiac ischemia	Mesenteric ischemia, renal ischemia, and ventricular pacing	Verdouw *et al.* [139]	Pig	Infarct size	Reduced infarct size
37	Myocardial PKC activation	Cardiac ischemia	Mesenteric	Wolfrum *et al.* [30]	Rat	Infarct size	Reduced infarct size
38	PKC and mitochondrial KATP channels	Cardiac ischemia	Mesenteric ischemia	Wang *et al.* (2002)	Rat	Infarct size	Reduced infarct size
39	Norepinephrine	Cardiac ischemia	Hindlimb	Oxman *et al.* [38]	Rat	Reperfusion, arrythmias	Decreased arrythmias
40	Norepinephrine	Cardiac ischemia	Cerebral ischemia	de Zeeuw *et al.* [119]	Pig	Infarct size	No change in infarct size
41	Suggested adrenergic Pathway and blockade by reserpine.	Cardiac ischemia	Hindlimb	Kharbanda *et al.* [20]	Pig	Infarct size	Reduced infarct size
42	Opioid delta 1 receptors	Global skeletal protection	Hindlimb	Addison *et al.* [47]	Pig	Ischemic necrosis	Skeletal protection
43	Opioid receptors	Cardiac ischemia	Coronary effluent of donor following cardiac ischemia	Dickson *et al.* [67]	Rabbit	Infarct size	Reduced infarct size
44	Opioid receptors and PKC; humoral pathway	Cardiac ischemia	Infrarenal	Weinbrenner *et al.* [78]	Rat	Infarct size	Reduced infarct size
45	Metenkephalins	Cardiac ischemia	Coronary effluent following cardiac ischemia	Dickson *et al.* [68]	Rabbit	Infarct size	Reduced infarct
46	Endogenous opioids	Cardiac ischemia	Mesenteric	Patel *et al.* [33]	Rat	Infarct size	Reduced infarct size
47	Hemoxygenase	Liver ischemia	Hindlimb ischemia	Lai *et al.* [136]	Rat	Liver function	Improved liver function

acute preconditioning of the intestine and brain remains unproven. IR injury impairs endothelial function in the initial phase primarily by impairment of formation and bioavailability of NO, which may explain the lack of effect of the early phase of RIPC in the brain and intestine. This may also be explained by different

protocols of preconditioning used for remote preconditioning in different organs and different criteria for assessment of preconditioning in the heart and muscle (cytoprotective effects) as against endothelial function in the intestine and brain. Therefore similar outcome measures are needed to investigate the role of NO.

What Induces NO?

In a mouse model increased NF-κB expression in the limb following RIPC induced NF-κB and iNOS in the heart, suggesting the role of cytokines in the signaling pathway for induction of NO [44]. In a rat model, brief hepatic ischemia increased gastric CGRP, increased mucosal flow [34], and reduced gastric mucosal erosions and this effect was abolished by L-NAME (NO blocker), suggesting that CGRP induces NO release and subsequent vasodilatory effects and increased flow. Evidence suggests release of ROS, cytokines, and NO into the venous effluent following intestinal ischemia reperfusion [91] activates NF-κB and PKC [92]. These signaling pathways induce iNOS [93] in the target organ. Peralta has shown adenosine increases NO formation and this effect is blocked by adenosine antagonists [94].

Pathway

The data from the studies discussed show that brief periods of ischemia reperfusion in RIPC induce NO pathways. Li *et al.* showed that RIPC by hindlimb ischemia increased NF-κB in skeletal muscle and heart and induced ischemic adaptation in the heart by iNOS formation [44]. In view of the short half-life of NO (5 s), it is unlikely for NO to be produced in the remote organ and reach the target organ to confer protection by the blood stream. Chen *et al.* [75] showed that RIPC of the limb reduced myocardial infarct size through NO production. Since hexamethonium (autonomic ganglion blocker) did not abrogate reduction in myocardial infarct size, it seems that NO pathways act through the bloodstream. In an animal model of warm hepatic IRI, Kanoria's group showed increased hepatic venous plasma nitrates/nitrites [77] and amelioration of hepatic IR following hindlimb RIPC. This provides further support for the argument in favor of the bloodstream being the NO pathway. Clearly, further clarification of this is needed by measuring systemic venous plasma nitrate/nitrite levels and plasma arginine levels (NO precursors) after RIPC. Also, knockout models of NOS would be specific for investigating the role of NO pathways in preconditioning.

Is NO a Trigger or Mediator?

Tokuno *et al.* demonstrated reduced myocardial infarct size following induced brief brain ischemia without an increase in cardiac iNOS; however; the protection was abolished in NOS knockouts, suggesting the role of NO as a trigger [23]. Blockade of NO prior to RIPC by L-NAME abolished myocardial protection, supporting the role of NO as a trigger [75]. Increased iNOS production 24 h after RIPC would suggest its role as a mediator and future studies by blocking NO after RIPC are needed to demonstrate its role as a mediator.

Effector Mechanism

IPC studies have demonstrated that NO modulates microvascular perfusion through its vasodilatory effect [95] and through its anti-inflammatory actions, including inhibition of stellate cell activation [96], neutrophil adhesion [88, 90, 97], and platelet aggregation. NO plays a key role in initiating and maintaining preconditioning. The early phase of preconditioning mediated by eNOS is through generation of cGMP [98], as shown by Lochner *et al.* in the myocardium [99] and subsequently inhibition of cAMP levels as well as reduction in energy demands. The late phase is protein synthesis dependent and is through activation of PKC, NF-κB, and transcription of iNOS [100]. In the liver NO mediates preconditioning by inhibitory actions on endothelin [14], activation of adenosine A2 receptors, and subsequent NO formation [94]. NO also has been shown to confer protection against cold ischemia [15] of liver. NO inhibits apoptosis of cells by inhibition of caspase activity, TNF-α, and up-regulation of Bcl-2 [101–103]. NO has a protective effect on intestinal microcirculation such as scavenging of oxygen free radicals, maintenance of normal vascular permeability, inhibition of smooth muscle proliferation, reduction of leukocyte adherence to the mesenteric endothelium, prevention of mast cell activation, and platelet aggregation [84, 86, 88, 104]. Kubes *et al.* [83] have demonstrated in the intestine that eNOS has a protective effect on intestinal mucosa, while increased iNOS has been shown to increase mucosal apoptosis by generation of free radicals such as peroxynitrate [105, 106]. However recent studies have shown that the production of peroxynitrate may be associated with loss of eNOS rather than increased iNOS production [107]. This observation suggests a dichotomous role for NO in IR injury with small quantities of NO produced by eNOS, reducing IRI, while excessive NO due to iNOS, causing deleterious effects.

Adenosine (Table 10, Studies 11–14)

Evidence

Adenosine is an extracellular molecule that is both a trigger and a mediator of IPC as demonstrated from past studies [108]. Adenosine is a hormone widely distributed in human tissues. Adenosine production occurs in myocytes, endothelial cells, and vascular cells. During ischemia of the heart, brain, and the kidney,

the imbalance between oxygen supply and demand results in net breakdown of adenosine triphosphate (ATP) and release of adenosine, which can increase up to 50 fold.

Three studies have shown the role of adenosine in RIPC of the heart by RAO and MAO. Two studies in a rabbit model [25, 27] showed RIPC by RAO (10 min); reperfusion (10 min) prior to CAO reduced myocardial infarct size, and 8-SPT (adenosine blocker) abolished the protective effects, while RIPC by single cycle of MAO (15 min) and reperfusion prior to CAO in a rat model increased plasma adenosine levels and reduced myocardial infarct size.

The following two studies demonstrated the role of adenosine in RIPC of skeletal muscle. Adenosine release has been shown to be an effector molecule in skeletal muscle IPC [109]. (1) RIPC increased adenosine plasma levels and the protective effect was partially blocked with reserpine. (2) Prior adenosine blockade [49] did not completely abolish latissmus dorsi flap protection by RIPC (limb ischemia, 3 $\times$ 10 min cycles); however, adenosine blockade (8-SPT) and free radical scavenger mercaptopropionyl glycine (MPG) completely abolished the RIPC effect, suggesting that adenosine plays a partial role in RIPC.

Trigger or Mediator

In a rabbit model adenosine blockade prior to RIPC abolished cardioprotection, demonstrating its role as a trigger of RIPC [27]. Adenosine blockade after RIPC before reperfusion abolished cardioprotection, suggesting its role as a mediator of RIPC also.

Pathway

Following ischemia-induced ATP breakdown, adenosine crosses the cell membrane and enters into the interstitial space by simple diffusion. From the interstitial space it escapes into the intravascular space by paracellular washout (slow, 10% under physiological conditions). It would seem that in RIPC adenosine produced in the remote organ would reach its target organ by the bloodstream; however, it has a half-life of 0.6–1.5 s as it is rapidly taken up by endothelial cells, RBCs, and pericytes, which contain nucleoside transporters and are responsible for rapid degradation of adenosine [110], making it unlikely for adenosine to reach its target by the circulation. There are no studies in RIPC that demonstrate modulation of adenosine degradation or activation of nucleoside transport inhibitors. Two studies showed a rise in plasma adenosine levels following preconditioning, suggesting that reperfusion and release into circulation is required for adenosine-induced RIPC [25, 27]. One study [32] supported a neurogenic pathway for adenosine in RIPC. They demonstrated abrogation of preconditioning of the heart by brief cycles of MAO and reperfusion due to prior ganglion blockade. To further clarify the pathway, they demonstrated that intramesenteric infusion with adenosine mimicked the effects of brief MAO and prior ganglion blockade abolished the protective effects of RIPC. Ganglion blockade after reperfusion did not abrogate the protective effects but adenosine receptor blockade abolished myocardial protection. These observations suggest that adenosine acts locally to stimulate afferent nerves in the mesenteric bed, which in turn activate adenosine receptors in the heart. This argument is supported by data demonstrating lack of preconditioning following intraportal or intracaval infusion of adenosine and effectively excludes effects of adenosine spillover during mesenteric infusion, which could potentially stimulate adenosine receptors in the liver and contribute to myocardial protection.

Effector Mechanism

8-SPT (adenosine receptor blocker) abolished RIPC by MAO. 8-SPT was given after reocclusion of mesenteric artery [32], preventing adenosine access to the mesenteric bed. This suggested the presence of adenosine receptors on the heart, the effect of which was blocked by 8-SPT.

Does Adenosine Modulate End Effectors K_{ATP} Channels?

In RIPC two studies (RAO reduced myocardial infarct size, rabbit model) [25, 27, 28] demonstrated that selective mitochondrial K_{ATP} channels blockers (5-HD) abolish the protective effect. The action potential of sarcolemmal K_{ATP} channels was unaffected. Modulation of K_{ATP} channels reduced ATP depletion, preserved intracellular pH, and enhanced recovery of ATP and phosphocreatinine levels during reperfusion [25]. Decreased acidosis reduced intracellular Ca accumulation and myocardial infarct size. These observations are supported by IPC studies [109] and suggest that KATP channels serve as end effectors in adenosine pathways.

IPC studies have shown that adenosine mediates both the early and the late phases of preconditioning via different end-organ receptors. In the heart adenosine acts via the A1 receptor. Adenosine receptors mediate anti-adrenergic effects indirectly by reducing C-AMP levels. The A1 receptor-mediated effects involve activation of PKC, tyrosine kinases [111], heat shock protein, and MAPK. Kinases modulate end effector mitochondrial KATP channels. A2 receptors act in the hepatic microcirculation as shown by Peralta *et al.* [94]. A2 receptors are linked to vasodilatation and antiplugging effects of adenosine. Activation of these receptors and their effects are due to endothelium dependent and independent mechanisms, indirectly through release of NO and through direct relaxation of vascular smooth muscles. A3 receptors are found in myocytes.

The effects of adenosine include vasodilatation [110],

inhibition of leukocyte adhesion, neutrophil and platelet function [112], and free radical production. These have to be clearly demonstrated in future studies in RIPC.

Cytokines (Table 10, Studies 3, 24, 25)

NF-κB

Li *et al.* in a mouse model showed RIPC (six cycles brief hind limb ischemia) prior to myocardial IR reduced infarct size. IRI activated NF-κB in hearts but prior RIPC attenuated activation of NF-κB in IR [44]. However RIPC in sham animals demonstrated NF-κB activation in both the limb and hearts. This study demonstrates a dual role for NF-κB. While excessive NF-κB activation in IR injury has deleterious effects and increases infarct size, activation of NF-κB following limb preconditioning led to an adaptive response in the heart, increased iκβ (inhibitory KB) expression, which attenuated NF-κB activation following sustained IR injury and reduced myocardial infarct size, attenuated decrease in left ventricular developed pressures (LVDF) and increases in left ventricular end-diastolic pressures (LVEDP) on reperfusion. In NF-κB and iNOS knockout models, the decrease in LVDF was attenuated but preconditioning did not confer any additional benefit. NF-κB and iNOS knockouts had less severe increase in LVEDP but no additional attenuation was conferred by preconditioning. NF-κB is induced by ROS and subsequently acts through activation of kinases in the preconditioning response. Preconditioning may down-regulate the inflammatory response during reperfusion as NF-κB activation increases its own inhibitor Iκβ (Tahepold) or it may act through a mediator such as iNOS, as suggested by data from this study.

TNF-α

Increases in TNF-α levels following hepatic IR injury led to remote organ injury (lung and kidney) and IPC of the liver reduced both TNF-α levels and remote organ injury as demonstrated by Peralta *et al.*[113]. This study suggested that TNF-α may have a role in remote preconditioning. In RIPC two studies have investigated the role of TNF-α. Ates *et al.* showed raised TNF-α levels following renal IR, which were reduced by preconditioning with brief hepatic ischemia and associated with improved renal function as compared to IR groups [24]. RIPC by brief hepatic ischemia prior to gastric IR (30 min) attenuated plasma TNF-α levels in a rat model [60]. Ren *et al.* showed in a rat model of cardiac IR reduced myocardial infarct size following IPC and in TNF-α knockout mice. The protective effect of the early phase of IPC was not abolished in TNF-α knockouts but that of the late phase of IPC was abolished in knockouts, suggesting that late IPC is TNF-α dependent. Both the early and the late phase of RPCT (abdominal incision prior to cardiac IR) in TNF-α knockouts further reduced myocardial infarct size, suggesting that remote preconditioning of trauma was mechanistically different from TNF-α ablation [63]. Clinical efficacy of cardioprotective strategies maybe maximized by using a combination of RPCT and TNF-α ablation. This study also showed that blockade of NF-κB and TNF-α was additive, suggesting NF-κB to be involved in the signaling pathway of TNF-α. The mechanism of TNF-α-induced RIPC and IR is unclear and future studies are needed to study the mechanism and signaling pathways involved.

IL-6

In lung IR injury secondary to remote limb ischemia reperfusion, blockade of interorgan inflammatory mediators such as cytokine IL-6 and levels of primed neutrophils by prior limb preconditioning confers protection with no significant difference in levels of TNF-α. Conversely lung protection against local IRI by remote brief limb ischemia is mechanistically different and entails incomplete blockade of systemic inflammatory response syndrome mediators such as IL-6 and circulating primed neutrophils and complete suppression of IL-1β, which is an early mediator of reperfusion injury. Therefore, despite incomplete blockade, it is likely that remote preconditioning reduces expression of adhesion molecules and neutrophilic infiltration in the lung.

IL-1β

RIPC has been shown to confer protection on the remote organ in lung dysfunction due to limb IR and local IR by blockade of IL-1β. The role of IL-1β was demonstrated by Harkin *et al.,* who clearly showed an increase in IL-1β in limb IR, which led to lung injury and deterioration of lung function [58]. In sustained limb IR, limb IPC protected lung function from remote IR injury by blocking the cytokine IL-1β. A similar rise in IL-1β was shown by Waldow in IRI of the lung. RIPC of the limb ameliorated the rise in IL-1β and conferred protection on the lung. Thus IL-1β has been shown to have a role as an interorgan mediator of IR injury and as a mediator of local IRI [59].

PKC (Table 10, Studies 37, 38)

Evidence

Wolfrum *et al.* showed that PKC was responsible for reduction in myocardial infarct size after mesenteric ischemia reperfusion and blockade of PKC by a highly selective inhibitor chelerythrine prevented reduction in infarct size [30]. They showed an increase in myocardial PKC following RIPC and blockade of PKC abolished the protective effect. Weinbrenner *et al.* showed RIPC by infrarenal aortic occlusion for 15 min followed

by reperfusion reduced myocardial infarct size, which was abolished by chelerythrine [78].

Pathway of Transduction

There is evidence that myocardial PKC undergoes activation following ischemic stimuli in IPC [114–116]. This leads to conversion of the cytosolic PKC to particulate PKC fraction, thereby increasing the ratio of the particulate to cytosolic fraction. Subsequent activation of mitochondrial PKC receptors results in activation of tyrosine kinases and MAP kinases, which lead to opening up of mitochondrial potassium receptor K_{ATP} channels. Mitochondrial K_{ATP} channels serve as end effectors in modulation of mitochondrial energy flow and preservation of mitochondrial membrane integrity. Both humoral and neuronal pathways have an important role in PKC-mediated RIPC. Wolfrum *et al.* demonstrated increased plasma bradykinin levels following mesenteric ischemic preconditioning, which activated myocardial PKC and blockade of bradykinin receptors with bradykinin antagonist HOE 140 abolished myocardial protection [30]. Ganglion blockade with hexamethonium did not alter the cytosolic to particulate ratio of PKC but prevented activation of PKC. These observations suggest that bradykinin-induced PKC activation is a prerequisite for the cardioprotective effect of RIPC, that activation of PKC is a decisive step in conferring cardioprotection, and that both the bradykinin-dependent humoral pathway as well as the neuronal pathway are essential for PKC activation.

Bradykinin (Table 10, Studies 18, 37)

Evidence

Shoemaker *et al.* [29] were the first to demonstrate that mesenteric preconditioning induced increased endogenous bradykinin levels, which had a remote preconditioning effect on the heart and reduced infarct size following coronary infarction. Blockade of bradykinin receptors was associated with increased myocardial infarct size. However, bradykinin blockade in non-preconditioned animals did not influence infarct size and in the absence of preconditioning there was no change in basal bradykinin levels [117].

What Is the Pathway?

A combined sensory neurogenic and humoral pathway is strongly suggested in the bradykinin-mediated RIPC [29]. Following mesenteric ischemia reperfusion, there is local release of bradykinins, which stimulates the sensory afferent nerves projecting on efferent nerves to the heart, which in turn precondition the heart. Bradykinin receptors B2 are involved in sensory nerve stimulation and bradykinin receptor antagonists HOE-140 (Hoechst-140) abolish the protective effect. This study also showed that bradykinin receptor blockade led to loss of protection in both direct and remote IPC but ganglion blockade abolished protection only in remote preconditioning. Thus direct preconditioning is associated with blood-borne kininogens in contrast to the complementary effect of humoral and neurogenic pathways in RIPC. Bradykinins activate intracellular transduction of PKC as demonstrated by Wolfrum *et al.* [117]. However, the pathway downstream of kinases remains unclear. Although modulation of K_{ATP} channels is suggested, this needs to be clarified in future studies.

Catecholamines (Table 10, Studies 39, 40, 41)

Toombs *et al.* showed IPC by 5 min of CAO, 10 min reperfusion in rabbits prior to 30 min CAO, and 120 min reperfusion reduced infarct size [118]. In reserpinized rabbits subsequent IPC failed to reduce infarct size, suggesting the role of noradrenaline release in IPC. In RIPC two studies provided evidence to support the role of catecholamines. Oxman *et al.* [38] showed an increase in cardiac norepinephrine release and increased baseline plasma levels after limb preconditioning due to a systemic stress response that was partially abolished by reserpine due to depletion of catecholamine stores. In reserpinized animals the antiarrhythmic effect of RIPC was blocked. Kharbanda *et al.* showed that sympathetic blockade abolished RIPC resulting in increased myocardial infarct size [20]. de Zeeuw *et al.* demonstrated that increase in cardiac interstitial norepinephrine levels following ischemia was cardioprotective but RIPC by cerebral ischemia was ineffective due to inadequate increase in norepinephrine levels [119]. The role of catecholamines in RPCT is only speculative and future studies are needed to clarify this.

Pathway and Effector Mechanism

In direct IPC endogenous catecholamines are known to act upon cardiac adenoreceptors, stimulating myocardial protein kinase eventually leading to preconditioning of the heart [120]. In RIPC the likely pathway seems to be the sympathetic nerve pathway as reserpine abolished protection. It is also possible that catecholamines released into the circulation may act on sympathetic nerve endings in target organs; however, this needs to be further clarified by measurement of plasma catecholamine levels and use of adrenergic receptor blockers. The postreceptor mechanisms are unclear and the role of catecholamines in activation of kinases as well as modulation of K_{ATP} channels as end effectors needs to be clearly demonstrated in future experiments.

Opioids (Table 10, Studies 42, 43, 44, 45, 46)

Evidence

IPC of the intestine (three cycles of 8 min ischemia, 10 min reperfusion) prior to intestinal IRI (30 min

MAO, 2 h reperfusion) reduced intestinal injury, edema, LDH, and malionaldehyde levels (markers of oxidative stress) [121]. Pretreatment with morphine mimicked the effects of IPC and naloxone abolished the protective effects of both IPC and morphine [121]. The intestine and colon are rich in opioid receptors and contain opioid peptides [121]. This study demonstrated an increase in endogenous opioid peptides in the effluent collected after IPC and suggested that opioids are released in response to oxidative stress to confer a protective effect against stress.

Opioid release from the stress of brief IR could result in a remote protective mechanism. Five studies have demonstrated the role of opioids in RIPC in intestinal, skeletal muscle, and heart tissue. (1) On the basis of the theory that opioids are released as a natural response to stress, are released irrespective of whichever organ is stressed, and act widely and ubiquitously on remote organs, Patel *et al.* [33] demonstrated in a rat model that RIPC (MAO, 15 min) prior to CAO reduced myocardial infarct size, which was abolished by naloxone. (2) Hindlimb RIPC (3 × 10 min IR cycles) prior to 4 h of muscle flap ischemia and 48 h reperfusion reduced latissmus dorsi, rectus abdominis, and gracilis flap infarct size. This study demonstrated the remote protection of all skeletal tissue by RIPC. RIPC (3 × 5 min IR cycles) prior to 30 min CAO and 120 min reperfusion in a rat model reduced myocardial infarct size and plasma LDH levels [122] and naloxone abolished these effects. Brief infrarenal aortic occlusion (IOA) 15 min prior to prolonged infrarenal aortic occlusion (30 min) protected myocardium [78]. Dickson *et al.* demonstrated a role for opioids in transferred preconditioning. Met and leu-enkephalins were liberated from the preconditioned donor rabbit heart into the coronary effluent, which subsequently elicited protection when given to virgin acceptor hearts. Addition of naloxone to the coronary effluent abolished opioid-induced protection. Based on evidence that opioids induce ischemic tolerance in the intestine in direct IPC and the presence of abundant opioid receptors in the intestine, Dickson *et al.* treated ischemic gut with coronary effluent from preconditioned hearts and showed that the recovery of maximal contractile force of gut after ischemia was enhanced by opioids [66–69].

Pathway

Opioid receptors are known to be present in neuromuscular regions, especially delta receptors [123], and previous studies have described the humoral action of opioid receptors in skeletal muscle by demonstrating [124] that B-endorphins released in the circulation stimulate glucose uptake in muscle. In RIPC (hindlimb) autonomic ganglion blockade by hexamethonium did not abolish the effects of opioids [49], suggesting the blood circulation to be the likely pathway of transmission of protection in opioid-induced RIPC. The need for reperfusion following IOA for cardioprotection and lack of protection in occlusion without reperfusion [78] and the evidence for transferred preconditioning in virgin acceptor hearts by opioids in coronary effluent from preconditioned hearts are observations that support a humoral pathway.

Effector Mechanism

Opioids act on receptors in the target organ. This has been shown in heart, skeletal muscle, and intestinal tissue [33, 49, 122]. Skeletal muscle flap protection by RIPC was abolished by selective δ1 opioid receptor antagonists and myocardial protection was abolished by selective κ1 receptor antagonists. δ1 receptors have been shown in all species and human cardiomyocytes [78]. Activated δ1 opioid receptors induce effects which mimic RIPC. In transferred preconditioning leu-enkephalins activate δ1 opioid receptors to induce ischemic tolerance in myocardium or gut [66].

Opioid-induced RIPC prior to skeletal muscle IRI reduced ATP depletion, lactate accumulation, neutrophil infiltration, and myeloperoxidase activity in preconditioned skeletal muscle [49], which was abolished by opioid receptor antagonists. These observations suggest that the energy-sparing effect coupled with attenuation of lactate accumulation during early reperfusion is triggered by activation of opioid receptors. This is supported by IPC studies ([125].

Do Opioid Receptors Modulate KATP Channels?

One study has shown the role of K_{ATP} channels in the end effector energy sparing effect [66, 122]. Coadministration of Glibenclamide to coronary effluent from preconditioned hearts abolished transferred preconditioning in ischemic gut [66]. RIPC by κ opioid receptors in the heart modulates mitochondrial pore mobility in the post receptor mechanism [122]. IPC studies have shown opioid receptors to act via inhibitory G-protein, activation of multiple kinases with modulation of mitochondrial and sarcolemmal K_{ATP} channels serving as final end effectors [122]. Future studies are needed to clarify postreceptor mechanisms in RIPC and resolve receptor subtypes.

Free Radicals (Table, Studies 10–27)

IPC studies have shown that free radicals can directly activate kinases leading to transcription of protective proteins [92, 126], and free radical scavenger MPG abrogates the protective effects of direct preconditioning. Weinbrenner *et al.* were the first to show that free radicals are key candidate molecules in RIPC [41]. A single cycle of 15 min of infrarenal aortic occlusion followed by 10 min reperfusion reduced myocardial infarct size significantly. MPG blocked the effects

of both RIPC and a single cycle of direct preconditioning but failed to block multiple cycles of direct preconditioning. This suggested that preconditioning is a graded phenomenon with multiple cycles producing a more robust preconditioning stimulus with free radicals being only partially involved in the mechanism of protection. This study did not demonstrate the source of free radicals. Recently, Patwell *et al.* demonstrated the appearance of hydroxyl free radicals in the circulation following IR of the limb [127], and the argument that free radicals may be produced from the ischemic limb in RIPC was supported by Chen *et al.* [128], who showed that myocardial infarction was reduced by four cycles of 10 min femoral artery occlusion-reperfusion associated with an elevation in whole blood free radical counts up to 2 h following RIPC. Since ROS levels were significantly low compared to the IRI group, this data suggest the role of low-dose ROS in inducing preconditioning. In addition, a period of reperfusion for generation and action of free radicals is needed. These findings collectively suggest that free radicals reach the remote organ via the bloodstream to induce a preconditioning effect. Conversely, in an animal model, occlusion of the coronary artery before preconditioning supported a nerve pathway of transmission of the preconditioning effect [128].

Effector Mechanism

Direct IPC studies have shown that ROS led to the release of triggers such as NO, catecholamines, adenosine, and bradykinin. ROS activates intracellular kinases directly [126] and induces synthesis of protective proteins. Also ROS activates cytokine NF-κB, which induces iNOS mRNA transcription 24 h later to confer delayed protection in the target organ. None of these pathways have been clearly demonstrated in RIPC. Chen *et al.* [76] showed that ROS induced elevation of heat shock protein and mitochondrial antioxidant enzymatic activity, which helped maintain mitochondrial function and reduce apoptosis. However, pretreatment with MPG abrogated HSP and antioxidant activity. Further clarification of the end effector mechanism of ROS in RIPC, the source of ROS, pathway, and the role of low-dose ROS in maintenance of mitochondrial membrane permeability as well as prevention of apoptosis is needed. Also quantification of the levels of ROS, which induce protective mechanisms need to be determined since high levels of ROS are the key elements in the initial cascade of IR injury.

K_{ATP} Channels (Table 10, Studies 31–36, 38)

Evidence

Moses *et al.* [47] showed that RIPC (three cycles hindlimb occlusion) reduced latissmus dorsi infarct size, which was abolished by nonselective blockers (glibenclamide) and selective mitochondrial channel blockers (5-HD). Kristiansen *et al.* showed RIPC of the donor heart by hindlimb IR (4 $\times$ 5 min cycles) was abolished by blockade of nonselective and mitochondrial K_{ATP} channels and increased infarct size in donor heart on implantation. Administration of diazoxide (selective mitochondrial k channel activator) prior to explant conferred protective effects similar to RIPC, suggesting that the effects of RIPC are memorized in the explanted hearts and are critically dependent on modulation of mitochondrial K_{ATP} channels [45]. Konstantinov *et al.* showed that blockade of K_{ATP} channels following RIPC in the recipient increased myocardial infarct size in the implanted heart [46]. Dickson *et al.* showed blockade of K_{ATP} channels abolished transferred preconditioning in virgin hearts conferred by coronary effluent from preconditioned hearts [66]. Mabanta *et al.* showed that remote microvascular preconditioning is mediated by K_{ATP} channels and blockade of K_{ATP} channels abolished preconditioning [129]. Pell *et al.* showed renal IR (10 min cycles) reduced myocardial infarct size and cardioprotection was abolished by K_{ATP} blockers (5-HD) [27]. Moses *et al.* showed remote skeletal protection by limb RIPC (3 $\times$ 10 min ischemia) was abolished by 5-HD (selective mitochondrial channel blocker) [47].

Pathway and Effector Mechanism

Two studies have demonstrated the modulation of IRI in denervated hearts in a transplant model; this suggests that the humoral pathway is involved in conferring protection. The role of opioids in transferred preconditioning supports the humoral pathway.

Are These K_{ATP} Channels Triggers of RIPC, Mediators, or End Effectors?

Moses showed blockade of K_{ATP} channels prior to RIPC abolished protection, suggesting their role in the trigger mechanism of RIPC [47]. Blockade of K_{ATP} channels after RIPC abolished protection; however, blockade 10 min prior to ischemia did not influence infarct size, indicating a role of K_{ATP} channels in the mediation of signal transduction. This study demonstrated that the critical time period for K_{ATP} channels to remain open after triggering was 10 min further, supporting a role in mediation of signal transduction. One study has shown the opening of mitochondrial K_{ATP} channels and ionic fluxes across mitochondrial K_{ATP} channels in the opioid pathway of RIPC [122] and one study showed ionic fluxes across K_{ATP} channels in RIPC by the adenosine pathway [27]. These observations suggest that K_{ATP} channels serve as end effectors in the pathway of RIPC and their role needs to be further clarified as end effectors for other candidate molecules.

Mitochondrial or Sarcolemmal K_{ATP} Channels?

All studies discussed above clearly demonstrate the role of mitochondrial K_{ATP} channels in RIPC as blockade with 5-HD (selective mitochondrial channel blocker) abolished RIPC. Pell *et al.* [27] showed that RIPC was abolished by 5-HD without affecting the action potential of sarcolemmal fibers or vasodilator effects of sarcolemmal K_{ATP} channels. The muscle used in the Moses model was surgically denervated, not associated with changes in action potential and muscle contractility, suggesting the unlikely role of sarcolemmal K_{ATP} channels in the energy-sparing effect. Two studies showed K flux across mitochondrial K_{ATP} channels after RIPC [27, 122].

Pell *et al.* [27] showed that K_{ATP} channels reduce ATP depletion and maintain intracellular pH and phosphocreatinine levels in heart muscle after RIPC. Moses *et al.* showed decreased neutrophilic infiltration, myeloperoxidase activity, and ATP-sparing effect in preconditioned skeletal muscle [47]. These data suggest that K_{ATP} channel opening reduces the rate of ATP hydrolysis or mitochondrial ATPase activity, thereby decreasing the rate of ATP depletion. Also, opening of mitochondrial K_{ATP} channels decreases mitochondrial calcium load, which preserves mitochondrial integrity. Mitochondrial volume is regulated by K_{ATP} channels and volume changes modify energy flow through the electron system, thereby influencing energy transfer between mitochondria and cellular ATPases. These studies showed that RIPC applied *in vivo* exerts protection upon skeletal muscle and heart after explantation from the body and has potential beneficial effects in relation to heart transplantation, cardiopulmonary bypass, and autologous skeletal muscle transplantation.

CGRP and Brain Gut Axis (Table 10, Studies 19, 20, 21, 22)

Evidence

CGRP is a neuropeptide and principal neurotransmitter found in capsaicin-sensitive sensory nerves. CGRP receptor antagonists abolished reduction in infarct size by IPC [130] in a rat model, suggesting the role of CGRP in mediation of IPC of the heart.

Five studies demonstrated the role of CGRP in RIPC by brief MAO. Tang, Xiao, Wolfrum *et al.* showed that brief MAO increased plasma levels of CGRP following RIPC and reduced myocardial infarct size and blockade of CGRP receptors, and ganglion blockade abolished RIPC [31, 34, 35]. Ganglion blockade did not affect CGRP release. Brzozowski *et al.* showed decreased mucosal CGRP and blood flow in gastric IR and RIPC by hepatic/coronary ischemia (2 cycles × 5 min IR) restored mucosal CGRP levels in gastric IR and gastric flow and prior treatment with CGRP receptor antagonist and capsaicin-induced sensory nerve deactivation abolished RIPC by MAO [61]. Administration of exogenous CGRP in denervated animals restored RIPC effects.

What Is the Pathway?

An increase in plasma levels of CGRP as shown in three studies [31, 34, 35] supports a humoral pathway. However, ganglion blockade abolished myocardial protection from brief MAO, demonstrating a neuronal pathway for CGRP in RIPC by MAO.

Capsaicin selectively depletes neurotransmitters in sensory nerves and is known to cause deactivation of sensory afferent neurons. Two studies in RIPC by MAO have shown that treatment with capsaicin prior to RIPC by MAO abolished both reduction in myocardial infarct size and increase in CGRP levels [34, 35]. Two studies in RIPC by brief hepatic artery occlusion and CAO [60, 61] showed that prior treatment with capsaicin abolished the increase in gastric mucosal CGRP, increase in gastric blood flow, and reduction in gastric erosions following RIPC. These observations strongly support the role of sensory neurons in RIPC. In addition Brzozowski *et al.* showed that vagotomy abolished RIPC from brief hepatic ischemia or CAO and reduced plasma levels of CGRP. This observation suggests that vagal efferents form a part of the effector pathway in CGRP-mediated RIPC and demonstrates the role of the brain gut axis in RIPC. Finally, L-name (NO blocker) abolished reduction of myocardial infarct size by CGRP-mediated RIPC, suggesting that CGRP acts via the NO pathway [34].

End Effector Mechanism

One study showed that in RIPC by MAO, CGRP activated myocardial PKC and reduced infarct size [31]. PKC activation and myocardial protection was abolished by both ganglion blockade and CGRP receptor antagonists. These data suggest that in RIPC, CGRP acts at the cellular level by activation of kinases. Another study showed the NO pathway as discussed above and NO may be the end effector responsible for CGRP effects. This argument is supported by past studies providing evidence for CGRP-related NO release [34]. One study showed the inhibition of cytokines (TNF-α and IL-1β) release in the target organ [60]. Further clarification of the pathway downstream is needed in future studies.

Prostaglandins (PGEs) (Table 10, Study 23)

Evidence

Brzozowski *et al.* showed that indomethacin (nonselective cyclooxygenase blockers), SC-560 (selective cox-1), and rofecoxib (selective cox-2) blockers abolished the gastric protective effects of IPC by two cycles of 5 min celiac artery occlusion, RIPC by 2 cycles of 5 min

hepatic ischemia, or CAO [61]. Concurrent treatment with exogenous PGE_2 counteracted the effects of Cox blockers and restored the hyperemic mucosal effects of RIPC. Brzozowksi *et al.* also showed increased gastric mucosal generation of PGE_2 in IPC and RIPC. These observations suggest that increased endogenous activity of prostaglandins particularly of the COX-2 variety could be involved in preconditioning and it is known from past studies that they have a protective effect in gastric ulcers.

Pathway and Effector Mechanism

Brozozowski *et al.* showed that functional ablation of sensory nerves by pretreatment with capsaicin abolished the protective effects in all preconditioned groups. This observation suggests the involvement of sensory afferent neurons in the pathway. Vagotomy abolished RIPC from brief hepatic/CAO, suggesting the role of vagal efferents and the brain gut axis in mediation of RIPC. RIPC was associated with increased CGRP and decreased TNF-α and IL-1β expression and release, suggesting amelioration of gastric IRI by prostaglandins through modulation of cytokine release and increased mucosal flow by vasodilatory effects of CGRP through NO release. These effects were attenuated by pretreatment with capsaicin and ablation of sensory nerves known to release NO and CGRP.

HSPs and Hemoxygenase (HO-1) (Table 10, Studies 28, 47)

HSP28, 40, 60, 70, 90, 104 have an important physiological and pathological role. One of the most important HSPs is HSP70, which has a cytoprotective role. The constitutive form of HSP is HSP73, which all cells express to some extent. The inducible form is HSP72.

HSP expression is more in myocytes compared to microvascular cells. Enhanced HSP expression may serve to protect the heart against ischemia reperfusion, hypoxia, and chemicals in addition to hyperthermia. HSP express themselves as early as 3 h post reperfusion in the myocardium, begin to decay from 42 h post reperfusion, and persist up to 72 h as detected by immunohistochemistry.

In three studies in RIPC, Tanaka *et al.* showed in a rabbit model RIPC by four cycles of 5 min CAO and 5 min reperfusion [21, 131] increased cardiac tolerance due to increased HSP expression in the ischemic and remote myocardium; Konstantinov showed RIPC by six cycles of 4 min femoral artery occlusion and 4 min reperfusion increased expression of HSP73 genes (anti-inflammatory) in the heart of preconditioned mice as compared to sham [40] and Chen *et al.* in a rat model showed increased myocardial HSP expression (HSP70) following hindlimb RIPC [76].

What Induces HSP?

HSP is induced by ischemia [131] and ROS [76]. Tanaka showed myocytes with immunoreactivity for HSP as early as 3 h and persistence of this immunoreactivity up to 72 h. This suggests that HSP expression may be associated with a delayed phase of protection. Mechanisms by which ischemia may induce HSP expression include enhanced tissue levels of catecholamines and angiotensins, which induce HSP expression, myocardial dyskinesia, which induces compensatory hypercontraction of non-preconditioned myocardium, increased workload and a modest increase in HSP in the nonischemic myocardium, and elevated end-diastolic pressure, which leads to increased workload and HSP expression. However these need to be clearly demonstrated in experimental settings.

What Is the Pathway and Effector Mechanism?

One study [40] showed that reperfusion stimulates HSP gene expression in myocardium following limb RIPC in a rat model. Chen *et al.* demonstrated that reperfusion induced myocardial HSP expression following limb RIPC and this was abrogated by free radical scavengers (MPG) [76]. These studies suggest that circulatory mediators stimulate HSP expression in remote tissue and free radicals are one of these mediators. The effector mechanism may involve scavenging of ROS and modulation of K-channels but this needs to be clearly demonstrated.

HO-1 (Table 10, Study 47)

HO-1 is a form of inducible HSP. It is an enzyme that mediates the rate-limiting step of breakdown of haem to biliverdin, CO, and iron. In this process it scavenges ROS and mitigates IRI. Previous studies have shown hemoxygenase to reduce hepatic IR and lung IR [132–135].

One study showed RIPC by limb ischemia [136] (four cycles of 10 min ischemia) reduced hepatic IRI and improved liver function. Hemoxygenase expression at 3 h of reperfusion suggested its role in amelioration of IR. Zinc protoporphyrin (HO-1 inhibitor) 1 h prior to IR abolished RIPC effects.

What Is the Pathway?

Thus far no studies have demonstrated HO-1 activity in serum following RIPC. It is likely that following RIPC peripheral breakdown of haem may lead to increased CO levels in the blood and CO has both vasodilatory and antiplugging effects in hepatic sinusoids. However no study has measured post RIPC serum carboxyhemoglobin levels to demonstrate this. Lai *et al.* [136] demonstrated no increase in HO-1 in peripheral macrophages following RIPC and therefore the role of peripheral macrophages as a pathway for HO-1 induction in the liver is unlikely. RIPC induces a low-grade oxidative stress in the limb that may lead to release of ROS and cytokines into the circulation, low-

grade oxidative stress in the liver, and increased hepatic HO-1 expression. This is hypothetical and needs to be proved by using ROS scavengers. HO-1 pathways scavenge ROS following IR, stabilize mitochondrial membrane permeability, and reduce apoptosis by decreased cytochrome C release as shown by previous studies. HO-1 also down-regulates iNOS and inhibits NF-κB expression. Future studies are needed to define the pathway and effector mechanism of HO-1.

Effect of RIPC on Inflammatory Gene Expression (Table 10, Studies 29, 30)

The first study to look at the effect of remote preconditioning on inflammatory gene expression modification in a human model showed that brief forearm ischemia led to suppression of proinflammatory genes [137] encoding proteins in circulating leukocytes, reduction in neutrophil chemotaxis, adhesion, and exocytosis. Studies by Huda *et al.* (RIPC MAO model) [140] showed expression of genes associated with organ protection following RIPC.

Clinical Trials

Kharbanda *et al.* conducted the first clinical trial in humans [20] and showed that forearm ischemic preconditioning is associated with diminished ischemia reperfusion injury in the contralateral arm as well as diminished endothelial injury. The mechanism was not clearly identified, although it was thought to be due to a substance released in the circulation. In a recent trial in children undergoing cardiac surgery for congenital heart defects on cardiopulmonary bypass, Cheung *et al.* demonstrated that four cycles of right hindlimb ischemia (5 min) followed by reperfusion (5 min) prior to cardiopulmonary bypass preconditioned the heart and reduced infarct size [73]. Thereafter Kristiansen *et al.* studied the effect of forearm ischemia on cardiac ischemia in cardiopulmonary bypass procedures and found it to be protective. This protective effect was attributed to KATP channels [45].

In humans, Konstaninov *et al.* [137] showed diminished expression of inflammatory genes in neutrophils following limb RIPC. Since neutrophils are one of the key mediators of the late phase of IRI responsible for oxidative stress and injury to organs, modulation of neutrophil activation would be of prime importance in reducing IRI.

CONCLUSIONS AND FUTURE WORK

Remote preconditioning is a novel method of preconditioning. Limb, mesentery, and kidney are organs that can effectively be used to induce remote protection and reduce IRI. Brief periods of cardiac ischemia confer protection on the lungs and *vice versa*. This may be of benefit in cardiovascular surgery and coronary bypass procedures.

Similar protection in musculoskeletal flaps and protection of the brain function needs to be evaluated in clinical trials. Adenosine, NO, PKC, bradykinins, catecholamines, opioids, HO-1 (HSP), free radicals, and cytokines are the candidate mechanisms that form part of a complex cascade involved in transduction of the preconditioning effect with overlap between neurogenic and humoral pathways. The RIPC stimulus requires reperfusion, providing unequivocal evidence of a humoral mediator. However, there are many circulating mediators such as NO, opioids, and adenosine which have undergone selective blockade to abrogate RIPC. The end effect of the remote stimulus is to reduce the mitochondrial permeability in the target organ, conserving cellular ATP levels and reducing apoptosis. Microarray analysis has shown transcription of anti-inflammatory genes and modulation of oxidative stress to be of prime importance in preconditioning. Application of remote preconditioning in clinical settings has thus far been limited and its use particularly in organ transplantation is of interest. Future studies are necessary to study the benefit of this method in a clinical setting. Modulation of gene expression to prevent IRI is a key area to be studied.

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ORIGINAL ARTICLE

Effect of remote ischemic preconditioning on hepatic microcirculation and function in a rat model of hepatic ischemia reperfusion injury

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Abstract

Background: Liver transplantation involves a period of ischemia and reperfusion to the graft which leads to primary non-function and dysfunction of the liver in 5–10% of cases. Remote ischemic preconditioning (RIPC) has been shown to reduce ischemia reperfusion injury (IRI) injury to the liver and increase hepatic blood flow. We hypothesized that RIPC may directly modulate hepatic microcirculation and have investigated this using intravital microscopy.

Methods: A rat model of liver IRI was used with 45 min of partial hepatic ischemia (70%) followed by 3 h of reperfusion. Four groups of animals (Sham, IRI, RIPC+IRI, RIPC+Sham) were studied (n = 6, each group). Intravital microscopy was used to measure red blood cell (RBC) velocity, sinusoidal perfusion, sinusoidal flow and sinusoidal diameter. Neutrophil adhesion was assessed by rhodamine labeling of neutrophils and cell death using propidium iodide.

Results: RIPC reduced the effects of IRI by significantly increasing red blood cell velocity, sinusoidal flow and sinusoidal perfusion along with decreased neutrophil adhesion and cell death.

Conclusions: Using intravital microscopy, this study demonstrates that RIPC modulates hepatic microcirculation to reduce the effects of IRI. HO-1 may have a key role in the modulation of hepatic microcirculation and endothelial function.

Keywords

ischemic preconditioning, remote ischemic preconditioning, hepatic ischemia reperfusion injury

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Introduction

Liver transplantation involves a period of cold and warm ischemia followed by reperfusion which initiates an inflammatory cascade resulting in organ non-function and dysfunction.[1–3] This is referred to as ischemia reperfusion injury (IRI).

IRI remains a major concern in liver surgery and transplantation as the incidence of primary non-function is 5–10% in liver transplantation and the problem is aggravated in fatty livers. The success and expansion of liver transplantation has resulted in a shortage of organ donors and the use of marginal grafts including steatotic livers. Preconditioning is an adaptive response which helps reduce the severity of IRI in an experimental setting and has the potential of increasing the donor pool by increasing tolerance of marginally fatty livers to IRI. Ischemic preconditioning (IPC) involves the interruption of blood flow to the liver for brief periods followed by reperfusion. Nitric oxide (NO)/adenosine release has been shown to regulate endothelial function and increase blood flow to the liver.[4–8] However, direct IPC produces trauma to major vessels and direct stress to the target organ. Remote ischemic preconditioning (RIPC) is a novel method which entails brief periods of ischemia followed by reperfusion of one organ and results in protection of remote organs from ischemia without direct stress or trauma to blood vessels. It is

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presumed that remote preconditioning acts by release of biochemical messengers into the circulation or by activation of nerve pathways which confer protection on the remote organ. Studies have been carried out to show the benefit to the heart, lung and kidney after brief ischemia in the mesentery,[9–12] limb[13–20] and kidney.

Previous studies (Menger *et al.*)[21]using intravital microscopy have shown that the hepatic microcirculation is a major target of hepatic IRI. Microvascular injury precedes parenchymal injury and is as a result of hypoxia secondary to lack of microvascular perfusion (no-reflow) and reperfusion-associated inflammatory response which includes activation of kupffer cells and neutrophils (reflow – paradox). No reflow in sinusoids is because of endothelial swelling and intravascular hemoconcentration. Reperfusion is associated with release of proinflammatory cytokines, oxygen radicals, up-regulation of endothelial and leukocyte adhesion molecules and interaction of leukocytes with endothelial lining of the hepatic microvasculature. Hepatic IR induces neutrophil adhesion in sinusoids and post-sinusoidal venules.[22,23]

Kanoria *et al.* demonstrated improved liver function using RIPC in hepatic IRI and increased hepatic blood flow by ICG and laser Doppler flow measurements.[13] Lai *et al.* demonstrated improved liver function and the role of hemoxygenase in hepatic protection.[24] Gustaffson demonstrated protection of hepatic function by remote preconditioning.[25] None of these studies addressed the *in vivo* microcirculatory changes seen in hepatic IR and the effect of RIPC on hepatic IR. We focused our study on the hepatic microcirculatory changes in hepatic IR and the modulation of the hepatic microcirculation by RIPC. We hypothesized that RIPC directly modulates hepatic microcirculation in IR injury and applied intravital microscopy to investigate the effects of RIPC.

Material and methods

Animals and surgical procedures

All experiments were conducted under a project license from the UK home office in accordance with the Animals' Scientific procedures act 1986. Male Sprague–Dawley rats, weighing 250–300 g, were used. Animals were kept in a temperature-controlled environment with a 12 h light/dark cycle and allowed tap water as well as standard rat chew pellets ad libitium. Animals were anesthetized with 4 l/min of isoflurane (Baxter, Norfolk, UK) and maintained with 1–1.5 l/min of O^2 and 0.5–1.0% 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, KY, USA).

Polyethylene catheters (0.76-mm inner diameter; Portex, Kent, UK) were inserted into the right carotid artery for monitoring of mean arterial blood pressure and the left jugular vein (0.40-mm inner diameter; Portex) for administering normal saline (1 ml/100 g/h body weight to compensate for intra-operative fluid loss). The animals were placed in a supine position on a heating mat (Harvard apparatus Ltd., Kent, UK) to maintain their temperature.

Animal model

Hepatic I/R

A standard model of lobar hepatic ischemia of the left lateral and median lobes (70% of liver) was used.[26] Laparotomy was carried out through a midline incision. The ligamentous attachments of the liver were cut and the liver was exposed. Ischemia was induced by clamping the corresponding vascular pedicle with an atraumatic microvascular clamp. This model avoids splanchnic congestion. Hepatic ischemia was for a period of 45 min followed by 3 h of reperfusion. All animals were given a bolus of heparin [20 U/kg, intravenously (i.v.)] prior to clamping to prevent hepatic artery thrombosis.

Limb preconditioning

The technique of remote ischemic preconditioning involved a hind limb tourniquet, which was applied around the thigh. Limb perfusion was monitored by a laser Doppler (Moor instruments, Surrey, UK); the tourniquet around the limb was tightened until no flow was detected. The procedure involved 5 min of ischemia followed by 5 min of reperfusion. This was repeated four times.[27]

Tissue and blood collection

After 3 h of reperfusion, the animals were killed by exsanguination and blood was collected in a BD vacutainer tube SST^{m}II advance 5.0 ml tubes for Serum, BD vacutainer tube LH 102 I.U> (6 ml) for citrated plasma and BD vacutainer K 2E 7.2 mg (4 ml) for ethylenediaminetetraacetic acid (EDTA) plasma and centrifuged at 1500 *g* for 10 min to sediment the red blood cells (RBC). Serum and plasma samples were snap frozen in liquid nitrogen and subsequently stored at −80°C. Liver tissue was also snap frozen and stored at −80°C. Tissues were also fixed in 10% formalin and stored for histology.

Experimental groups (n = 6 in each group)

Four groups of animals were studied. Group one (Sham) in which animals were subjected to laparotomy only and underwent an identical experimental protocol without clamping. In group two (IRI) the animals were subjected to 45 min of liver ischemia followed by 3 hours of reperfusion. Ischemia was induced by a microvascular clamp applied across the vascular pedicle supplying the left and median lobes of the liver (70% ischemia). Group three were preconditioned immediately prior to the IRI (RIPC+IRI) group as shown in Fig. 1. In group four (RIPC+Sham) preconditioning was done in the Sham group. Standard protocols for preconditioning and inducing ischemia as described above were used.

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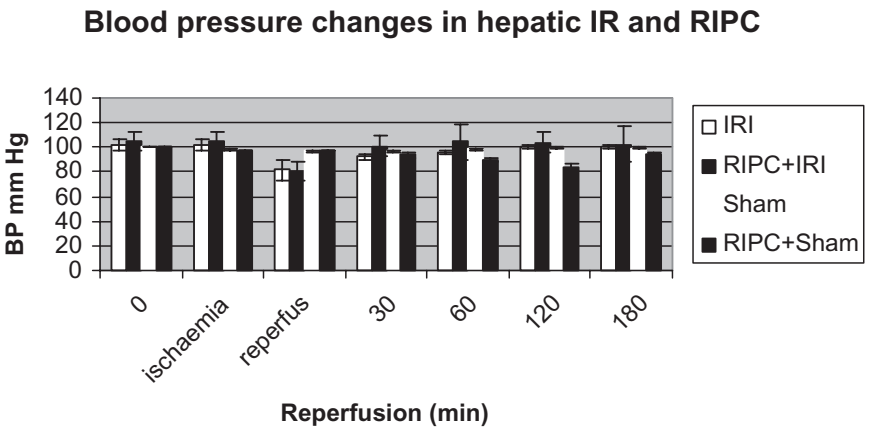
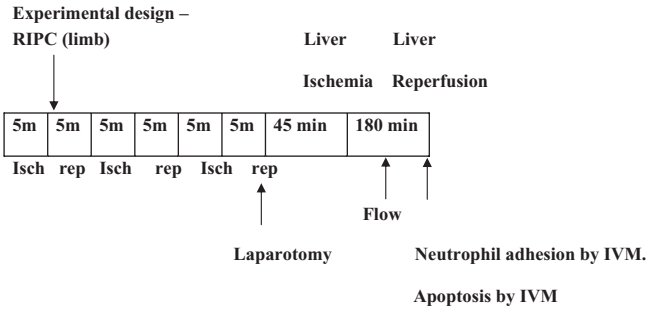


Figure 1 Changes in blood pressure seen in hepatic ischemia reperfusion (IR) and effect of preconditioning. Both IR and (RIPC)+IR show a similar fall in blood pressure; however, RIPC prior to IR rapidly restores blood pressure to baseline compared with IR only. Values expressed as mean + SEM. $P < 0.05$



Intravital videofluorescence microscopy

The animals were maintained under anesthesia with isoflurane and oxygen .Their temperature was maintained by a warming mat (which was regulated by a thermostat).

The liver was exposed, placed upon a glass mount and covered with a cover slip. The liver was continuously irrigated with normal saline. A drop of saline was placed on the cover slip and the tip of the microscope lens was immersed in the saline drop.

A Nikon (Tokyo, Japan) microscope (Nikon epi-illumination system with filter block set suitable for Texas Red, FITC and DAPI dyes) coupled to a CCD camera [JVC TK-C1360β (Osaka, Japan) colour video camera] was used. The power of magnification was 10× and 40×. The microscopy images were recorded for offline analysis. Quantitative assessment of microcirculatory parameters was performed both during the experiment and offline using frame-by-frame analysis of the recorded images.

Microcirculation was assessed by evaluating acinar perfusion in 10 randomly chosen acini and leukocyte endothelial interaction in 10 post-sinusoidal venules.[21,22,23,28] LUCIA (lab universal computer image analysis; Nikon) software was used to analyze the images.

RBC velocity (V)

FITC (Fluoroisothyocyanate; Sigma-Aldrich, Dorset, UK) labeled red cells were prepared from rat blood suspended in glucose saline buffer solution (20 mg FITC/ml of RBC).[29] Labeled RBCs (0.5 ml) were administered i.v. to assess the velocity of RBC flow. Ten randomly chosen non-overlapping rappaport acini were assessed at each time point and the mean value was calculated. The RBC velocity was calculated by assessing the distance of RBC movement (microns) in each sinusoid in subsequent frames. Twenty-five frames were captured per second. The velocity was calculated using the formula = D × 25/number of frames moved.

Sinusoidal perfusion and perfusion index (PI)

The sinusoidal perfusion index was calculated as the ratio of perfused hepatic sinusoids. Continuous perfusion has been described as continuous flow of red cells through hepatic sinusoids for at least 1 min. Intermittent perfusion has been described as occasional flow of RBCs in sinusoids. Perfusion index = (Scp + Sip/Scp+Sip+Snp). [Continuous perfusion (Scp) + intermittent perfusion (Sip) to the total visible sinusoids which includes non perfused sinusoids (Snp)].[22]

Sinusoidal diameter (D)

Sinusoidal diameter was assessed by measuring the length across 10 randomly chosen hepatic sinusoids at 30, 60, 120 and 180 min of reperfusion and the mean value was expressed in microns.

Sinusoidal blood flow

The sinusoidal blood flow was calculated using the formula: (V) ×22/7× (D/2)2.

V = Velocity, D = diameter.

Neutrophil adhesion

Rhodamine 6 G (0.3 mg/kg; Sigma)[30] was given i.v. after 180 min of reperfusion to assess neutrophil adhesion. The numbers of leukocytes adherent to the sinusoidal endothelium and venular endothelium were counted as those stationary for a period of 30 s under green filter light and expressed as leukocytes/mm^2. The area

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of the vessels was calculated using the product of diameter and length assuming cylindrical geometry (3.14 × D × L).

Cell death

Hepatocellular death was assessed by i.v. injection of Propidium iodide (0.05 mg/kg i.v.)[31] at 180 min of reperfusion prior to termination of the animal. The numbers of nuclei stained with the dye per high power field were counted. Ten randomly chosen high power fields were assessed.

Histology

At the end of the experiment, liver tissue was fixed in 10% formalin and embedded in paraffin in preparation for light microscopy analysis. Sections were cut at 5μ and stained with hematoxylin and eosin (H&E) for histological analysis. The histologic changes in the routine (H&E) stained sections were graded using the modified Suzuki's criteria.[32] In this classification system, sinusoidal congestion, hepatocyte necrosis and hepatocyte vacuolation were graded on a score of 0–4. No changes were scored as 0 whereas severe congestion, extensive vacuolation and greater than 60% of lobular necrosis was awarded a score of 4.

Numerical assessment	0	1	2	3	4
Sinusoidal congestion	None	Minimal	Mild	Moderate	Severe
Vacuolation	None	Minimal	Mild	Moderate	Severe
Necrosis	None	Single cell	30%	60%	>60%

Statistical analysis

All data are presented as mean and standard error of the mean (SEM). Differences were considered significant for $P < 0.05$. Comparisons between groups were performed using one-way analysis (ANOVA). Bonferroni's correction was applied for ANOVA.

Results

Hemodynamic parameters

Blood pressure (MAP)

There was no significant difference in the sham group between baseline and 180 min demonstrating a hemodynamically stable model. The blood pressure reduced transiently in the IR and RIPC+IR groups but this was insignificant. However, the recovery to baseline was more rapid in the RIPC group (Fig. 1).

Pulse rate

No significant difference was seen between the groups.

Oxygen saturations

The fall in saturations in both the IR group and RIPC+IR group was not statistically significant.

Hepatocellular injury (liver function tests)

After 180 min of reperfusion, the difference between the Sham and IRI groups was statistically significant ($P < 0.05$). Preconditioning prior to IRI reduced serum transaminase levels although the difference was not statistically significant. Preconditioning in Sham animals did not show any significant elevation in transaminases.

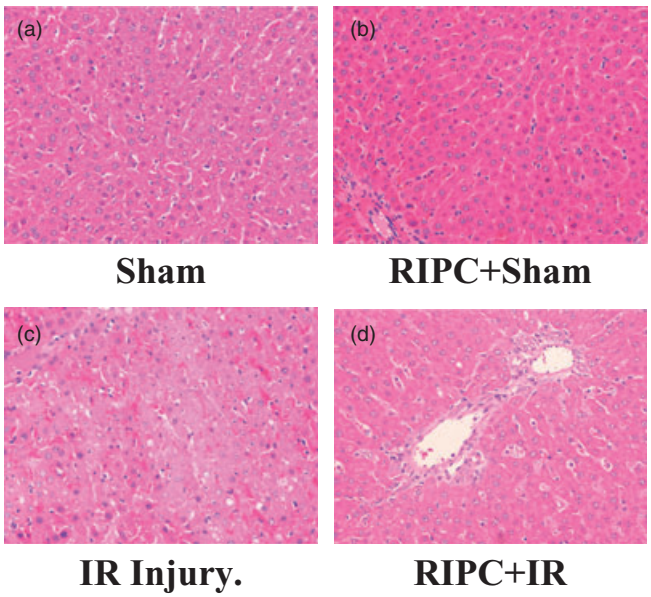

Figure 2 Histology (a) remote ischemic preconditioning+ischemia reperfusion (RIPC+IR): the hematoxylin and eosin (H&E) section shows sinusoidal congestion, some hepatocyte vacuolation but no significant necrosis. (b) IR: the H&E section shows large areas of necrosis and sinusoidal congestion, normal residual hepatocytes noted at bottom of the frame. (c) Sham: the H&E section reveals no significant damage. (d) RIPC+Sham: the H&E section reveals a congested central vein but no other significant change

	IR	RIPC+IR	Sham	RIPC+ Sham
ALT (IU/ml)	2079 ± 322.3	1398 ± 460.8	118 ± 22.37	132 ± 25.49

Histologic investigation

Histology of the liver tissue revealed that IR injury produced significant periportal congestion in association with severe necrosis in zones 2 and 3 and also sub capsular necrosis. The changes were diffuse. RIPC+IR showed less necrosis and damage compared with the IR group. Sham animals revealed minimal changes. Congestion was present in both portal vein and central veins. Hepatocytes showed vacuolation. RIPC+Sham animals showed minimal changes, some vacuolation, but no necrosis, similar to the Sham group (Fig. 2)

Histological scoring table

	IR	RIPC+IR	Sham	RIPC+Sham
Suzuki score	8.83 ± 0.7	6.2 ± 0.58	4 ± 0.31	1.5 ± 0.34

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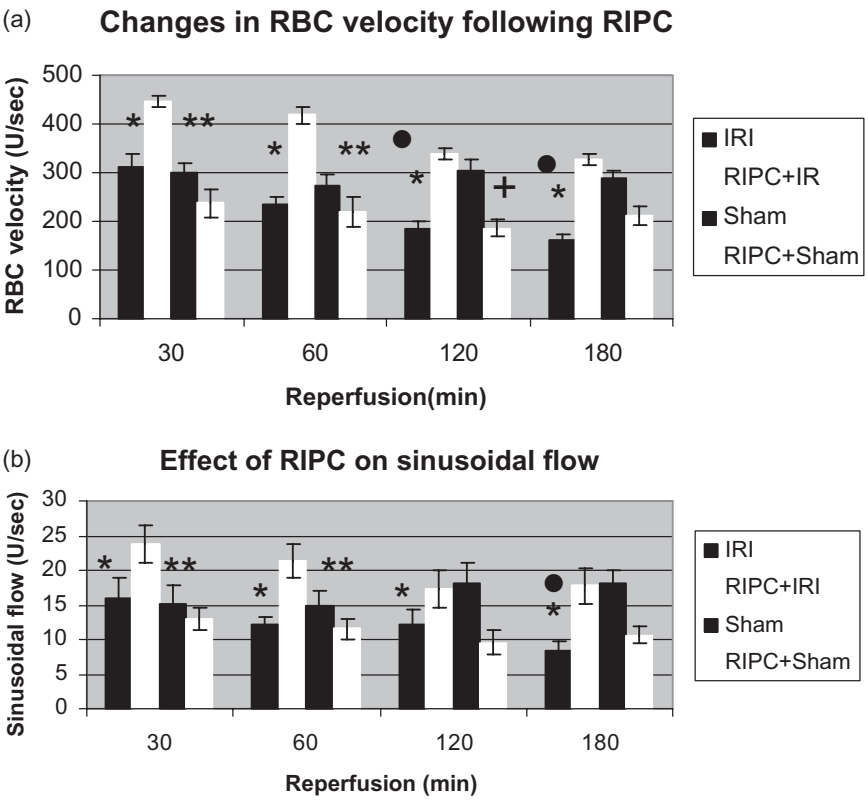

Figure 3 (a) Velocity of red blood cell (RBC) flow in Sham animals remained unchanged throughout 180 min of observation, although there was a significant increase in velocity in preconditioned animals prior to ischemia reperfusion injury (RIPC+IRI) as compared with IRI at 30, 60 and 120 min of reperfusion. Values expressed as mean ± SEM. *$P <$ 0.05 (RIPC+IR/IR). **$P < 0.05$ (RIPC+IR/Sham). ●, $P < 0.05$ (Sham/IR). +, $P < 0.05$ (Sham/RIPC+Sham). (b) Sinusoidal flow: $V \times (D/2)2 \times \pi = V$ is velocity of RBC, D is sinusoidal diameter. There was a significantly better flow in preconditioned animals (RIPC+IRI) as compared with non-preconditioned (IR). Values expressed as mean ± SEM. *$P < 0.05$ (RIPC+IR/IR). **$P < 0.05$ (RIPC+IR/Sham). ●, $P < 0.05$ (Sham/IR)

The Suzuki score was significantly reduced in the preconditioned group compared with the IR group ($P < 0.001$).

Intravital microscopy results

Intravital microscopy demonstrated significant changes to the hepatic microcirculation after IR and modification by RIPC.

Velocity of RBC flow

The mean RBC velocity in the Sham group was constant throughout the time of observation. IRI led to decreased RBC velocity whereas preconditioning significantly increased RBC velocity (Fig. 3a).

Sinusoidal blood flow

The sinusoidal blood flow was significantly better in the RIPC+IRI group compared with the IRI group (Fig. 3b).

Diameter of sinusoids

The mean diameter showed no significant difference between the RIPC+IR group and the IR group. The difference between the Sham and RIPC+IR and between the Sham and IRI groups was not statistically significant.

Perfusion of sinusoids

Preconditioning significantly improved sinusoidal perfusion being significantly high compared with the IRI group. These data show no significant difference between RIPC+ IRI and Sham groups at all time points (Fig. 4).

Neutrophil adhesion

The number of adherent neutrophils was significantly lower in the RIPC+IRI group as compared with the IR group and similar to the Sham group. In the sinusoidal bed, neutrophil adhesion was significantly reduced in the preconditioned group as compared with the IR group (Figs 5a,b,6).

Hepatocellular death

No significant difference was found between the Sham and RIPC groups but both had a significantly lower number of apoptotic cells compared with IRI (Fig. 7).

Discussion

New findings

This study demonstrates that RIPC modulates microcirculatory disturbances in hepatic IR leading to increased RBC velocity, sinusoidal perfusion, decreased sinusoidal and venular neutrophil adhesion, improved hepatic microcirculation and decreased hepatocyte cell death. This is the first *in vivo* study to

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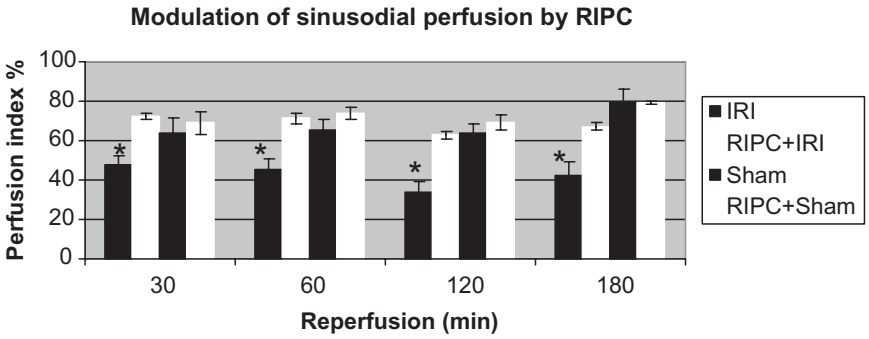

Figure 4 Sinusoidal perfusion index (PI): the PI remains unchanged in Sham animals throughout 180 min of observation. The PI in remote preconditioned animals (RIPC+IRI) is significantly higher than non-preconditioned animals (IRI). The PI in RIPC+IR is not different from Sham. Values expressed as mean ± SEM. *$P < 0.05$

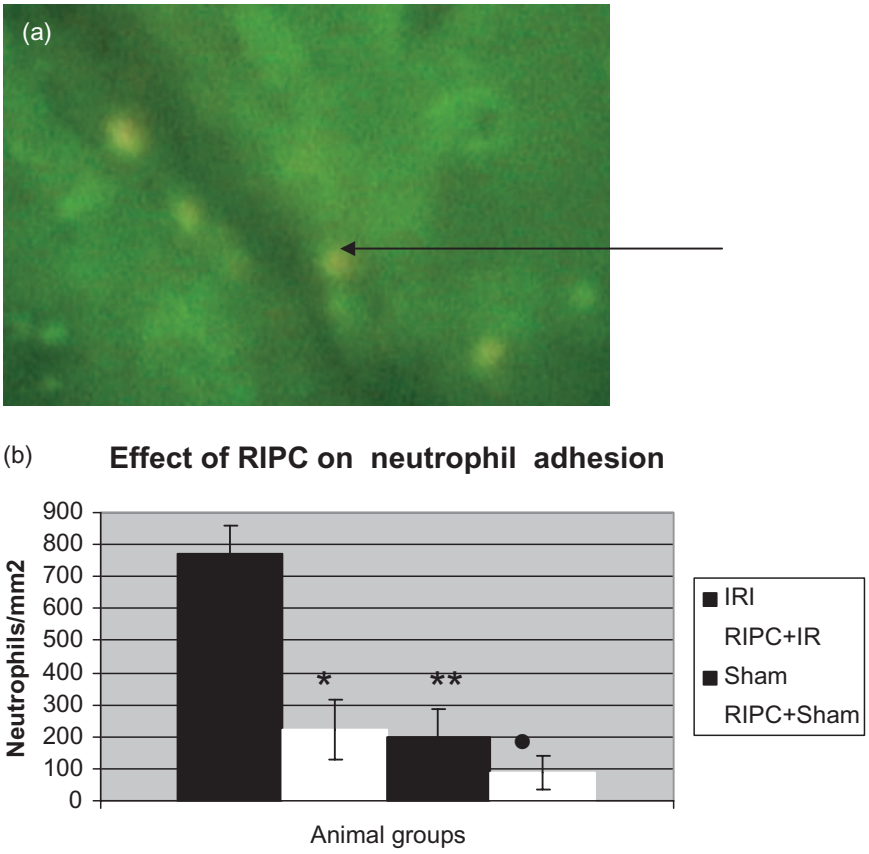

Figure 5 (a) Neutrophils stained using rhodamine are seen adherent to post-sinusoidal venular endothelium. The number of adherent neutrophils divided by the area of endothelial surface ($\pi \times D \times L$) gives the number of neutrophils/mm^2. D, sinusoidal diameter, L, length of segment along adherent neutrophils. (b) Significantly reduced venular neutrophil adhesion in the preconditioned (RIPC+IRI) group compared with the non-preconditioned group (IRI). Values expressed as mean ± SEM. *$P < 0.05$ (IR/RIPC+IR), **$P < 0.05$ (IR/Sham), ●, $P < 0.05$ (IR/RIPC+Sham)

demonstrate microcirculatory effects of RIPC in hepatic IR by intravital microscopy and suggest that improvement of hepatic microcirculation is a key mechanism responsible for hepatic protection by RIPC in IRI. Recently RIPC has been shown to modulate pancreatic microcirculation and reduce pancreatic IRI.[33]

Adequacy of model

The experimental model used is a previously well-described hepatic lobar ischemia model of warm IR. In the model 45 min of partial ischemia (70% of the liver) was induced followed by 3 h of reperfusion.[26] Intravital microscopy has shown that IRI significantly reduced velocity of flow and perfusion as compared with Sham animals. Therefore the model was considered suitable to study the effects of RIPC. As this study was limited to investigating the effects of warm IR and the effects of RIPC in warm IR, a partial hepatic IR model was chosen as against a transplant model. RIPC was produced using a tourniquet around the hind limb for four cycles of 5-min ischemia followed by reperfusion. This technique of RIPC has previously been used in the modulation of cardiac IRI after transplantation.[25] Three or more cycles of preconditioning have been shown to be more effective than a single cycle in previous experimental studies[27,34,35] and this formed the basis for four cycles of RIPC used in the current study.

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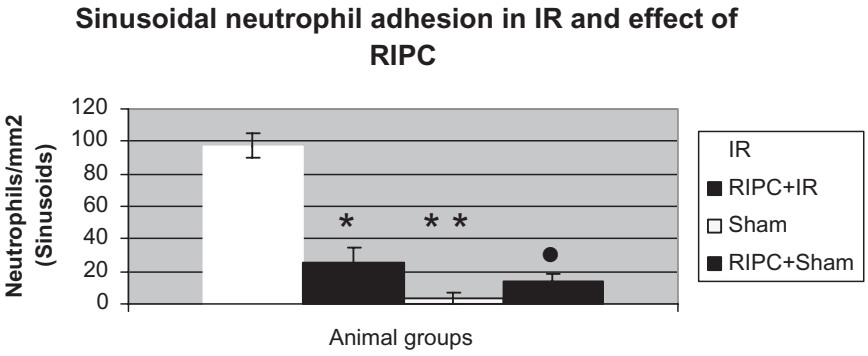

Figure 6 Significantly reduced sinusoidal neutrophil adhesion in the preconditioned group (RIPC+IRI) compared with non-preconditioned group (IRI). Values expressed as mean ± SEM. *$P < 0.05$ (IR/RIPC+IR), **$P < 0.05$ (IR/Sham), ●, $P < 0.05$ (IR/RIPC+Sham)

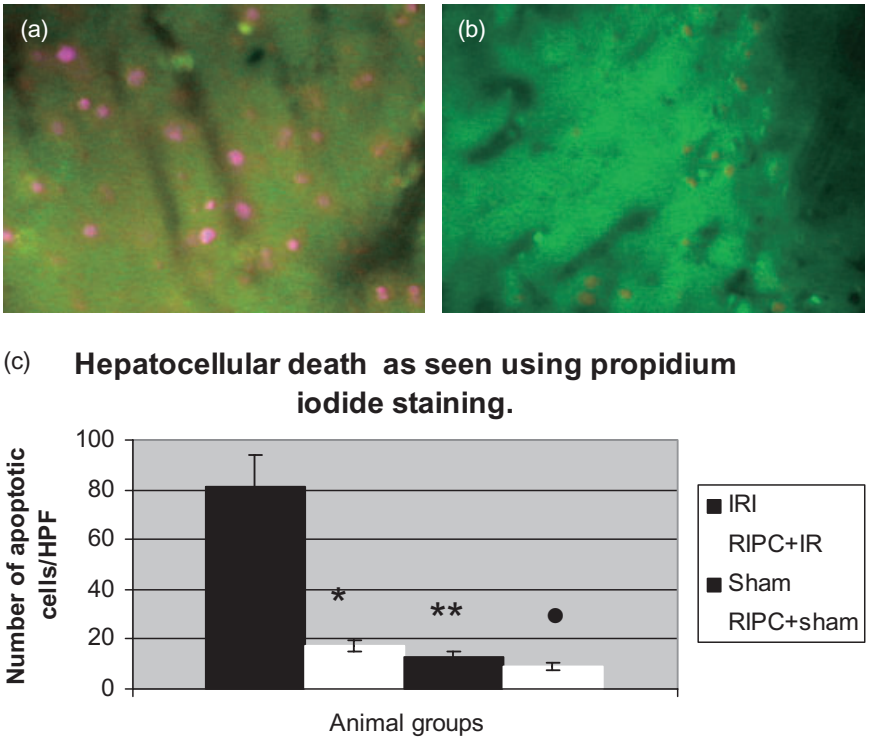

Figure 7 (a) Hepatocellular cell death in ischemia reperfusion injury (IRI) using propidium iodide staining (IVM). The dead cells appear pink stained by propidium iodide. The number of cells divided by the surface area of the field above gives the number of cells/mm^2. (b) Hepatocellular cell death seen in remote ischemic preconditioning (RIPC)+ IR using IVM. (c) Hepatocellular cell death in the preconditioned group (RIPC+IRI) was significantly less compared with the non-preconditioned (IRI) group. Values expressed as mean ± SEM. *$P < 0.05$ (IR/RIPC+IR), **$P < 0.05$ (IR/Sham), ●, $P < 0.05$ (IR/RIPC+Sham)

Systemic hemodynamics

Shams were hemodynamically stable throughout the course of the experiments. Limb preconditioning in Sham animals caused a transient insignificant fall in blood pressure during preconditioning with rapid recovery to baseline levels suggesting no major effects of Sham laparotomy, anesthesia or RIPC alone. A transient fall in blood pressure and oxygen saturations associated with onset of reperfusion was seen in both the IR and RIPC+IR groups. However, recovery of blood pressure to normal levels was rapid in the RIPC+IR group as compared with the IR group only. These observations have been observed in preconditioning models of lung IRI.[36,37] Kanoria *et al.* have demonstrated a fall in blood pressure after hepatic IR in a rabbit model and rapid recovery of blood pressure in the group which was preconditioned.[13]

Histologic findings and limitations of the scoring system

The RIPC+IR group showed less necrosis, vacuolation and congestion compared with the IR group and objective scoring showed a significantly lower score. However, it was difficult to arrive at an objective score if the changes noted were focal/patchy. There were several sections with only sub capsular infarction/necrosis. Scoring these sections were difficult using the criteria.

Hepatic microcirculatory changes

Sham

The Sham group showed constant velocity, flow and perfusion over the 180 min of observation. This suggests a stable model for the study.

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Effects of IRI on hepatic microcirculation

Our study shows a significant fall in sinusoidal perfusion and velocity of flow in the IRI group which was observed at all time points. Increased venular neutrophil adhesion, hepatocyte cell death, sinusoidal neutrophil stasis in the IR group with significantly increased serum transaminase levels in IRI and a significantly lower perfusion index compared with Sham animals were observed. Leukocyte adhesion causes endothelial injury, RBC stasis, modulation of sinusoidal tone and perfusion in hepatic IR.[22,23] Mechanisms which contribute to failure of perfusion include endothelial swelling and injury, leukocyte endothelial interaction, leukostasis, alteration in RBC flow and increased viscosity of blood.[22,23,38–40] Both Perfusion failure and venular adhesion have been shown to correlate with hepatic dysfunction, reduced bile flow and increased transaminases.[39] These studies support our observations in the hepatic IR model in this study. Reduced RBC velocity seen in hepatic IR in our study is explained by previous studies which suggest that free radical release during IRI leads to erythrocyte damage, increased deformity and sluggishness of RBC causing reduced RBC velocity, erythrocyte flux and increased viscosity of blood.[39]

Effects of RIPC on hepatic microcirculation in IRI

This study demonstrates that RIPC increases RBC velocity and erythrocyte flux across sinusoids, significantly reduced hepatocyte cell death, increased sinusoidal perfusion and sinusoidal flow as compared with the IR group. The serum transaminase levels in the RIPC+IR group were lower than IRI. These observations suggest an improvement in liver function in the RIPC+IR group as compared with IR group. Interestingly there is a significant initial rise in RBC velocity and flow in the RIPC+IR group as compared with Sham and IR although the initial velocities in Sham and IR are not significantly different. However, at 120-min reperfusion a significant drop in velocity is seen in the IR group as compared with Sham whereas the velocity in RIPC+IR is similar to the Sham group. These observations suggest that the initial rise in baseline velocity in the RIPC+IR group acts as a protective mechanism against the deleterious effects of IR and prevents a fall in velocity and sinusoidal perfusion below Sham values as reperfusion injury progresses over a period of time. The initial molecular pathway responsible for the increased baseline velocity is unclear but it is likely to be induced by RIPC in the hepatic ischemia period itself as the velocity of flow is seen to be high at 30 min of reperfusion.

A significant reduction in both venular neutrophil adhesion and sinusoidal adhesion implies modulation of neutrophil activation by RIPC leading to decreased oxidative stress, IRI and perfusion failure. Previous studies[41] have shown ischemic preconditioning to reduce hepatic IR-induced perfusion failure and preservation of mitochondrial redox state but none have demonstrated the role of RIPC in hepatic IR. Studies have shown that reduction in RBC velocity occurs because of leukocyte adhesion, endothelial injury and perfusion impairment (no reflow phenomenon) and increased RBC damage as a result of free radical release during IR. The observations in our study suggest that RIPC modulates hepatic microcirculation and may induce protective mechanisms which protect RBC against free radical induced damage; however, this needs to be investigated further.

Effects of RIPC on sham

Remote ischemic preconditioning in the normal Sham group showed a reduced RBC velocity and sinusoidal flow at 120 min as compared with 30 min and recovery to baseline at 180 min. This change in velocity was not statistically significant. This maybe as a result of initial preconditioning induced oxidative stress followed by development of a tolerance to the low-grade oxidative stress. This observation is supported by Chen *et al.*[20] who demonstrated that limb RIPC produced an increase in free radical levels in the blood of preconditioned animals which served as a trigger to induce heat shock protein expression in remote skeletal tissues and confer protection against skeletal IR. In comparison with the Sham group, the velocity of flow and sinusoidal flow in RIPC+ Sham was less at 120 min and 180 min as a result of low-grade oxidative stress induced in a normal liver.

Potential pathway and mechanisms in remote ischemic preconditioning of the liver

Studies have shown RIPC-induced NO in the heart to reduce myocardial IRI which was not abolished by neurogenic blockade,[19] increase in plasma free radicals after brief limb RIPC which induced heat shock proteins in remote skeletal muscle[20] and increased hemoxygenase (HO-1) in hepatic macrophages after limb preconditioning the source of which was not peripheral lymphocytes.[24] These data suggest that brief limb ischemia leads to release of biochemical messengers such as free radicals in the blood which may induce oxidative stress in the remote organ and HO-1 expression. HO-1 is known to promote degradation of hem, increase CO production and scavenge free radicals resulting in improved hepatic function. *In vivo* studies of the liver have shown cells, which are hepatic sinusoid-associated pericytes primarily responsible for CO-mediated blood flow regulation in sinusoids.[42,43] The role of free radicals as messengers in inducing HO-1 in the liver, the pathway for HO-1 induction in the liver after brief limb ischemia and the role of HO-1 in modulation of hepatic microcirculation and endothelial function needs to be investigated further. The findings in this study suggest decreased neutrophil activation, decreased endothelial adhesion and injury resulting in improved perfusion and flow in the hepatic microcirculation. These data suggest that RIPC may induce a decrease in endothelial adhesion molecule expression through HO-1. HO-1 is known to reduce endothelial ICAM expression[30,42,44] and this needs further investigation in animal models of remote ischemic preconditioning.

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Conclusion

RIPC protects the liver from IRI by modulation of the hepatic microcirculation. Our study using intravital microscopy demonstrates that increased hepatic blood flow after RIPC is as a result of increased RBC velocity, sinusoidal perfusion and decreased neutrophil adhesion and cell death. Future animal studies need to focus on the role of RIPC in animal transplant models and the use of HO-1 knockout models to clarify the role of HO-1 pathways in the regulation of endothelial function. Clinical trials need to investigate the role of RIPC in reducing morbidity and mortality in patients undergoing liver transplant and resection surgery.

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Conflicts of interest

None declared.

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