HAEMOPERFUSED PORCINE KIDNEY MOI

Thesis submitted for the degree of

Doctor of Medicine

at the University of Leicester

by

Simon John Francis Harper

University of Leicester

2008

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

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ABBREVIATIONS

8-oxo-dG 8-Hydroxy-2'-deoxyguanosine

8-isoPGF$_2\alpha$ 8-isoprostane

2-MAC 20-methyl aminochroman

AChE Acetylcholinesterase

AE-ITU Aminoethyl-isothiourea

AIF Apoptosis inducing factor

AMC Amino-4-Methyl Coumarin

AMP Adenosine monophosphate

ANOVA Analysis of variance

ATN Acute tubular necrosis

ATP Adenosine triphosphate

AUC Area under the curve

CAN Chronic allograft nephropathy

CIT Cold ischaemic time

CoPP Cobalt protoporphyrin

cNOS Constitutive nitric oxide synthase

CS Cold storage

DFO Deferoxamine

DGF Delayed graft function

DIC Disseminated intravascular coagulation

DNP Dinitrophenylhydrazine

ECMO Extracorporal membranous oxygenation

EMS Exsanguinous metabolic support

ET-1 Endothelin-1

GFR Glomerular filtration rate

GSH Reduced glutathione

GST Glutathione-S-transferase

Hb Haemoglobin

HBD Heart-beating donor

Hcrit Haematocrit

HES Hydroethylstarch

HO-1 Haem-oxygenase 1

ICAM 1 Intercellular adhesion molecule 1

IFN γ Interferon γ

iNOS Inducible nitric oxide synthase

IOPS Isolated organ perfusion system

IP Ischaemic preconditioning

IRI Ischaemia-reperfusion injury

LDB Leucocyte depleted blood

L-NAME N^G-nitro-L-arginine methyl ester

L-NIL L-N^6-(1-iminoethyl)lysine

L-NMMA N^G-monomethyl L-arginine

MAC Membrane attack complex

MAP Mean arterial pressure

MBL Mannose binding lectin

MHC Major histocompatibility complex

MIP-2 Macrophage inflammatory protein 2

MP Machine perfusion

NAD Nicotinamide adenine dinucleotide

NAG N-Acetyl-β-D glucosaminidase

NKC Natural killer cell

NHBD Non-heart-beating donor

NOS Nitrogen oxide synthase

PARP Roly (ADP-ribose) polymerase

PCT Proximal convoluted tubule

PFR Plasma flow rate

PGE1 Prostaglandin E1

RBC Red blood cell

RBF Renal blood flow

RVR Renal vascular resistance

PNF Primary non-function

ROS Reactive oxygen species

TNF α Tumour necrosis factor α

UNOS United Network of Organ Sharing

UW University of Wisconsin

VCAM 1 Vascular cell adhesion molecule 1

VS Viability score

vWF Von Willebrand factor

WB Whole blood

WIT Warm ischaemic time

PUBLICATIONS AND PRESENTATIONS GENERATED BY THIS RESEARCH

Harper, S, Hosgood, S, Kay, M, Nicholson, M (2006). Leucocyte depletion improves renal function during reperfusion using an experimental isolated haemoperfused organ preservation system. *Br. J. Surg.* **93**: 623-629

Harper, S, Hosgood, S, Waller, H, Yang, B, Kay, M, Goncalves, I, Nicholson, M (2008). The effect of warm ischaemic time on renal function and injury in the isolated haemoperfused kidney. *Transplantation. (in press)*

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ABSTRACT

Ischaemia reperfusion injury (IRI) is a major contributor to short and long-term renal allograft dysfunction, particularly in kidneys from non-heart-beating donors (NHBD). The increasing use of NHBD kidneys has generated renewed interest in isolated organ perfusion as a means of graft resuscitation and evaluation. The aim of this study was to design and validate an isolated kidney haemoperfusion system while investigating the effects of three key factors influencing early graft injury and function; leucocyte activity, warm ischaemic time (WIT) and perfusion pressure.

Porcine kidneys were perfused with normothermic oxygenated autologous blood on an isolated organ perfusion system (IOPS) designed using cardiopulmonary bypass technology. Physiological and biochemical parameters were measured throughout the 6 hour perfusion period. Interval serum, urine and tissue samples were taken for physiological analysis, histological evaluation and assays measuring oxidative tissue injury, apoptosis and endovascular injury.

Kidneys perfused with leucocyte-depleted blood functioned significantly better than those perfused with whole blood in terms of creatinine clearance, oxygen consumption, acid-base homeostasis and renovascular haemodynamics. Haemoperfused kidneys demonstrated functional deterioration in parallel with increasing periods of warm ischaemia (7, 15, 25 and 40 minutes). Increasing WIT was also associated with elevated serum markers of oxidative protein and lipid injury and these correlated accurately with functional parameters. In contrast, elevated caspase 3 activity was associated with better renal function. A higher perfusion pressure of 95mmHg was associated with significantly improved renal function compared to sub-physiological pressures without increasing endovascular injury.

The IOPS represents a reliable and versatile model of IRI and as such has demonstrated that leucocyte depletion, WIT and perfusion pressure significantly affect early graft injury and function. The system offers extensive scope as a tool for evaluating IRI ameliorating interventions and in clinical organ viability assessment and preservation.

ACKNOWLEDGMENTS

I would like to thank all the staff in the Transplantation Department at the University of Leicester for their help and support.

I am extremely grateful to Sarah Hosgood for the enormous amount of skill and effort she put into setting up and running the model and for her help and friendship during this study. I would also like to thank Helen Waller and Bin Yang for their great help with the oxidative and apoptotic assays.

I am very grateful to my fellow registrars Atul Bagul, Mark Kay, James Yates and Monika Kaushik for being superb colleagues.

My sincerest thanks and appreciation go to Professor Nicholson for the exemplary supervision he provided. His knowledge, leadership and enthusiasm played a major part in making my time in the transplant department so enjoyable and rewarding.

I would like to thank my family for all their love and support.

My deepest thanks go to my wonderful wife Inês for all her patience, encouragement and intelligence in helping me with this work.

CHAPTER 1

Ischaemia Reperfusion Injury in Renal Transplantation

Introduction

Ischaemia-reperfusion injury (IRI) is a key event in numerous human pathophysiological processes. IRI arises from the paradox that following a period of ischaemia, tissue reperfusion with oxygenated blood, although generally favourable, can cause significant damage through generation of reactive oxygen species (ROS)[1]. The investigation, prevention and treatment of IRI remain major areas of interest after decades of scientific research. Clinical situations influenced by IRI are wide ranging and include coronary artery disease, stroke and organ transplantation.

Ischaemia-reperfusion injury is a central process in renal transplantation. All renal allografts sustain variable periods of both normothermic and hypothermic ischaemia prior to reperfusion following implantation. The early pathological clinical manifestations of allograft IRI are delayed graft function (DGF) and rarely primary non-function (PNF). Primary non-function is disastrous as recipients undergo a surgical procedure offering no benefit and they are often immunologically sensitised for future transplantation. Delayed graft function is associated with prolonged hospital stay, additional invasive procedures, ongoing dialysis costs and psychological morbidity in the recipient[2]. In addition, delayed graft function is also associated with higher rates of acute rejection and acceleration of chronic allograft nephropathy[3, 4]. A number of risk factors for DGF have been identified including donor age and co-morbidity[5], pre-transplant haemodialysis[6], calcineurin inhibitor-based immunosuppression[7] and cold ischaemic time[8]. However post-transplant IRI is of particular significance in grafts from non-heart-beating donors (NHBD) when compared to heart-beating

cadaveric and live donors due to the prolonged period of primary warm ischaemia they sustain[9].

Non-heart-beating donor kidney transplantation

Renal allografts from NHBDs are associated with a higher incidence of DGF and PNF but their increasing importance in transplant programmes is driven by the growing disparity between the renal transplant waiting list and available donor organs[10]. The number of brainstem death donors has fallen significantly worldwide due to factors including improved road safety and changes in neurosurgical practice. In particular, the increased use of decompression techniques, such as free-floating scalp flap elevation, for patients with major head injury has lead to a degree of shift in donor demographics from HBD to NHBD[11]. In this context, several transplant centres report that an increase in NHBD activity serves only to maintain as opposed to expand transplant activity[12, 13]. In some countries such as Spain and Japan however, extensive NHBD programmes have been found to significantly reduce transplant waiting lists[14, 15].

The use of NHBD to provide much needed organs must however, be justified through clinical outcomes following transplantation. Nicholson analysed pooled data from 7 comparative studies published between 1990 and 2002 and found that the rate of PNF in NHBD was 5.8% compared to 1.3% in HBD transplants[16]. This higher incidence of PNF in NHBD kidneys is often seen as the most important barrier to wide spread acceptance of NHBD programmes. In a review of European activity the reported incidence of PNF in NHBD grafts varied significantly between 4 and 14%[11]. Furthermore, a review of NHBD outcomes in

the United Kingdom found the incidence of PNF in controlled NHBD kidneys (Maastricht Category III / IV) was 0% compared to 19.5% in uncontrolled (Maastricht Category I / II) (*Table 1*). Together these findings indicate that the higher incidence of PNF is potentially avoidable, primarily through judicious selection of NHBD kidneys used for transplantation by avoiding those with irreversible ischaemic injury[17]. Several groups have reported significant reduction in PNF as a result of organ viability assessment mainly centred on the use of organ perfusion to analyse criteria such as flow dynamics and perfusate enzyme concentrations[18, 19].

The other key concern over NHBD organs is that the incidence of DGF remains significantly higher compared to HBD kidneys, in the range of 50 – 100%[20]. In HBD grafts, DGF is associated with impaired graft function, reduced graft survival and higher rates of acute and chronic rejection[3, 4, 21]. However DGF appears not to affect long term function in NHBD kidneys in the same way. The Leicester group found that despite significantly differing early function there was no significant difference in serum creatinine levels at 1 yr or in 5 year graft survival when comparing living donor, HBD or NHBD transplant recipients[22]. A similar pattern is observed by other centres using both controlled and uncontrolled NHBD, reporting 5 year survival in the range 54-78% for NHBD compared with 55-85% in HBD[14, 23-25]. There are now several groups reporting comparable 10 year graft survival for NHBD and HBD kidneys[9, 26, 27]. The greater susceptibility of HBD kidneys to long-term dysfunction following DGF is thought to relate to the deleterious effect of brainstem death. Brainstem death is associated with significant physiological changes leading to renal injury including autonomic storm causing haemodynamic instability, renal afferent arteriole spasm and significant hormonal and electrolyte imbalance[28]. In addition there may be significant coagulopathy leading to disseminated intravascular coagulation (DIC) and pro-inflammatory cytokine release[29].

Uncontrolled donors also benefit from the absence of the morbidity associated with a prolonged intensive care admission.

The evidence relating to acute rejection rates in NHBD grafts is unclear. Certainly DGF in general is associated with an increased risk of acute rejection relating to cytokine and ROS generated upregulation of MHC class II molecules during IRI[30, 31]. A report looking at UNOS (United Network of Organ Sharing) data in the US showed a higher but acceptable incidence of rejection in NHBD (19% vs 14%)[32]. In a review of the European experience, only Pacholcyzk et al from Poland reported a significant difference in the incidence of AR in NHBD vs. HBD grafts (66% vs 46%)[11, 33]. In addition, in a study comparing mainly Category I NHBD with HBD kidneys, donor brainstem death was in fact the variable most strongly associated with increased risk of acute vascular rejection[15].

Chronic allograft nephropathy (CAN) is the leading overall cause of renal allograft loss and is associated with prolonged warm ischaemia and reperfusion injury, such as that sustained by NHBD grafts[34, 35]. However comparable long-term survival data comparing NHBD and HBD organs would suggest little difference in the incidence of CAN[9, 26, 27]. Baines et al compared biopsies 1 year post-transplantation in NHBD and HBD organs and found no significant difference in graft fibrosis, the key mode of injury leading to chronic allograft dysfunction[36].

The key variable peculiar to NHBD organs is the sustained warm ischaemic time but a clear relationship between WIT and graft outcome has not been established. A broad range of acceptable WIT limits of 30 to 150 minutes are advocated in different centres[37, 38]. Castelao et al found that grafts from controlled NHBDs had a similar rate of DGF to HBD (34%) but those from uncontrolled had a much higher rate (88%) suggesting that the difference in WIT

influenced early function[25]. The effect of WIT on the incidence of PNF remains unclear. Shiroki et al found that WIT greater than 30mins was associated with higher rates of PNF in a series of NHBD transplants[39]. Takai et al also reported significantly longer WIT in grafts with PNF although the sole risk factor for DGF was in fact prolonged CIT[40]. In contrast other groups found no relationship between WIT and PNF[14, 41].

The increasing use of NHBD kidneys has led to much investigation into approaches to improve outcome through amelioration of IRI. Various organ retrieval techniques have been advocated including total body donor cooling with cardiopulmonary bypass and extracorporal membranous oxygenation (ECMO)[42, 43]. The minimisation of nephrotoxic immunosuppression for recipients of NHBD kidneys has also been shown to significantly improve early graft function and many protocols have been established[44]. A key area of interest is the use of hypothermic and normothermic machine perfusion to evaluate organ viability and resuscitate damaged grafts prior to transplantation (see next chapter). Finally, a range of interventions under investigation, aimed at ameliorating IRI in allografts, are likely to provide most benefit in the context of NHBD transplantation.

Pathophysiology of renal allograft ischaemia-reperfusion injury

Histopathology

Renal IRI following transplantation is histopathologically akin to acute tubular necrosis (ATN) in the allograft. The focus of most injury is in the proximal tubule and renal microvasculature as a result of oxidative injury, inflammation and cell loss secondary to

necrosis and apoptosis. A variable degree of irreversible injury to the distal segment (S3) of the proximal tubule is seen during ischaemia-reperfusion. This results in shedding of necrotic tubular cells into the lumen causing impaired filtrate flow and back pressure which in turn reduces glomerular filtration rate (GFR)[45]. Impedance to flow is increased further as a result of the gelatinisation of Tamm-Horsfall protein in the loop of Henle. A significant change in the solubility of this protein secreted by the ascending limb occurs as the result of elevated sodium concentration in the post-ischaemic nephron[46]. Endothelial injury, microvascular spasm and sludging of erythrocytes and leucocytes lead to capillary occlusion, particularly in the outer medullary inner stripe[47]. The resultant reduction in glomerular transcapillary pressure reduces GFR further and slows clearance of tubular debris and recovery of renal function.

Ischaemia

A number of changes occur during tissue ischaemia leading to direct injury and priming for additional injury on reperfusion. Cellular ATP levels fall due to reduced production and rapid dephosphorylation to adenosine monophosphate (AMP) in the context of impaired oxidative metabolism. AMP is further degraded to hypoxanthine via adenosine and inosine[48]. Depleted ATP leads to ATP-dependent ion channel dysfunction and loss of electrolyte homeostasis. Inward diffusion of Na^+ and water causes cell swelling and elevation of intracellular Ca^{++} concentration stimulating increased phospholipase and protease activity[49]. In particular, calcium-dependent cleavage of xanthine dehydrogenase to produce xanthine oxidase increases significantly[50]. Anaerobic glycolysis during ischaemia leads to formation of metabolites such as lactic acid. The subsequent fall in intracellular pH causes lysosomal

membrane instability with activation of lytic enzymes and inhibition of free metal ion binding to carrier proteins[51, 52].

Reperfusion and reactive oxygen species

The accumulation of xanthine oxidase, hypoxanthine and free metal ions during ischaemia serve to create an environment in which, on reperfusion, an array of reactions lead to the generation of reactive oxygen species[53]. The reaction of hypoxanthine with xanthine oxidase to form xanthine and uric acid in the presence of oxygen generates superoxide ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2)[54]. Superoxide is also generated from leakage of electrons from the mitochondrial electron transport chain causing univalent reduction of oxygen. The inflammatory response associated with reperfusion includes recruitment of cells capable of generating $O_2^{\cdot -}$ through phagocytic respiratory burst[55]. Ischaemia itself is associated with accumulation of $O_2^{\cdot -}$ and H_2O_2 due to reduced activity of energy-dependent manganese superoxide dismutase which converts $O_2^{\cdot -}$ to H_2O_2 and glutathione which reduces H_2O_2 to water[56, 57].

Superoxide and H_2O_2 are relatively low energy ROS but are responsible for production of highly reactive species. Free intracellular iron (Fe++) generated through impaired carrier protein binding, reduces H_2O_2 to the highly reactive hydroxyl radical ($OH^{\cdot}$)[58]. The importance of this reaction is supported by the ability of iron chelators such as desferrioxamine to reduce IRI[59]. Generation of nitric oxide (NO), itself a ROS, occurs through upregulation of nitric oxide synthase (NOS) during IRI[60]. Nitric oxide can react with $O_2^{\cdot -}$ to eventually produce a non-radical nitrate ion (NO_3^-) and thus provide a protective role[61]. However the same reaction

can generate peroxynitrite ($ONOO^-$), a reactive species shown to induce lipid peroxydation and cell death and produce $OH^{\cdot}$ on decomposition[62, 63]. Neutrophils, in addition to producing $O_2^{\cdot -}$, also generate reactive hypochlorous acid (HOCl) from H_2O_2 using myeloperoxidase. HOCl can also react with $O_2^{\cdot -}$ to produce further highly injurious $OH^{\cdot}$[64].

Reactive oxygen species exert direct injury through reaction with lipids, proteins and DNA causing cell dysfunction and loss through necrosis and apoptosis via several mechanisms. DNA strand breakage has been shown to activate poly (ADP-ribose) polymerase (PARP) and deplete nicotinamide adenine dinucleotide (NAD^+), an important co-factor for glycolysis, and ATP leading to cell death[65]. Cell protein denaturation and dysfunction occurs particularly through tyrosine nitration by peroxynitrate[66]. In renal IRI peroxynitrate also impairs the adhesion of the tubular epithelial cells to the basement membrane contributing to tubular obstruction[67]. Lipid injury is primarily through peroxidation and leads to distruption of cell membrane structure and function. In addition to direct injury ROS play a key role in upregulation of the inflammatory process central to IRI.

Leucocytes

Neutrophils have been identified as the initial mediators of the inflammatory component of renal IRI. Generation of ROS and subsequent lipid peroxidation in endothelial and parenchymal tissues leads to the rapid production of cytokines and chemokines which attract neutrophils and monocytes to tissues sustaining IRI[68]. Early upregulation of chemoattractants including interleukin-8 and macrophage inflammatory protein 2 lead to neutrophil infiltration within 1 hour of reperfusion[69, 70]. Leucocytes are then recruited to sites

of inflammation through upregulation of adhesion molecules driven by endothelial activation by local ROS and cytokines including tumour necrosis factor α (TNF α), interferon γ (IFN γ) and interleukins 1, 2 and 8[75]. Initial rolling adhesions are formed by the selectins (E, P and L-selectin) allowing close association of leucocytes to endothelial cell chemokines. Leucocyte immobilisation and diapedesis are then mediated by the immunoglobulin-like molecules intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) binding to ligands β1-integrin and β2-integrin on leucocytes[71].

A second phase of chemokine expression involving interferon-gamma inducible protein 10 and chemoattractant protein 1 facilitates further recruitment of neutrophils and also monocytes, natural killer cells (NKC) and T cells[68]. In addition, components of the activated complement cascade such as C5a, act as potent chemoattractants for leucocytes. Neutrophils themselves perpetuate the process through further production of superoxide, nitric oxide, hypochlourous acid and cytokines[72]. The subsequent inflammatory response leads to direct tissue damage through release of proteases and upregulates apoptosis through cytokines interleukin 10 and TNF α[73]. In addition to this it is thought that the magnitude of leucocyte adhesion to the endothelium contributes to microvascular occlusion during reperfusion, the so-called 'no reflow' phenomenon[74]. Furthermore, vasoactive factors such as leucotrienes produced by leucocytes are thought to contribute to microvascular spasm and impaired perfusion[75].

Complement

Complement has been shown to be an important mediator of renal IRI[76-78]. The complement cascade is activated via three pathways: the classical (activated antigen-antibody interaction), the alternative pathway (activated by microbial products) and the mannose binding lectin (MBL) pathway (activated by mannose present on microbial surfaces). These pathways converge at the cleavage of C3 leading to the activation of C5, generation of anaphylatoxins C3a and C5a and the membrane attack complex (MAC) or C5b – C9. Factors C3a and C5a mediate recruitment and activation of neutrophils and macrophages to produce cytokines and adhesion molecules[79]. In addition C5a acting through the C5a receptor (C5aR) has been shown to induce apoptosis directly[80]. The MAC, in addition to causing cell death through both necrosis and apoptosis, has been shown to attract and activate neutrophils, generate cytokines and upregulate adhesion molecules[81-83].

Experimental evidence lends support to the alternative and MBL pathways being predominant in renal IRI although the classical pathway is strongly implicated in IRI involving other tissues[84]. C4-deficient mice, in contrast to C3-deficient mice and mice with no functional alternative pathway (factor B -/- mice), were not protected from renal IRI. De Vries et al demonstrated that MBL deposition preceded and co-localised with components of the MAC in murine and human renal IRI[77]. In addition they found that complement activation could mediate renal IRI through neutrophil-dependent and -independent mechanisms[80].

Nitric oxide

Nitric oxide plays a key role in normal renal physiology and is thought to have both potentially adverse and protective effects during IRI[85]. It is formed from L-arginine by two isoforms of nitric oxide synthase (NOS); constitutive NOS (cNOS) generally localised to the pre-glomerular vasculature and macula densa, and inducible NOS (iNOS) more widely distributed with the exception of renal vasculature and macula densa[86]. Nitric oxide produced by iNOS appears to contribute to renal IRI whereas cNOS provides a protective role. Several animal studies have shown that selective inhibition or absence (using iNOS knockout mice) of iNOS can significantly reduce the severity of renal IRI[62, 87, 88]. In contrast non-selective inhibition of both isoforms of NOS with N^G-monomethyl L-arginine (L-NMMA) are associated with increased vasoconstriction and microvascular thrombosis as the protective role of cNOS upregulation in maintaining blood flow is impaired[89-91]. Furthermore selective inhibition of cNOS with N^G-nitro-L-arginine methyl ester (L-NAME) was found to worsen renal perfusion function in a rodent model of endotoxic shock[92]. The pathological role of NO produced by iNOS relates to formation of the reactive species peroxynitrite (and subsequently hydroxyl) through reaction with superoxide. In addition nitric oxide has been shown to increase formation of cytokines, upregulate adhesion molecule expression, increase neutrophil infiltration and increase endothelial permeability[93, 94]. It is also suggested that the dual role for NO during IRI may be biphasic. NO appears to be predominantly cytotoxic shortly after reperfusion (through iNOS) but essential in maintaining blood flow to ameliorate secondary injury over the longer term (through cNOS)[95].

Microvascular endothelium

Elevated renovascular resistance particularly across the afferent arterioles is an important pathophysiological element to renal IRI. This is in part secondary to the fall in the glomerular transcapillary hydraulic pressure gradient and back pressure from damaged and obstructed proximal tubules[96, 97]. However, direct injury to endothelial cells in IRI leads to elevated local levels of thromboxane and increased production of endothelin-1(ET-1), both potent vasoconstrictors. In addition to vasospasm ET-1 has been shown to upregulate leucocyte adhesion molecules and activate complement[98, 99]. Elevated levels of ET-1 are found in patients with delayed graft function and increased expression of ET-1 mRNA and protein in peritubular capillaries of post-ischaemic kidneys[100, 101].

Apoptosis

Apoptosis plays a significant role in renal IRI and is mediated via two main convergent pathways mediated by a cascade of proteolytic enzymes, the caspases[102]. The death receptor dependent pathway is activated by extrinsic ligands produced during inflammation such as TNF- α and Fas-ligand and is mediated through caspase 8[103]. The death receptor independent pathway is activated by release of intra-cytosolic factors cytochrome c and apoptosis inducing factor (AIF) leading to activation of caspase-9[104]. The release of cytochrome c and AIF is controlled by the mitochondrial Bcl-2 protein family within which there are stimulatory factors including Bax and Bak and inhibitors such as Bcl-2[105]. The caspase cascade is activated through step-wise cleavage of proenzymes converging on effector caspases, in

particular caspase 3 and 7[106]. Proteolytic effector caspases disrupt cytoskeletal and nuclear architecture leading to characteristic changes of cell shrinkage, nuclear fragmentation and plasma membrane blebbing. This ultimately leads to cell dissolution and formation of apoptotic bodies which are phagocytosed. Apoptotic bodies have also been shown to initiate an inflammatory response during renal IRI[107]. Furthermore, caspase-1 has been shown to modulate the inflammatory response directly through the maturation of Il-1β and Il-18[108, 109].

It has been demonstrated that apoptotic tubular cells can be identified in renal allografts as early as 20 minutes after reperfusion and the degree of apoptosis in transplanted kidneys has been shown to predict early renal function[110, 111]. Administration of specific caspase inhibitors has been shown to reduce inflammation and ameliorate tissue injury in small animal studies of renal IRI[107]. Salahudeen et al found that although necrosis of human renal PCT cells predominated during cold storage, on re-warming a significant proportion of cell loss was apoptotic[112]. However it remains unclear whether apoptotic signalling is primed during cold ischaemia prior to activation on reperfusion[113, 114]. Certainly the length of cold ischaemia has been shown to correlate with the degree of apoptosis in cadaveric renal allografts following reperfusion[115]. The balance between necrosis and apoptosis causing cell loss in renal allografts appears to relate to the severity of IRI, the later being associated with milder injury[116].

Methods to attenuate renal ischaemia reperfusion injury

Anti-oxidants

A range of anti-oxidant approaches have been applied to ameliorating renal IRI although promising experimental findings have rarely been mirrored in clinical trials. Organ preservation solutions in current clinical use contain various anti-oxidants but their precise benefit is often unclear. University of Wisconsin (UW) solution for example, contains glutathione among numerous components aimed at reducing IRI[117]. However it has been shown that reduced glutathione (GSH) added to UW is rapidly oxidised following manufacture and is thus likely to offer little benefit during organ storage[118]. Allopurinol, a xanthine oxidase inhibitor, is also widely used in preservation fluids as a logical choice of pharmacological intervention, although animal models examining effects on renal IRI in isolation have shown contradictory results[119, 120].

Numerous anti-oxidants have been investigated in experimental models aimed at clinical application. Potent antioxidants 20-methyl aminochroman (2-MAC) and deferoxamine (DFO) were evaluated as additives to UW solution used to preserve primary human proximal tubular cells in a model of renal IRI. Cell loss through both necrosis during cold storage and apoptosis during reperfusion was significantly reduced by both compounds[112]. However the clinical application of DFO has been hindered by a short half-life and toxicity, despite attempts to modulate this by binding to compatible polymers[121]. Anti-oxidant Lazaroid U74006F has been shown to improve renal function in animal transplant models by reducing expression of cytokines and iNOS and attenuating iron-mediated lipid peroxidation[122, 123]. In

addition, N-acetylcysteine, an effective ROS scavenger, has also been shown to reduce renal IRI in rats although neither has been trialled in clinical transplantation[124]. Superoxide dismutase conferred significant protection against renal IRI in rats but when applied clinically, a randomised trial using human recombinant superoxide dismutase demonstrated no reduction in DGF following renal transplantation[125, 126]. One example of a clinically proven anti-oxidant is propionyl-L-carnitine, an acyl anti-oxidant involved in mitochondrial ATP generation, having been shown to ameliorate post transplant IRI in the laboratory and clinical transplantation[127].

With the increasing awareness of the role of NO in IRI, selective inhibition of iNOS has been identified as a potential therapeutic target. L–N^6-(1-iminoethyl)lysine (L-NIL) and aminoethyl-isothiourea (AE-ITU) are relatively selective inhibitors of iNOS and reduce severity of renal IRI in a rat model[87]. In addition these inhibitors can exert other non-specific anti-oxidant and anti-inflammatory actions such as inhibition of PMN infiltration and reduction in endothelin-1 release[128]. More recently the highly specific iNOS inhibitor GW274150 has been shown to reduce post ischaemic renal injury, improve function and reduce formation of peroxynitrate[129].

Anti-inflammatory

Extensive investigation into the potential beneficial effects of attenuating the inflammatory response during renal IRI has been undertaken. Leucocyte depletion in animal models has been shown to reduce the severity of renal IRI. Klausner et al found that neutrophil depletion using a polyclonal rabbit anti-rat neutrophil serum following 45 minutes of renal

ischaemia and reperfusion improved renal function and reduced structural damage[75]. In an *ex-vivo* rabbit kidney model filtering of white cells from the haemoperfusate significantly improved renal function and reduced IRI injury[130]. Clinical trials examining the effect of leucocyte depletion on IRI have focused on myocardial ischaemia and the results have been equivocal[131] despite promising results in animal models[132].

Inhibition of adhesion molecules has been highlighted as a potential therapeutic target. Mice deficient in ICAM-1 have been shown to be protected against renal IRI[133] and blockade of ICAM-1 expression with antisense deoxynucleotides reduced early and late sequelae of renal IRI in rats[134]. However enlinomab, a monoclonal antibody to ICAM-1, did not reduce the incidence of DGF compared to placebo in a large randomised control trial[135]. Treatment with soluble P-selectin glycoprotein ligand has also been shown to reduce leucocyte infiltration, reduce injury and improve renal function in rat models of renal ischaemia and transplantation[136, 137]. In addition, numerous small animal studies have shown attenuation of renal IRI following inhibition/blockade of cytokines including TNF-α[138], macrophage inflammatory protein 2(MIP-2)[69], monocyte chemoattractant protein 1[139] and interleukin 8[20].

Inhibition of complement factor activation in animal studies has had equivocal results. In a murine model of renal IRI de Vries et al demonstrated that blockade of factor C5 activation with the BB5-1 antibody significantly reduced inflammation and late apoptosis[78]. In contrast, Park et al reported that C3 convertase inhibition did not protect against IRI in their murine model[140].

Pharmaceutical intervention

A number of drugs, often licensed for treatment of unrelated pathology, have been investigated for use in ameliorating renal IRI. Clinical studies have shown addition of a calcium antagonist to preservation fluid or recipient post-transplant treatment regimen significantly improves early graft function[141, 142]. Proposed mechanisms of action for calcium blockade include inhibition of calcium dependent conversion of dehydrogenase to oxidase, inhibition of phospholipase and protease activity and antagonism of ET-1 induced vasoconstriction[49, 143].

There has been increasing interest in the potentially beneficial effects of statins through mechanisms unrelated to lipid lowering properties and these may extend to ameliorating IRI[144, 145]. Cerivastatin has been shown to improve outcome following acute ischaemic renal failure through inhibition of nuclear factor κB and protein 1 activation and upregulation of anti-inflammatory cytokine interleukin 6[146]. Statins have also been found to attenuate endothelial MHC class II expression and increase endothelial NOS and fibrinolytic activity during inflammation[147].

The role of vasodilators, particularly those derived from the cyclo-oxygenase pathway, has been investigated. Kidneys perfused with Iloprost, an prostacycline analogue, prior to transplantation had a lower rate of PNF[148]. Prostaglandin E1 (PGE1) incorporated into pulsatile hypothermic perfusion has also been shown to improve early graft function compared to conventional cold storage[149]. In addition to vasodilatation, PGE1 may confer protection against IRI through stabilisation of endothelial cell membranes and inhibition of platelet aggregation and neutrophil activation[150, 151].

Ischaemic Pre-conditioning

Ischaemic preconditioning (IP) is a process by which a planned short period of ischaemia is employed to upregulate intrinsic protective mechanisms within tissue prior to a major ischaemia-reperfusion insult. Various IP protocols have been successfully applied to cardiac IRI for many years and more recently this approach has been extended to other domains including models of renal transplantation[152, 153]. The underlying mechanisms of IP are complex and numerous mediators have been implicated including NO, Mitochondrial K_{ATP} and heat shock proteins, in particular haem-oxygenase 1[154].

Haem-oxygenase 1 (HO-1) is an inducible heat shock protein shown to ameliorate IRI in models of heart, liver and renal transplantation[155-157]. HO-1 expression is upregulated by a number of stimuli including endotoxaemia, hyperthermia and ischaemia-reperfusion. The rate limiting step in the oxidative degeneration of haem to biliverdin, free iron and carbon monoxide (CO) is catalysed by the HO-1 isoform[158]. Biliverdin and subsequent reduction product bilirubin have an anti-inflammatory action through the inhibition of the complement cascade[159]. Increase in free iron stimulates production of ferritin, a protein with anti-oxidant activity[160]. Carbon monoxide has generated significant interest as a potential ameliorator of IRI having been shown to inhibit inflammatory signalling, inhibit platelet aggregation and cause vasodilatation[161]. Otterbein *et al* demonstrated that CO inhibits the expression of lipopolysaccharide-induced pro-inflammatory cytokines such as tumor necrosis factor-α, interleukin-1β, and macrophage inflammatory protein-1β while simultaneously increasing expression of the anti-inflammatory cytokine interleukin-10[162]. CO has also been shown to

inhibit apoptosis via a p38 mitogen-activated protein kinase dependant pathway involving caspase 3 during IRI[162].

Anti-apoptotic

A range of endogenous and exogenous compounds have been shown to have anti-apoptotic activity which may protect against IRI. In particular growth factors such as IGF-1 have been shown to reduce apoptosis in animal models of renal IRI although without conferring any benefit to patients with acute renal failure[107, 163]. Another approach shown to reduce apoptosis during IRI in a murine model, is removal of death receptor ligand TNF-α using a monoclonal antibody[73]. However, non-specific inhibition of apoptosis could potentially be deleterious in the context of transplantation. Impaired removal of allogenic T-cells may predispose to allograft rejection[164] and the deletion of excessive, damaged or non-functioning renal cells and inflammatory cells by apoptosis may be important for repair of allografts following IRI[165]. More specific caspase inhibitors Z-VAD-FMK and B-D-FMK have been shown to reduce acute and chronic renal injury in small animal models of IRI and may therefore provide effective treatments[166].

Gene Transfer

Transfer of several protective genes including Il-10, ICAM antisense and HSP 20 using adenoviral vectors have been shown to ameliorate cardiac IRI in experimental models[167-169]. In a rat model of lung transplantation transfer of recombinant cNOS genes to donors prior to

transplantation reduced neutrophil infiltration and improved oxygenation in recipient allografts. Transfer of the CTLA4-IG gene was successful in improving survival in a liver transplant model[170]. In the context of renal transplantation, gene transfer using adenoviral vectors has been used to successfully transfer the Fas-ligand gene in rat renal allografts[171]. Limiting the conversion of this approach into clinical practice is the time required for transfected donor organs to transcribe enough effector protein product prior to transplantation. A possible solution is gene transfer during prolonged warm perfusion of organs and this has been successfully performed in canine kidneys after 24 hours using an acellular perfusate[172].

Conclusion

Ischaemia reperfusion injury is a central process influencing short- and long-term outcome following renal transplantation, particularly in the context of non-heart-beating donors. The underlying mechanisms are complex and include multiple cell types involved in an array of intra- and extra-cellular signalling and effector pathways. The understanding of renal allograft IRI therefore continues to require extensive investigative experimentation but also provides numerous targets for therapeutic intervention.

Figures and Tables

I	**Dead on arrival to hospital**
II	**Unsuccessful resuscitation**
III	**Awaiting cardiac arrest – In-patient (withdrawal of support)**
IV	**Cardiac arrest after brain-stem death**
V	**Cardiac arrest in a hospital inpatient**

Table 1: Maastricht Classification of Non-heart-beating donors

CHAPTER 2

Isolated Kidney Perfusion

Introduction

Isolated kidney perfusion has been used extensively in research and clinical transplantation. The use of machine perfusion (MP) for renal allograft preservation was predominant until the early 1980s when increasing evidence suggested cold storage (CS) was equivalent or possibly better[173, 174]. Renewed interest in recent years relates to increased use of marginal organs needing pre-transplant evaluation and resuscitation (potentially through *ex-vivo* perfusion). This is coupled with a greater understanding of the pathophysiology behind IRI and how it might be ameliorated during the preservation period. The isolated perfused kidney has also been used as an investigative tool in a range of research specialties including cardiopulmonary bypass physiology, toxicology and ischaemia-reperfusion[175-177]. Perfusion with preservation solution under hypothermic conditions has been applied to organ transplantation for decades. Normothermic perfusion with acellular solutions and blood or partial blood suspensions, currently remains predominantly experimental but there is increasing interest in applying it to clinical organ preservation.

Hypothermic perfusion

The key principle of cold storage relies on the reduced consumption of ATP under hypothermic conditions. Metabolic rate slows by 1.5 – 2 fold with each drop of 10^0C[178]. However even at temperatures below 4^0C tissue ATP loss is considerable, for example cellular ATP drops by 84% in the rabbit liver after 6h cold storage[179]. In addition, it is likely that core cooling of organs during cold storage is often substandard. Active perfusion during

hypothermia may enhance parenchymal cooling through improved perfusion into the microvasculature and continuous replenishment of cold perfusate. A further benefit of perfusion may be more effective flushing of donor blood components and microthrombi from the renal vasculature and reduction in vasospasm. A common finding during organ perfusion is a significant fall in resistance to flow during the perfusion period[180]. Furthermore, it has been shown that improved microvascular flushing through the addition of streptokinase to organ perfusate significantly improved early renal function in a porcine and human allografts[181, 182]. Several groups report evaluation of graft viability, as opposed to improved preservation, during perfusion as the principle advantage of hypothermic perfusion, having a significant impact on graft selection[18, 19]

Despite the potential advantages of hypothermic perfusion over static cold storage it remains contentious whether these lead to overall improvement in graft outcome. Wight et al[183] performed a meta-analysis of studies comparing MP and CS and found that there was a 20% reduced risk of DGF in MP compared with CS. This difference applied to HBD and NHBD kidneys equally but did not correlate with any difference in 1 year graft survival. The quality of the evidence was found to be low and the studies highly heterogenous in terms of perfusion solutions, machines and protocols. There are several studies reporting no significant difference between CS and MP in transplant outcomes[184-188]. A study comparing 10 paired grafts assigned to MP or CS found no significant difference in need for dialysis within 2 weeks post transplant or 1 year graft survival[186]. A larger prospective study of 25 kidney pairs found that 14 (56%) did not require dialysis within the first month post transplant in both MP and CS groups[185]. Mendez et al found a trend to improved rates of DGF (34.6% vs. 65.4%) and 1yr graft survival (73.1% vs. 57.7%) in MP vs. CS but these were not statistically

significant. The largest of these studies of 96 paired HBD kidneys demonstrated no significant difference in DGF (CS 54%, MP 43%), 3 month graft survival or 1 year graft survival[188].

The majority of evidence supporting no difference between CS and MP comes from earlier studies and it has been proposed that differences in practice, particularly longer perfusion preservation periods, may have biased outcomes in favour of CS[180]. However there is some early contemporaneous evidence in support of MP. A trial involving 29 kidney pairs found a significantly higher incidence of DGF in CS (62%) compared with MP (17%) although there was no difference in 1 year graft survival[189]. In contrast, Heil et al[190] in a randomised trial comparing 27 paired grafts did find significantly better 1 year graft survival in MP (89%) versus CS (74%). A larger trial involving 194 allografts found a significantly lower incidence of DGF in the MP group although again no significant difference in graft survival[191]. There are also a number of more recent studies reporting better outcomes in renal transplantation following MP preservation[192-195]. A retrospective review of 25 matched allograft pairs found a significantly lower incidence of DGF in MP vs. CS although both groups had 100% 1 year survival[193]. A larger prospective study again identified more ATN in CS compared with MP (45% vs. 29%) but no difference in acute rejection episodes or 1 year graft survival[194].

A role for MP in specifically improving outcome from high risk or marginal organs has been outlined. It has been proposed that MP maybe particularly superior over CS in the context of prolonged cold ischaemic time (CIT). Jaffers et al found a significantly higher rate of ATN in kidneys undergoing CS for greater than 24 hours when compared to CS for less than 24 hours and MP for any period of time[196]. In a study showing significantly reduced DGF in MP grafts, it was also noted that in CS but not MP grafts, DGF occurs more frequently with

increasing CIT[189]. A similar pattern was found in a large randomised controlled trial despite finding no overall difference between CS and MP in terms of DGF and graft survival[188]. In the wider context of marginal donors Burdick et al found that use of MP for kidneys from donors older than 55 years significantly reduced the requirement for dialysis following transplantation[197]. The authors proposed that machine perfusion may confer most benefit in the context of NHBD renal transplantation. Early studies involving NHBD reported no significant difference in outcome following CS or MP, in a similar pattern to HBD studies[198]. However more recently there is mounting evidence that MP can reduce ATN and improve graft function and survival in NHBD allografts[199, 200].

Normothermic perfusion

Cold ischaemia has been shown to enhance graft inflammatory and immunogenic responses and augment injury following reperfusion[201]. In addition hypothermia causes stiffening of the microvasculature causing endothelial damage and increased vascular resistance[202]. Normothermic perfusion could be used to avoid these complications by excluding cold ischaemia, providing more physiological preservation conditions and greater scope for graft assessment prior to transplantation. In a canine auto-transplant model significantly better renal function was seen following implantation of organs following 18 hours of acellular normothermic perfusion compared to 18 hours static cold storage[203]. Metcalfe et al compared prolonged normothermic haemoperfusion of porcine kidneys with hypothermic perfusion[204]. Kidneys were then assessed using haemoperfusion and they found that the normothermic organs had greater ability to concentrate creatinine and conserve

sodium. A further benefit of warm perfusion may be the ability to store organs for longer time periods of time as metabolic demands can be met throughout preservation. It is well recognised that cold ischaemic times longer than 24 hours detrimentally affect graft outcome. In contrast, successful models of normothermically perfused canine and human kidneys were extended to 48 hours without significant renal injury[205].

Normothermic preservation could also be used to reverse the detrimental effects of retrieval related ischaemia prior to transplantation. Brasile et al found that a period of perfusion with acellular, oxygenated solution at 32^0C could ameliorate ischaemic injury in a canine model of NHBD transplantation. This applied to models encompassing both prolonged cold ischaemic time (18 hours CIT following 30 min WIT) and significant warm ischaemia (up to 2 hours)[203, 206]. It is proposed that a key element of this effect is re-oxygenation in an environment deplete in components of an inflammatory response. In addition erythrocyte sludging within the microvasculature, known to occur on reperfusion of a hypothermic kidney, is avoided[207]. Interestingly, normothermic perfusion prior to cold storage has also been shown to reduce renal IRI in clinical NHBD transplantation. This was performed using 1 hour normothermic in-situ perfusion with cardiac bypass followed by gradual cooling and then conventional organ retrieval and storage. The incidence of PNF and DGF was significantly reduced when compared to hypothermic in situ perfusion alone[208].

Normothermic perfusion may confer other specific benefits over hypothermic preservation in terms of graft resuscitation. A number of interventions to ameliorate IRI will often rely on normal cell metabolism to exert their effect. Induction of potentially protective elements, such as heat shock proteins, requires normal cell signaling, gene expression and protein transcription. Brasile et al found that heme oxygenase-1(HO-1) could be generated

successfully using cobalt protoporphyrin (CoPP) in acellular normothermic perfusion but not during cold perfusion[161]. Gene transfer using viral vectors during reperfusion has been shown to be effective following 24 hours warm perfusion but not during hypothermic perfusion due to the reduction in cell metabolism and division[172]. Addition of fibroblast growth factor during 24 hours of normothermic perfusion was shown to potentiate active cellular repair, maintain cytoskeletal integrity and reduce cell loss by necrosis and apoptosis[209].

Optimal conditions for isolated organ perfusion

Oxygen Delivery

The potential for oxygen delivery during hypothermic perfusion may represent a significant advantage over static storage and due to lower oxygen requirements during hypothermia an oxygen carrier in the perfusate may not be required. Minor et al[210] demonstrated superior organ preservation with oxygenated hypothermic low flow perfusion versus cold storage in a porcine model of NHBD transplantation. Perfused grafts had significantly higher concentrations of ATP in tissue biopsy homogenates at the end of perfusion indicating improved oxygen delivery and significantly better renal function compared with static storage.

Oxygenation is not essential during hypothermic perfusion. However normothermic perfusion without adequate oxygenation provides inferior organ preservation in comparison to cold storage as this equates to prolonged warm ischemia[211]. Perfusion with simple oxygenated buffer solutions tend to require high perfusion pressures shown to be injurious to

microvasculature and when lower pressures are used preservation is compromised[212]. Hochel et al compared normothermic perfusion with tyrode solution, a simple buffer solution, with haemoperfusion in an isolated canine kidney model and found that GFR, urine output and oxygen consumption were significantly higher in the kidneys perfused with blood[213].

The ideal oxygen carrier for warm perfusion has yet to be identified. Red blood cells (RBC) would theoretically be the obvious choice and have the added advantage that they are capable of scavenging reactive oxygen species and ameliorating IRI[214]. Whole blood has been used to perfuse kidneys and livers for limited periods[215, 216]. Szajer et al found they could successfully perfuse porcine kidneys *ex-vivo* using whole blood through a Waters RM3 renal preservation system for 2 hours[217]. However development of clinically applicable preservation systems using whole blood may be hindered by practical issues relating to obtaining adequate blood volume, system occlusion and haemolysis. The use of solutions containing suspended RBCs or dilution of blood with preservation solutions has been identified as a potential approach. Pegg et al reported an ideal perfusate haematocrit of 20% for optimal flow dynamics and oxygen delivery in this context[218].

Cell-free perfusates have been investigated in several animal models as another alternative to whole blood. In the context of renal allograft perfusion, a perfusate containing perfluorocarbon, tissue culture medium and albumin was successfully used to maintain normal renal function for 3 hours in a NHBD porcine kidney model[219]. Perfluorocarbons are hydrocarbons in which hydrogen atoms are replaced by fluorine and form a liquid capable of dissolving high concentrations of oxygen. This liquid also has a low oxygen-binding constant and a linear relationship between oxygen saturation and partial pressure making it an effective medium for oxygen carriage and tissue delivery. The Maastricht group has developed a

normothermic perfusion model, the exsanguinous metabolic support (EMS) system, on which they have successfully perfused canine and human kidneys for up to 48 hours. The novel perfusate used contains pyridoxilated bovine haemoglobin as an oxygen carrier, suspended in a tissue culture-like medium[205].

Perfusate Viscosity

It has been suggested that the viscosity of perfusion solution may be important. There has been some concern over adequate microcirculatory perfusion in highly viscous solution at low temperatures[220]. In contrast use of colloid containing solutions may be important in reducing mechanical endothelial injury during pressure driven perfusion[221]. However no difference was seen in renal function in a porcine NHBD model following low flow preservation with high viscosity Belzer MPS or low viscosity HTK[210]. Hauet et al using an isolated porcine normothermic perfusion model, found that the type of colloid added to perfusate may influence quality of perfusion[222]. Addition of hydroethylstarch (HES) to normothermic perfusate lead to increased sodium and glucose excretion in the PCT, reduced plasma flow rate (PFR) and glomerular filtration rate (GFR), an effect not seen with addition of albumin or gelatin.

Pulsatile versus Non-pulsatile Perfusion

Several studies have attempted to clarify whether pulsatile or continuous renal allograft perfusion is most beneficial. Studies comparing techniques for extra-corporal by-pass organ

perfusion found that pulsatile flow lead to greater oxygen consumption and GFR and lower vascular resistance[223]. It is not clear whether in the absence of autonomic regulation, a similar benefit from pulsatile flow exists in the isolated organ. In the context of hypothermic perfusion Kozaki et al found continuous better than pulsatile flow in perfusion of marginal cadaveric renal allografts[19]. In contrast, Booster et al found pulsatile flow superior in resuscitating ischaemically injured kidneys[224]. Paquet compared pulsatile and non-pulsatile haemoperfusion of isolated pig kidneys but no clear difference in function was identified[176]. It has been proposed that the pump mechanism may be important. In a hypothermic kidney perfusion model, a valved tubular vacuum pump producing a more physiological pulse wave was found to be superior to a classic roller pump[225]. However in a rabbit renal haemoperfusion model the pulsatile pump mechanism caused significant haemolysis compared to a roller pump[226].

Perfusion Pressure

Pressures used for machine perfusion vary greatly and particularly in normothermic perfusion it remains unclear whether physiological pressure is the ideal. Subphysiological pressures may lead to a loss of autoregulation and glomerular filtration, but higher pressures may cause endothelial injury[227].

In a model of normothermic perfusion of porcine kidneys with whole blood a continuous sub-physiological pressure of 60-90mmHg was used to maintain flows of 70 - 150mL/min[217]. Urine output was maintained throughout and biopsies at the end of the 2 hour perfusion period showed no ischaemic injury. Furthermore, successful prolonged maintenance

of haemoperfused porcine kidneys for 16 hours at mean pressures of 50mmHg was performed in a study comparing normothermic preservation techniques[204]. Similar results were obtained using an acellular perfusate supplemented with bovine haemoglobin at a pressure of 50/30 mmHg to perfuse canine kidneys for 18 hours[206]. Despite this, the majority of models have used physiological pressures in order to improve renal function, seemingly at the expense of limiting the perfusion period to a few hours[216, 226, 228, 229]. One notable exception is the six-day preservation of canine kidneys using a combination of hypothermic and normothermic haemoperfusion at a pressure of 100mmHg[230]. However it is important to note that the haemoperfusion period was limited to 3 hours and canine kidneys are particularly resistant to ischaemic insult.

Perfusate Formulation

The formula of the ideal perfusate remains unknown and will depend significantly on the mode of perfusion. Hypothermic perfusion solutions remain similar to those used for static storage but components aimed at ameliorating IRI may confer greater benefits when added to perfusates. A study comparing pulsatile perfusion preservation of cadaveric kidneys with Vasolol and Belzer MPS found that the incidence of DGF was significantly reduced in the former[231]. Vasolol is a novel adaptation of MPS with the addition of factors including N-acetylcysteine, L-arginine and alpha-ketoglutarate. However addition of specific anti-oxidants during static storage has also been shown to improve post-transplant function. Trimetazidine, a mitochondrial membrane stabiliser, added to Euro-Collins solution significantly reduce renal IRI in a porcine model of prolonged cold static storage[175]. In contrast to hypothermic

preservation, normothermic preservation does not rely on the principle of cell membrane stabilisation and maintenance of an isoosmolar environment in the context of slowed metabolism. Normothermic perfusates aim primarily to provide adequate substrates and oxygen for active metabolism and are thus generally based on tissue culture mediums containing a specific oxygen carrier.

Viability Assessment

The ability to assess viability of renal allografts during perfusion is one of the key arguments that protagonists use in support of widespread use of organ perfusion technology. A number of markers have been used experimentally and clinically to evaluate kidneys during perfusion. The Maastricht group analysed several markers during work with machine preservation of NHBD kidneys. Lactate dehydrogenase measured in the graft perfusate was found to be elevated in non-functioning kidneys that were subsequently transplanted[232]. Further work from the same group also identified significantly higher levels of glutathione-S-transferase (GST), a lysosomal enzyme from PCT cells, in grafts with PNF compared with functioning grafts[233].

The high proportion of NHBD transplants performed in Japan has lead to investigation of assessment of viability through flow dynamics during hypothermic perfusion. Kozaki et al[19] have established perfusion criteria for viable organs: (1) perfusion flow at least 0.4mL/min/g at perfusion pressure 30-50mmHg and (2) perfusion flow increasing with pressure decrease or plateauing during perfusion. One hundred and thirty kidneys were assessed and 120

transplanted with 100% graft function. The remaining 10 grafts were transplanted at other centres and all had PNF.

The Newcastle group has developed a local viability assessment protocol during machine perfusion combining perfusate markers and flow dynamic evaluation[18]. Kidneys are only transplanted if the perfusate GST levels remain below 200 U/litre/100g and a flow rate greater than 50ml/min/100g at a pressure of 60mmHg. The primary non-function rate since adopting this program has fallen dramatically from an unacceptable 55% to 7%.

Normothermic perfusion may provide superior graft assessment under more normal physiological conditions. The Leicester group evaluated graft function in porcine kidneys subjected to several different warm ischemic times prior to perfusion at 32^0C using acellular perfusate[219]. Several parameters, including vascular resistance, urine output and sodium loss, correlated strongly with increasing WIT. Stubenitsky et al developed a Viability Score (VS) based on oxygen consumption, flow dynamics and perfusate platelet concentration during normothermic perfusion of porcine kidneys following warm and cold ischaemia[234]. Allografts with a lower VS had milder ATN, better early creatinine clearance and shorter periods of DGF following transplantation.

Conclusion

The need to continually improve outcomes in renal transplantation in the context of using increasingly marginal donor organs drives expanding interest in isolated organ perfusion. Hypothermic and normothermic perfusion systems are being developed as potential methods of reducing preservation injury which influences early and late allograft function. A

key advantage of perfusion preservation appears to be the ability to assess organ viability prior to transplantation. This finding underpins another important application of the isolated organ perfusion system, namely the development of experimental models of organ reperfusion following transplantation. In this context the isolated kidney provides a vital tool allowing investigation of the mechanisms of ischaemia-reperfusion within a controlled but clinically applicable model.

AIMS

Thesis Aims

The aims of this study are:

1) To design a system for perfusing isolated porcine kidneys with autologous blood while maintaining viability and function over a period of several hours.

2) To develop the perfusion system to provide a reliable and representative model of renal allograft reperfusion following retrieval and transplantation, particularly in the context of NHBD organs.

3) To validate the model as a sensitive tool for investigating factors that may influence the mechanisms and severity of renal ischaemia-reperfusion injury.

4) To investigate the effect of leucocyte depletion on the severity of IRI during reperfusion in order to elucidate the importance of leucocytes in this context.

5) To investigate the effect of WIT on the severity of renal IRI and early graft function.

6) To investigate the relationship between IRI injury at the cellular level and graft function and identify potential biochemical markers for evaluating the severity of IRI.

7) To investigate the effect of perfusion pressure on isolated graft function and endovascular injury in the context of identifying optimal organ perfusion conditions.

CHAPTER 3

The Effect Of Leucocyte Depletion On Renal Function And Injury During Reperfusion Using An Isolated Organ Haemoperfusion System

Introduction

The underlying mechanisms of renal IRI are complex and the role of leucocytes, and in particular neutrophils, remains controversial[143,235]. In myocardial IRI there is increasing evidence that neutrophils play a less significant role than other factors such as complement activation[131], but the situation in renal IRI remains less clear. In vitro and small animal perfusion studies suggest a key role for white cells in renal IRI but this has yet to be confirmed by large animal studies or clinical trials[235,69,138].

The first part of this study was designed to elucidate the importance of leucocytes in renal ischaemia-reperfusion injury and validate a new model of renal allograft reperfusion. The model chosen for this investigation used isolated *ex-vivo* blood perfused porcine kidneys as these organs are known to respond to warm and cold ischaemic insults in a way that closely mirrors the human situation.

Methods

Kidney Retrieval

Large white pigs (60 – 70kg) were sacrificed by electrocution followed by exsanguination via an incision in the external jugular vein and approximately 1 litre of blood was collected into a sterile receptacle containing 25,000 units of heparin (Multiparin ; CP Pharmaceuticals, Wrexham, UK). A rapid retrieval technique was then employed, involving *en bloc* resection of intra-peritoneal organs (by a professional slaughterman) and then

simultaneous removal of both kidneys by two surgeons ensuring preservation of vascular and ureteric pedicles. The kidneys were immediately transferred to hyperosmolar citrate solution (Soltran; Baxter healthcare, Norfolk, UK) at a temperature of 4 °C and flushed with 250ml of the solution at a hydrostatic pressure of 100 cm water. The organs were stored on ice in hyperosmolar citrate solution and transported to the perfusion laboratory.

Isolated Organ Perfusion

The renal artery, vein and ureter were cannulated and the kidneys flushed with a plasma substitute (Gelofusine; B. Braun, Sheffield, UK) at 4 °C immediately prior to reperfusion on the Isolated Organ Perfusion System (IOPS). The IOPS was designed using commercially available clinical-grade cardiopulmonary technology (Medtronic, Watford, UK) and consisted of a centrifugal blood pump (550 Bio-pump), a heat exchanger (Grant, GD120, Cambridge, UK), a 5L venous reservoir (Medtronic) and a minimax plus membrane oxygenator (Medtronic). The circuit hardware included a speed controller, a TX50P flow transducer, a pressure transducer and a temperature probe (Cole-Parmer, London, UK). Two PC- 2 Gemini infusion pumps (Alaris, Basingstoke, UK), a urinometer (Bard, Crawley, UK) and two modified leukoguard RS white cell filters (Pall, UK & Phoenix cardiovascular systems, Preston UK) were incorporated into the system (*Fig. 1*).

The circuit was primed with 500 ml Ringer's solution containing mannitol 10mg (Baxter, Healthcare, Norfolk, UK), dexamethasone 10mg (Organon Labs Ltd, Cambridge, UK), cefuroxime 750mg (Britannia Pharmaceuticals Ltd, Surrey, UK) and 12ml sodium

bicarbonate 8.4% (Fresenius Kabi, Warrington, UK). Some 500ml of heparinized whole blood was added to the circuit after priming and allowed to circulate at a temperature of 37°C.

The perfusate was also supplemented with a nutrient solution (Nutriflex B; Braun Sheffield, UK) to which 100 units insulin (Actrapid; Noro Nordisk, Denmark, UK) and 25ml sodium bicarbonate 8.4% (Fresenius Kabi) were added. The nutrient solution was infused at a rate of 20 ml/hr. A vasodilator, Sodium Nitroprusside 25 mg, (Mayne pharma PLC, Warwickshire, UK) was administered during the first hour of reperfusion at 25ml/hr, after which 5% glucose solution (Baxter) was infused at 7ml/hr. Ringer's solution (Baxter) was used to accurately replace urine output. Creatinine (Sigma, Germany) was added to the perfusate to achieve an initial circulating concentration of 1000μmol/L.

Experimental Protocol

Porcine kidneys were randomized (using a computer based random assignment program) into two groups and perfused either with leucocyte-depleted blood (LDB; n= 6) or whole blood (WB; n = 6) for a period of 6 h. The renal blood flow, pressure and resistance were continuously monitored and the data downloaded to a laptop computer. Biochemical analysis of serum and urine samples was carried out at hourly intervals and used to calculate creatinine clearance (urinary creatinine U_{cr} x urinary volume V / plasma creatinine P_{cr}) as an estimate of glomerular filtration rate (GFR).

Oxygen consumption in ml/min/g was calculated from arterial and venous blood gas sampling {(PO_2 in arterial blood – PO_2 in venous blood) x flow rate/weight}. The haemoglobin, haematocrit and leucocyte count were measured before and after reperfusion.

Histology

Needle core biopsies were taken before and after perfusion, fixed in 4% formaldehyde and embedded in paraffin wax. 4μm sections were cut and stained with haematoxylin and eosin. Morphology was assessed semi-quantitatively using the following six parameters of renal injury: tubular dilation; tubular debris; nuclear shrinkage; nuclear absence; cytoplasmic vacuolation; vascular dilation. Two assessors blinded to the treatment group scored each parameter over 5 fields using a semi-quantitative severity score of 1 to 4 and a mean total severity score was calculated from these values.

Statistical analysis

Values are presented as mean ± s.d. Continuous variables such as serum creatinine were plotted as level *versus* time curves for each kidney and the area under each curve calculated using Microsoft ®Excel software (Reading, UK). Area under the curve (AUC) values were compared using the Mann-Whitney U test. Other continuous variables were compared using two-way analysis of variance. Discontinuous data was compared using the Fisher's exact test. $P \leq 0.05$ was taken as significant.

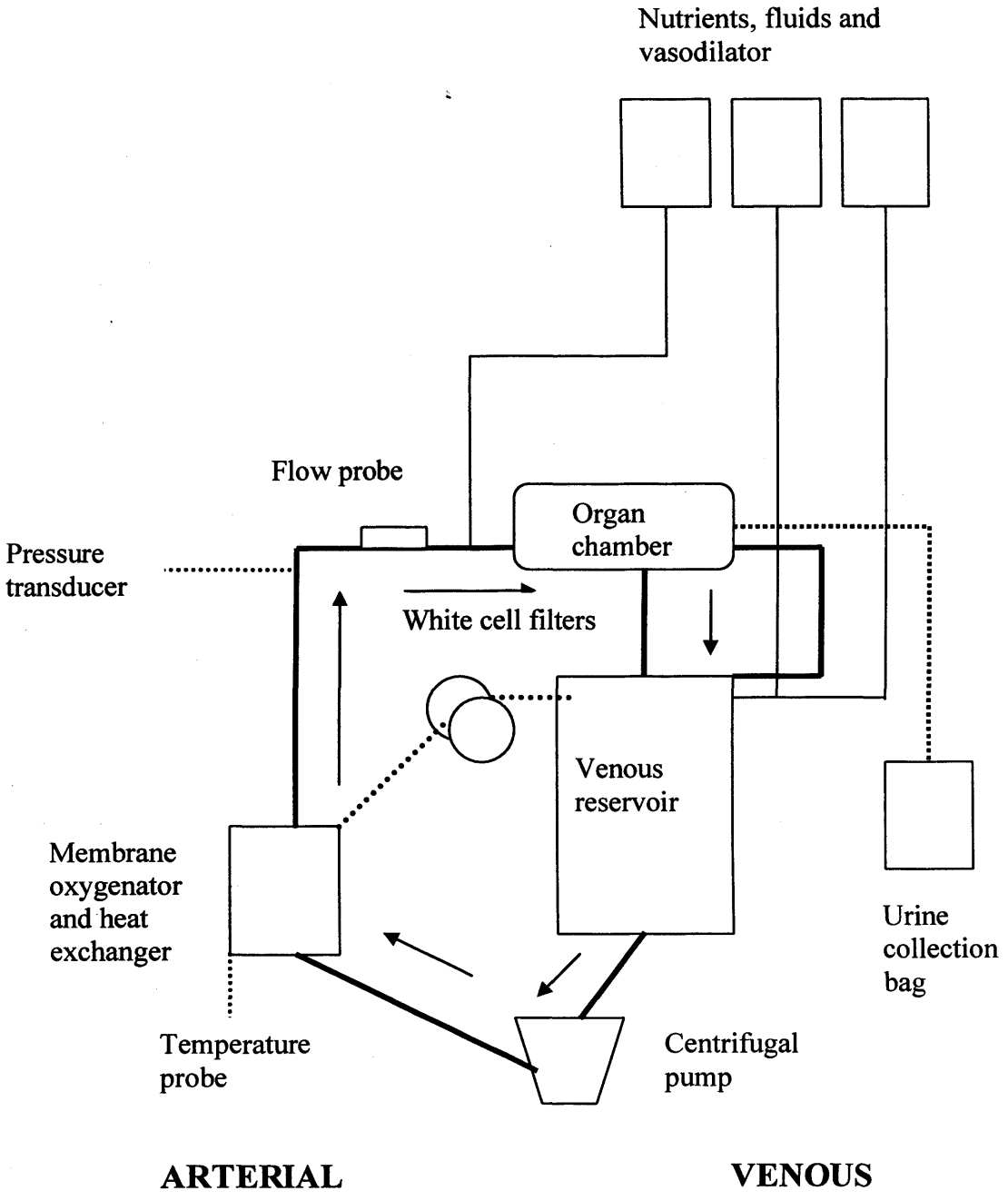


Figure 1a: Diagram of the Isolated Organ Perfusion System (IOPS)

Figure 1b: Photograph of the Isolated Organ Perfusion System (IOPS)

Results

Perfusion parameters (Table 2)

There was no significant difference in kidney weight between the two groups. Warm ischaemic time was limited to 6-7 minutes and cold storage time to just over two hours with no difference seen between the groups. Perfusion pressure, temperature and starting haematocrit were very similar for all experiments. The pre-perfusion white cell counts confirmed marked leucocyte depletion in the group perfused with filtered blood.

Renal Function

The leucocyte-depleted group demonstrated better renal function with serum creatinine levels falling by 95 ± 1 % compared to 72 ± 12 % in the whole blood group (P = 0.002) after 6 h perfusion. The mean AUC for serum creatinine was significantly lower in LDB at 1286 ± 214 compared with 2627 ± 418 in WB (P = 0.002; *Fig. 2*). Creatinine clearance was significantly higher in LDB throughout the perfusion period with a mean creatinine clearance of 10.6 ± 2.8 ml/min/100g compared with 1.9 ± 1.0 ml/min/100g in WB (P = 0.002; *Fig. 3*). In LDB creatinine clearance peaked to 16.1 ± 7.9 ml/min/100g at 3 h, which was markedly greater than the maximum clearance in WB (3.1 ± 3.3 ml/min/100g), reached after a 2 h (P = 0.002). The mean AUC for creatinine clearance was also markedly higher for LDB compared with WB (59 ± 18 *versus* 10 ± 5; P = 0.002).

The leucocyte-depleted group maintained excellent urine output during perfusion with a mean urine flow rate of 191 ± 75 ml/hr which was maximal in the first hour at 263 ± 79ml/hr and gradually tailed off over the perfusion period (*Fig. 4*). Urine output in the whole blood group followed a similar pattern but was significantly lower with a maximum flow rate of 115 ± 79 ml/hr and a mean flow rate of 70 ± 32 ml/hr (P = 0.002). The mean AUC for urine output was also significantly greater in LDB at 946 ± 395 compared with 344 ± 144 in WB (P = 0.002).

Haemodynamics

Renal blood flow steadily improved in both groups with flow reaching 73 ± 17 ml/min/100g in LDB and 53 ± 13 ml/min/100g in WB (*Fig. 5*). Renal vascular resistance was significantly higher in WB kidneys at the start of the perfusion period but fell within the first hour to a comparable level with LDB after which it remained steady in both groups. Both groups of kidneys had a similar percentage weight gain during the perfusion period, 21 ± 12% in LDB and 22 ± 8% in WB (*Table 3*).

Parameters	LDB	WB	P value
Kidney weight (g)	218 ± 20	213 ± 35	0.63
Warm ischaemic time (min)	6 ± 1	7 ± 1	0.064
Cold storage (hr)	2.2 ± 0.4	2.1 ± 0.2	0.413
Mean arterial pressure (mmHg)	55 ± 4.3	53 ± 1.8	0.31
Temperature (°C)	37.4 ± 0.5	37.1 ± 0.7	0.52
Haematocrit (l/l)	0.24 ± 0.06	0.22 ± 0.03	0.931
White cell count ($x10^9$/l)	0.7 ± 0.5	6.7 ± 1.4	0.004

Table 2: Mean parameters for kidneys perfused with leucocyte depleted blood (LDB) and whole blood (WB)

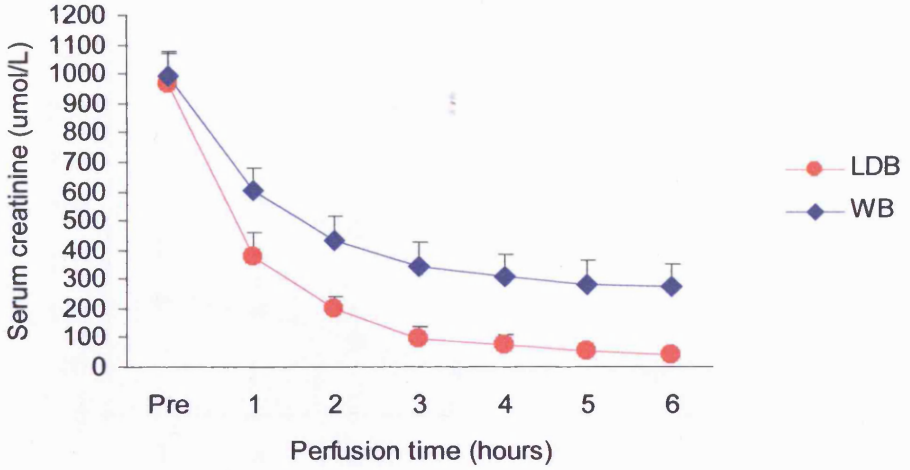


Figure 2: Mean serum creatinine levels over 6 hours.

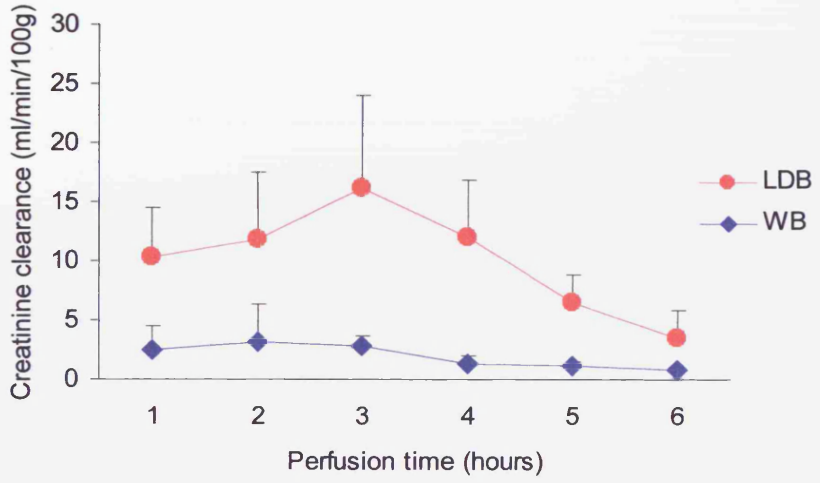


Figure 3: Mean creatinine clearance levels during 6 hours of reperfusion

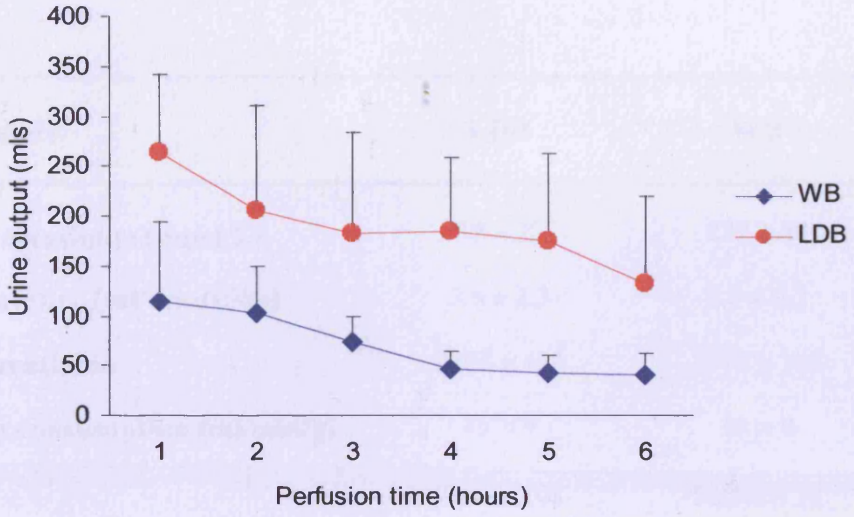


Figure 4: Mean hourly urine output during the perfusion period

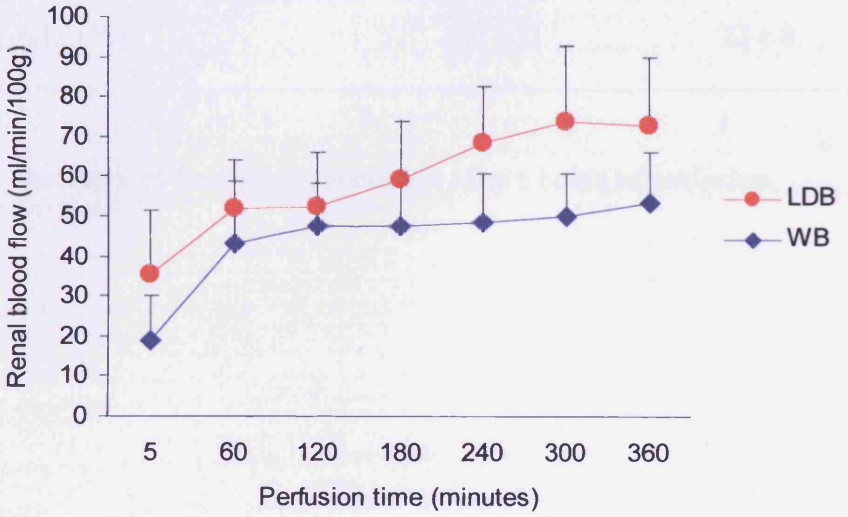


Figure 5: Mean renal blood flow over 6 hours

Parameters	LDB	WB	P value
Serum creatinine (μmol/L)	39 ± 12	272 ± 80	0.002
Cr. clearance (ml/min/100g)	3.6 ± 2.3	0.8 ± 0.2	0.002
AUC creatinine	2593 ± 418	1374 ± 168	0.016
Oxygen consumption (ml/min/g)	45 ± 9	33 ± 8	0.028
pH	7.44 ± 0.08	7.23 ± 0.12	0.017
Serum bicarbonate (mmol/L)	25 ± 3	17 ± 7	0.082
Base excess	0.3 ± 4.1	-6.6 ± 8.3	0.178
RBF (ml/min/100g)	73 ± 17	53 ± 12	0.092
RVR (mmHg)	0.4 ± 0.05	0.5 ± 0.11	0.065
Total urine output (ml)	1144 ± 450	422 ± 192	0.002
Weight gain (%)	20 ± 11	22 ± 8	0.937

Table 3: Summary of functional parameters after 6 hours of perfusion.

Acid-Base Homeostasis and Oxygen Consumption

The leucocyte-depleted group demonstrated significantly better acid-base homeostasis. The pH in LDB remained stable over 6 h with an initial and final serum pH of 7.44 ± 0.04 and 7.44 ± 0.08 respectively. In contrast, WB showed worsening acidosis during perfusion with a fall in the pH from 7.48 ± 0.08 to 7.23 ± 0.12 (P = 0.017 *versus* LDB; *Fig. 6*). In keeping with these findings, normal bicarbonate concentration was maintained in LDB with a final concentration of 25 ± 3 mmol/L but fell in WB from 26 ± 1 mmol/L to 17 ± 7 mmol/L, a change that reached only marginal significance (P = 0.065; *Fig. 7*). Base excess fell in WB from 0.7 ± 1.6 to -6.6 ± 8.4, whereas there was a non-significant improvement in LDB with a rise from -2.3 ± 1.0 to 0.3 ± 4.2 (P= 0.132, and 0.125 respectively; *Table 3*).

Oxygen consumption was greater in LDB compared with WB throughout perfusion becoming statistically significant at 6 h (46 ± 9 ml/min/g *versus* 33 ± 8 ml/min/g (P=0.028; *Table 3*).

Histology

There were no clear differences between the pre-perfusion biopsy severity scores for all parameters in LDB compared with WB. Both groups showed deterioration in overall morphology after 6 h perfusion and this was more marked in WB although the difference was not statistically significant (*Table 4; Fig. 8*).

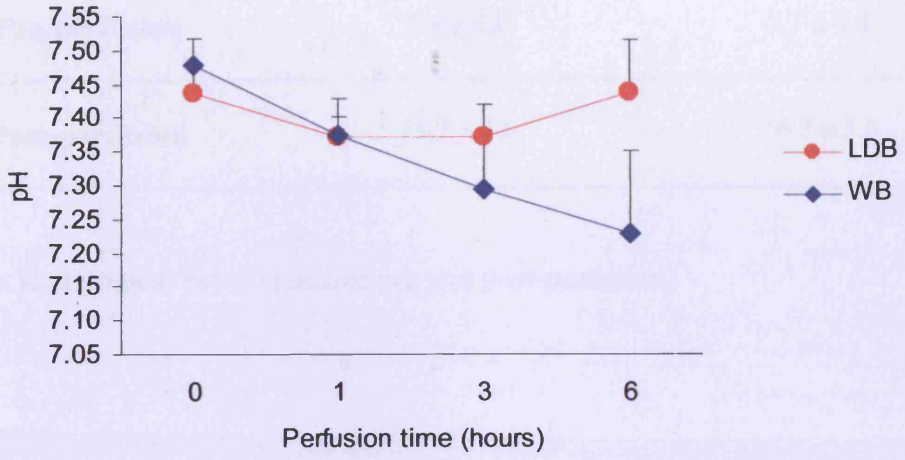


Figure 6: Mean pH levels over 6 hours

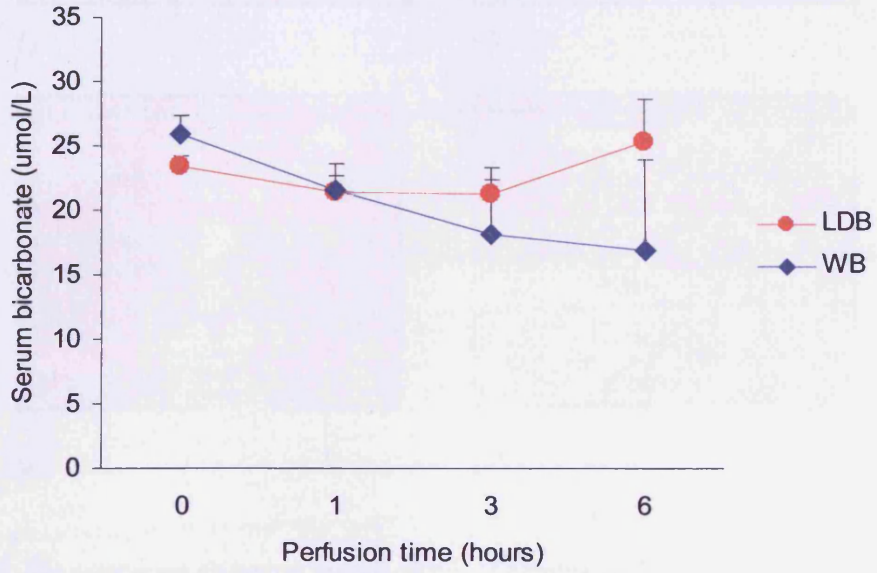


Figure 7: Mean bicarbonate levels

	LDB	WB
Pre-perfusion	7.8 ± 1.0	7.7 ± 1.4
Post-perfusion	13.7 ± 1.6	16.3 ± 3.6

Table 4: Histological severity scores pre and post-perfusion

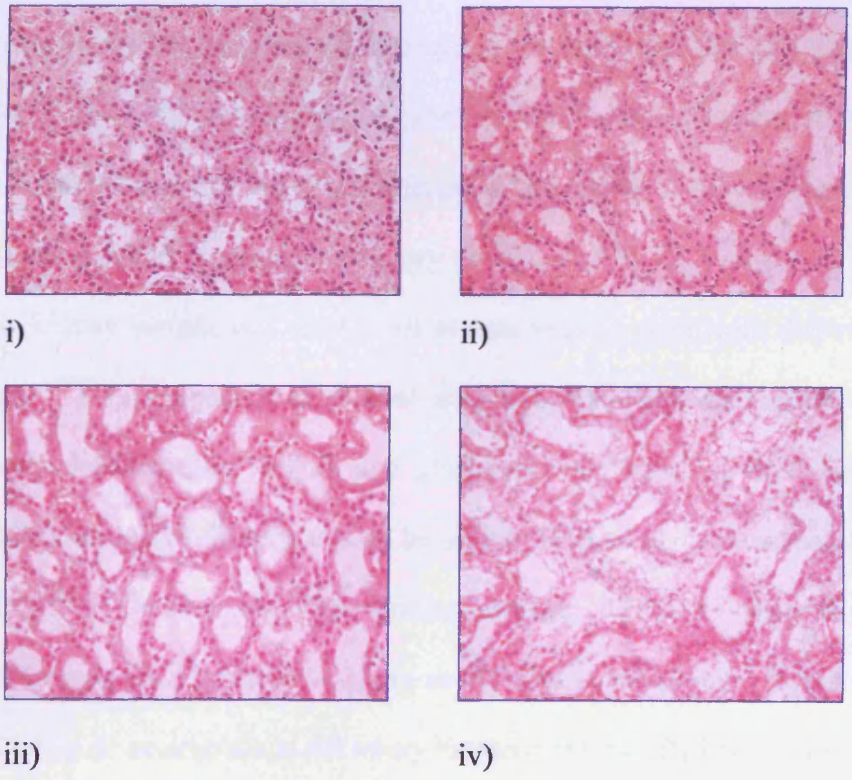


Figure 8: Photographs showing morphological changes (X 200). Photographs i) and ii) are LDB and WB pre-perfusion biopsies respectively and iii) and iv) 6 hour biopsies LDB and WB. Pre-perfusion biopsies were similar for both groups but post-perfusion tubular dilatation and debris and vascular dilatation were more marked in WB versus LDB.

Discussion

This study demonstrates that perfusion of isolated porcine kidneys with leucocyte-depleted blood leads to significant improvement in post-ischaemic renal function indicating an important role for white cells in the pathogenesis of renal ischaemia-reperfusion injury. Leucocyte depletion not only improved creatinine clearance and urine output but was also associated with improved renal haemodynamics, oxygen consumption and acid-base homeostasis. In addition to this the histological findings mirrored functional differences showing a reduction in acute tubular damage in the leucocyte depleted group. Nonetheless, leucocyte depletion did not completely abolish histological evidence of early graft injury. This may relate the fact that white cell filtering is incomplete or may indicate the presence of a neutrophil independent mechanism of injury as suggested by De Vries *et al*[80]. Furthermore, an increase in kidney weight was seen in all organs with no significant difference between the two groups. This is important as studies using a rabbit model demonstrate that weight gain closely paralleled impaired sodium and glucose tubular handling and overall renal injury. Histological assessment showed this to be mainly interstitial fluid accumulation rather than cell swelling[228]. Despite this, these findings provide further evidence for a vital role for leucocytes in early graft reperfusion injury resulting in impaired renal function.

The role of neutrophils in I/R injury has been extensively investigated. Neutrophils are recruited to reperfused tissue by free radical related upregulation of endothelial adhesion molecules, such as P-selectin and ICAM and also through endothelial cell and complement cascade derived chemotactic factors[236]. Neutrophils themselves perpetuate the process through further production of ROS, cytokines and nitric oxide (NO)[72]. The subsequent

inflammatory response leads to direct tissue damage and subsequent necrosis through release of proteases and has been shown to upregulate apoptosis through stimulatory cytokines[73]. Extensive leucocyte adhesion to the endothelium and cytokine generated vasospasm has also been shown to contribute to microvascular occlusion during reperfusion[74].

It has been demonstrated that neutrophil depletion reduces renal IRI but the extent of this effect remains controversial[237]. Rat *in vivo* models of renal IRI examining the effect of monoclonal and polyclonal anti-neutrophil antibodies showed no reduction in IRI despite producing significant neutropenia[238, 239]. Contrary to this Klausner *et al* concluded that neutrophil depletion using a polyclonal rabbit anti rat neutrophil serum following 45 minutes renal ischaemia and reperfusion improved renal function and reduced structural damage[75]. In addition, numerous small animal studies have shown that attenuation of neutophil infiltration by inhibiting important proinflammatory factors such as ICAM[133], TNF-α[138], MIP-2[69] and P-selectin[137] significantly reduce severitiy of IRI. Specific inhibition of lymphocyte trafficking by immunosuppressant agent FTY 720 has also been shown to ameliorate ATN and improve initial allograft function in a rat transplant model[240]. A similar improvement was obtained in this model using pre-treatment with ICAM-1 antisense oligonucleotides but only a mild additive effect observed with combined treatment. This is important because it has been suggested that leucocyte depletion studies using inhibition of adhesion molecules may inaccurately assign IRI amelioration to impaired leucocyte infiltration alone. It is thought adhesion molecules may also contribute to renal IRI independent of leucocyte activity, for example through cell-cell adhesion of sloughed epithelial cells causing tubular obstruction[241].

Clinical trials looking at the effect of leucocyte depletion on IRI focus on myocardial ischaemia and the results have been equivocal[131] despite evidence for a central role for

neutrophils in animal models[132]. In light of this it has been suggested that complement, generated mainly through the immune complex mediated classical pathway, may have a more crucial role in I/R than neutrophils[84]. In renal IRI a crucial role for complement in mediating late apoptosis and inflammation has been suggested by De Vries *et al* using a murine model[78]. Furthermore, in a similar model they found that neutrophil depletion did not affect the significant protective role of a C5 receptor antagonist[80]. It was concluded that complement could mediate IRI through both neutrophil dependent and independent pathways. In contrast to this, Park et al reported that in their murine model, C3-convertase inhibition did not protect against IRI[140].

In the context of renal perfusion models, Pacini and Bocci found that the filtering of white cells from blood used in a rabbit *ex-vivo* perfusion model greatly improved renal function and reduced injury[130]. Nunley et al found that porcine kidneys haemoperfused with white cell depleted blood for 10 minutes prior to transplantation functioned significantly better than those not perfused or those perfused with whole blood[242]. The findings of this porcine haemoperfusion study concur with these studies and indicate a significant role for neutrophils in early renal IRI. It is important to note that leucocyte filtration leads to depletion of other important cells such as macrophages and monocytes. These cells are also thought to play a key role IRI, in particular in the secretion of proinflamatory cytokines in response to ischaemia[243]. However, this lack of specificity is also a limitation of other methods of leucocyte depletion. It is well recognised that polyclonal and monoclonal antibodies against neutrophils have been shown to deplete monocytes and platelets and bind to epitopes on macrophages[235].

The findings of this study suggest that leucocytes play an important role in renal reperfusion injury. The data presented using isolated perfused porcine kidneys broadly agrees with the findings of previous studies using small animal kidneys. However, the porcine kidney is recognised to have anatomical and physiological characteristics that closely resemble the human situation and as such the current model is likely to provide data that is more representative than that from small animal models[130, 244]. The pig kidneys used were taken from animals weighing 60-70 kg and this provided kidneys of similar size to adult human kidneys. The model used was both reliable and reproducible. The use of modern cardio-pulmonary bypass technology prevented the development of damaging haemolysis and haematuria. The system allowed for the continuous measurement of physiological parameters and in parallel with this provided sequential biopsy material for the assessment of structural damage. As such the IOPS appears to be a versatile method of further investigating renal IRI.

CHAPTER 4

The Effect Of Warm Ischaemic Time On Early Graft Function And Injury During Perfusion On An Isolated Haemoperfused Porcine Kidney Model

Introduction

Renal allografts from NHBD are associated with an increased incidence of delayed graft function (DGF) and primary non-function (PNF) due to the prolonged warm ischaemic time (WIT) these kidneys are subjected to during retrieval[13]. The precise relationship between the extent of WIT and the incidence of DGF and PNF remains unclear and is difficult to evaluate in the clinical situation. This is reflected in the broad range of acceptable upper limits of WIT used by renal transplant programmes.

The aims of this study were:

1) To investigate the effect of increasing warm ischaemic time on early graft function and injury using the isolated haemoperfused porcine kidney model and

2) To describe the relationship between WIT and biochemical markers of renal oxidative damage in order to assess their usefulness in the prediction of graft outcome.

Physiological perfusion parameters and histological findings were used as indicators of early reperfusion injury following their validation by the leucocyte depletion study. In addition, biochemical markers of renal oxidative damage and apoptosis were analysed to investigate direct evidence of the effect of WIT on IRI. Small animal studies have identified quantitative markers of protein, lipid and DNA oxidative injury during renal IRI. Carbonyl content, commonly used as a biomarker of protein oxidation, was shown to increase in rat kidney tissue following IRI[245]. Similar models have demonstrated an increase in urinary 8-isoprostane (8-isoPGF$_2\alpha$), a product of ROS dependent breakdown of arachidonic (a marker of lipid peroxidation) and tissue accumulation of the oxidative DNA lesion 8-hydroxy-2'-dexoyguanosine (8-oxo-dG)[246]. Finally, a rat model has also been used to demonstrate

morphological and biochemical evidence of increased apoptosis during reperfusion following brief periods of ischemia[247].

Methods

Retrieval and Perfusion

Kidneys from large white pigs were retrieved and haemoperfused for 6 h as previously described in Chapter 3.

Experimental protocol

Renal blood flow, perfusion pressure and intrarenal resistance and urine output were measured continuously. Serum and urine biochemical analysis were performed at regular intervals allowing calculation of creatinine clearance, oxygen consumption and acid/base homeostasis. Porcine kidneys were divided into 4 groups (n=6) and subjected to 7 (control), 15, 25 and 40 min WIT prior to cold flushing and storage.

Urine samples at 1 and 6 h reperfusion were stored for the measurement of N-Acetyl-β-D glucosaminidase (a marker of tubular damage) using colorimetric assay (Bio-Quant, San Diego, USA) and also for 8-oxo-dG assay. Needle core biopsies were taken immediately prior to reperfusion, at 30 min and at 6 h, rapidly frozen using liquid nitrogen and stored at -80^{0}C. Protein carbonyl and caspase-3 activity detection was performed using this tissue. Blood was sampled immediately prior to reperfusion, at 3 h and 6 h. Plasma was separated at 1120g for

20 min at 4^0C and 500µl aliquots were stored at -80^0C and used for the determination of carbonyl and 8-isoPGF$_2\alpha$.

Histology

Needle core biopsies were taken pre and post perfusion, fixed in 4% formalin, dehydrated and embedded in paraffin wax. Sections were cut at 4µm then stained with haematoxylin and eosin for evaluation using light microscopy. Sections were scored by two independent observers over 5 fields assessing changes in seven morphological parameters. The samples were scored 0, 1, 2, 3 indicating the level of damage (0 normal, 1 mild, 2 moderate and 3 severe morphological changes) and mean severity scores calculated.

Measurement of protein carbonyl

Protein carbonyls were measured in plasma and tissue using an enzyme immunoassay (ELISA) kit (Zenith Technology Corp Ltd, Dunedin, NZ). Needle biopsy (20µg) homogenates were prepared using an eppendorf homogeniser in only 25µl of ultra-pure water to maximise protein concentration. Plasma and homogenates were centrifuged at 10,000*g* for 2 min and the supernatant taken for analysis. Standards and samples were derivitised with excess of dinitrophenylhydrazine (DNP) for 45 min at RT. For tissue samples twice the recommended volume was taken at the derivitisation stage to further increase protein concentration. Derivitised samples were applied to an ELISA plate and incubated O/N at 4°C. The plate was further developed according to assay instructions; washed and blocked for 30 min at RT,

washed and incubated with anti-DNP-biotin antibody for 1 h at 37°C, washed and incubated with streptavidin-HRP for 1 h at RT. After a final wash the chromatin reagent was applied. The reaction was stopped after 7 min and the absorbance was read at 450nm.

Determination of 8-isoPGF$_2\alpha$

The levels of plasma 8-isoPGF$_2\alpha$ were also determined by ELISA (Cayman Chemical Co, MI, USA). Plasma samples were centrifuged at 10,000*g* for 2 min and the supernatant taken for analysis. Plasma was diluted 10 fold prior to analysis. The sample and standards were added in triplicate to the ELISA plate together with an 8-isoPGF$_2\alpha$-anticholinesterase (AChE) conjugate and incubated for 18 h. During incubation the 8-isoPGF$_2\alpha$ present in the sample competed with the 8-isoPGF$_2\alpha$-AChE conjugate for 8-isoPGF$_2\alpha$-rabbit antiserum binding sites on the pre-coated plate. The plate was washed and then developed by addition of the substrate to AchE. The plate was read at 450nm after the colour development for 60 min.

Detection of 8-oxo-dG

Urinary 8-oxo-dG was detected using the Japan Institute for the Control of Aging new 8-oxo-dG check enzyme immunoassay kit (Gentaur, Legerlaan, Br). Urine samples were centrifuged at 10,000*g* for 2 min and were applied undiluted. Standards and samples were added to the ELISA plate in triplicate together with the 8-oxo-dG monoclonal antibody and incubated for 1 h at 37°C. This antibody reacted competitively with 8-oxo-dG in the samples

and 8-oxo-dG bound on the plate. The plate was washed and then incubated with the secondary antibody for 1 h at 37°C. The plate was washed and then developed by addition of the enzyme substrate. The plate was read at 450nm after the colour development for 15 min.

Measurement of caspase-3 activity

Caspase-3 activity was detected by a modified Fluorometric CaspACE™ Assay System (Promega, Southampton, UK) using 100 μg protein of renal tissue. This assay is based on the ability of caspase-3 to cleave a fluorogenic substrate of Ac-DEVD-7-Amino-4-Methyl Coumarin (AMC). The specificity of the assay was determined using a caspase-3 inhibitor Ac-DEVD-CHO. The fluorescence of this reaction was monitored at 360nm excitation and 460nm emission using Mx4000™ Multiplex QPCR System. Fluorescence intensity was calibrated against standard concentrations of AMC and caspase-3 activity was expressed as pmol AMC liberated per minute per μg protein at 30°C.

Statistical analysis

Values are presented as mean ± s.d or median with ranges. Continuous variables such as serum creatinine were plotted against time and the area under the curve (AUC) for individual perfusion experiments calculated using Excel software (Microsoft, Reading, UK). Analysis of data was carried out using GraphPad Instat Version 3 (GraphPad Software Inc., San Diego, USA). Differences between data collected at multiple time points within groups

were compared by either non-parametric one-way ANOVA with Tukey-Kramer post test or Kruskal-Wallis with Dunn's post test. Pearson and Spearman r values were used to describe correlations. $P \leq 0.05$ was taken as significant.

Results

Perfusion parameters

Perfusion pressure, temperature and kidney weight were very similar for all the groups (*Table 5*). There was no significant difference in starting perfusate haematocrit or white cell count. Cold storage time was just over 2 h for all groups. Kidneys in the control group (WIT7) sustained the shortest practicable warm ischaemic time in a NHBD rapid retrieval model which was found to be 7 minutes. The other three groups (WIT15, WIT25 and WIT40) sustained precisely 15, 25 and 40 min warm ischaemic time respectively.

Renal function

Renal function deteriorated sequentially with increasing WIT although the most significant difference was seen between WIT7 and WIT40 (*Table 6*). The percentage creatinine fall for WIT7, 15, 25 and 40 after 6 h perfusion were 76 ± 14%, 57 ± 17%, 52 ± 17% and 43 ± 15% respectively with WIT25 and 40 being significantly different from WIT7 (P=0.041, P=0.008; Fig. 9). The area under the curve for serum creatinine over 6 h was 2160 ± 487, 2772 ± 245, 3018 ± 679 and 3456 ± 781 for WIT7, 15, 25 and 40 respectively, although

only WIT7 and 40 were statistically significantly different (P=0.002; *Fig.10*). Creatinine clearance was significantly better again only in WIT7 compared with WIT40 throughout the entire perfusion period. However a marked increase in creatinine clearance was seen in WIT15 between 3 and 6 h perfusion remaining significantly different from WIT40 for this period (*Fig. 11*). The area under the curve for creatinine clearance showed a similar pattern being 14.4 ± 11.9, 10.5 ± 7.3, 4.8 ± 4.2 and 2.4 ± 2.6 for WIT7, 15, 25 and 40 (WIT7 *versus* WIT40, P=0.004; WIT15 *versus* WIT40, P=0.016). Total urine output was significantly greater in the WIT7 group versus WIT15, 25 and 40 groups being 536 ± 221 ml/hr compared to 241 ± 219 ml/hr, 250 ± 186 ml/hr and 151 ± 136 ml/hr respectively (P=0.04). The mean AUC for urine output for WIT7, 15, 25 and 40 were 432 ± 175, 202 ± 185, 179 ± 128 and 154 ± 127 respectively P=0.06).

Oxygen consumption, haemodynamics and acid-base homeostasis

Oxygen consumption at 6 h was lower with increasing WIT being 31 ± 6 ml/min/g, 21 ± 10 ml/min/g, 18 ± 13 ml/min/g and 16 ± 10 ml/min/g for WIT7, 15, 25 and 40 respectively; only WIT40 was statistically significantly different from WIT7 (P=0.018; *Fig. 12*). WIT40 had significantly poorer renal blood flow after 6 h compared with WIT7, 26 ± 15 ml/min/100g versus 50 ± 10 ml/min/100g (P=0.009), and higher renal vascular resistance at 1.0 ± 0.3 mmHg versus 0.5 ± 0.09 mmHg(P=0.004; *Fig. 13, Fig. 14*). Renal blood flow was also lower in WIT15 (34 ± 18 ml/min/100g) and WIT25 (29 ± 22 ml/min/100g) at 6 h compared to WIT7, although these were only of marginal significance (P=0.06). A gradually increasing

acidosis during perfusion was observed in all kidneys with no difference between the four groups (*Fig. 15, Fig. 16*).

Parameters	WIT7	WIT15	WIT25	WIT40	P value
Temperature (^{0}C)	37.3 ± 0.5	36.7 ± 0.6	36.4 ± 1.4	35.9 ± 2.7	0.49
White cell count (x10^9/L)	6.4 ± 1.3	6.4 ± 1.4	6.0 ± 0.4	6.2 ± 1.4	0.93
Cold storage (min)	2.0 ± 0	2.1 ± 0.1	2.2 ± 0.2	2.1 ± 0.1	0.08
Mean art. pressure (mmHg)	54 ± 1	54 ± 1	53 ± 1	54 ± 1	0.25
Haematocrit L/L	0.2 ± 0.02	0.19 ± 0.01	0.2 ± 0.02	0.2 ± 0.03	0.85
Warm ischaemic time (min)	7.0 ± 1.0	15	25	40	<0.0001

Table 5: Perfusion parameters

Parameters	WIT7	WIT15	WIT25	WIT40	P value
% creatinine fall	76 ± 14*[1,2]	57 ± 17	53 ± 17*[1]	40 ± 16*[2]	*[1] 0.041 *[2] 0.008
AUC creatinine	2160 ± 487*[1]	2772 ± 245	3018 ± 679	3456 ± 781*[1]	*[1] 0.002
AUC cr. clearance	14.4 ± 11.9*[1]	10.5 ± 7.3*[2]	4.8 ± 4.2	2.4 ± 2.6*[1,2]	*[1] 0.004 *[2] 0.016
O2 consum.(ml/min/g)	31 ± 6*[1]	21 ± 11	18 ± 13	16 ± 10*[1]	*[1] 0.018
pH	7.2 ± 0.08	7.2 ± 0.09	7.1 ± 0.16	7.1 ± 0.03	0.18
RBF (ml/min/100g)	26 ± 15*[1]	34 ± 18	29 ± 22	50 ± 10*[1]	*[1] 0.009
AUC RBF	226 ± 55	168 ± 65	152 ± 79	137 ± 69	0.19
RVR (mmHg)	0.5 ± 0.09*[1,2]	1.1 ± 0.9*[1]	2.2 ± 2.5	1.0 ± 0.3*[2]	*[1] 0.004 *[2] 0.004
Tot. urine output (ml)	536 ± 221*[123]	241 ± 219*[1]	250 ± 186*[2]	151 ± 136*[3]	*[12] 0.04 *[3] 0.015
AUC urine output	432 ± 175	202 ± 185	179 ± 128	154 ± 127	0.06

Table 6: Summary of functional parameters after 6 h of perfusion.

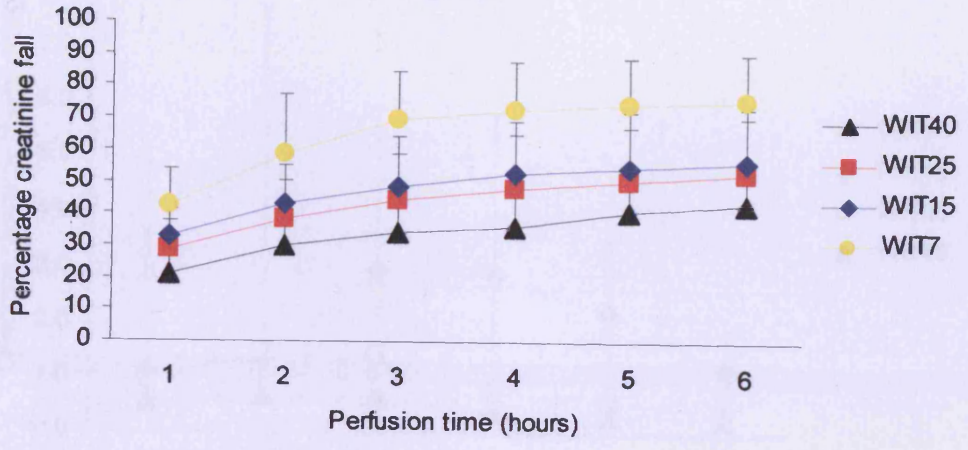


Figure 9: Percentage creatinine fall during perfusion

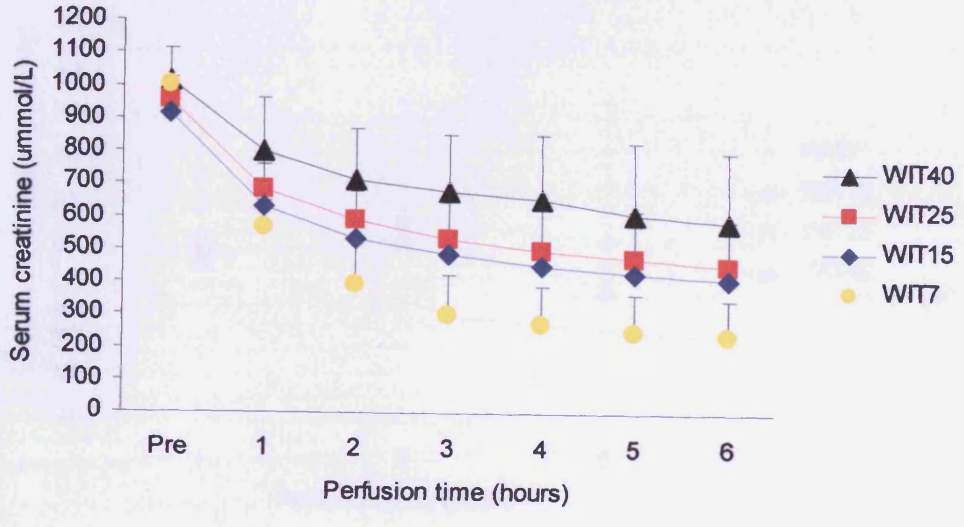


Figure 10: Serum creatinine over 6 h perfusion

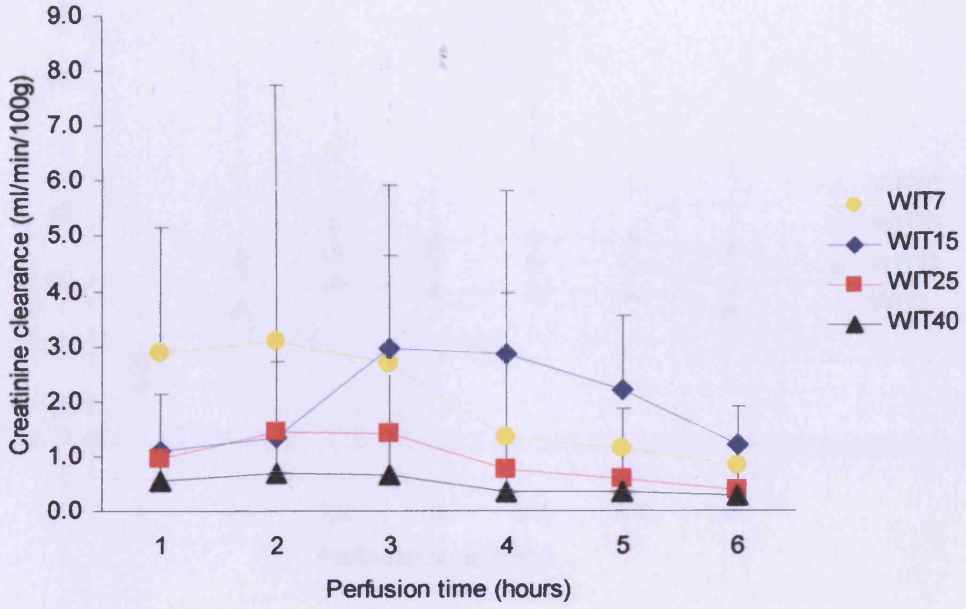


Figure 11: Creatinine clearance over 6 h perfusion

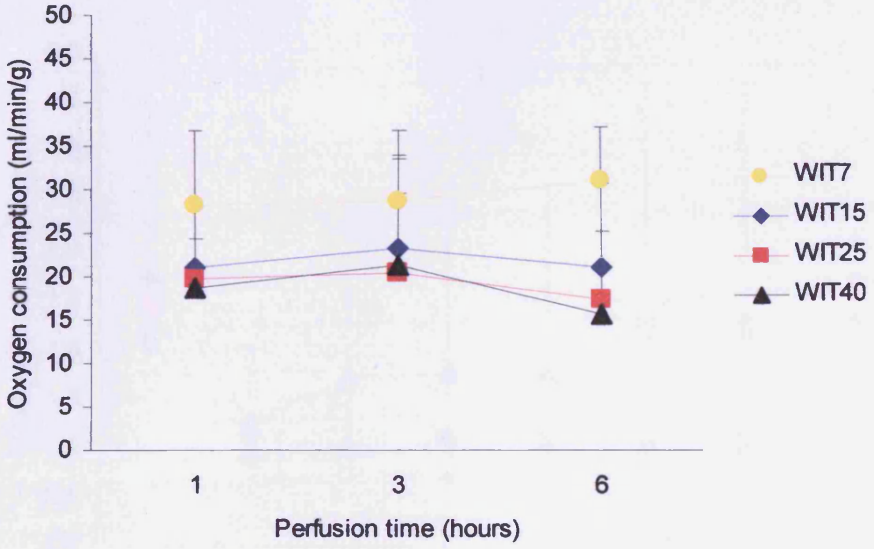


Figure 12: Oxygen consumption over 6 h perfusion

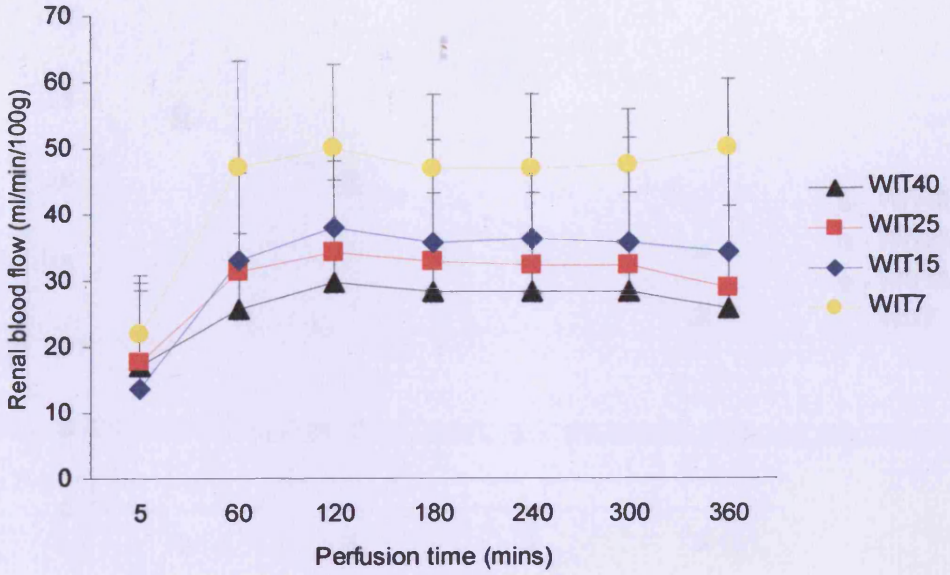


Figure 13: Renal blood flow over 6 h perfusion

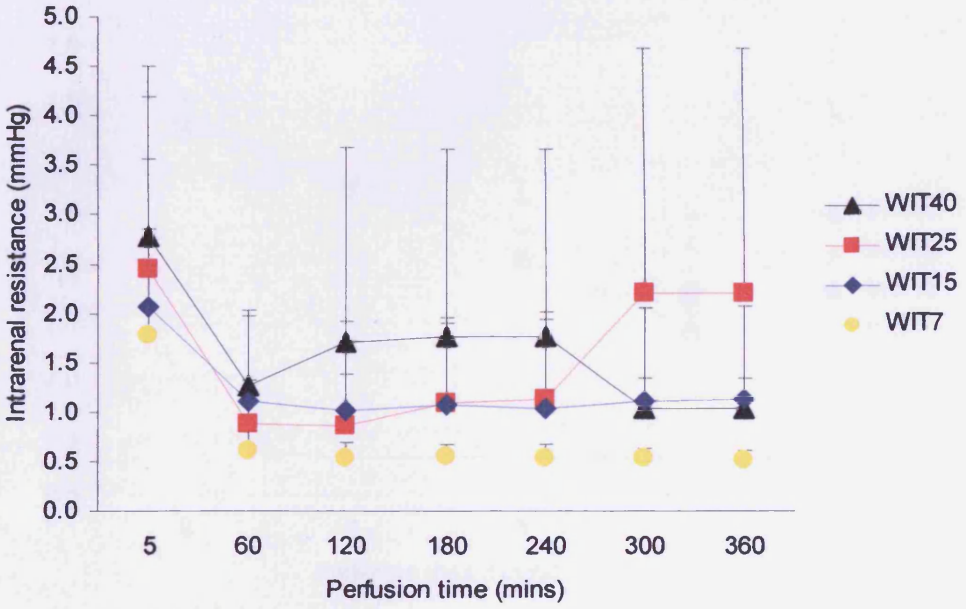


Figure 14: Intrarenal resistance over 6 h perfusion

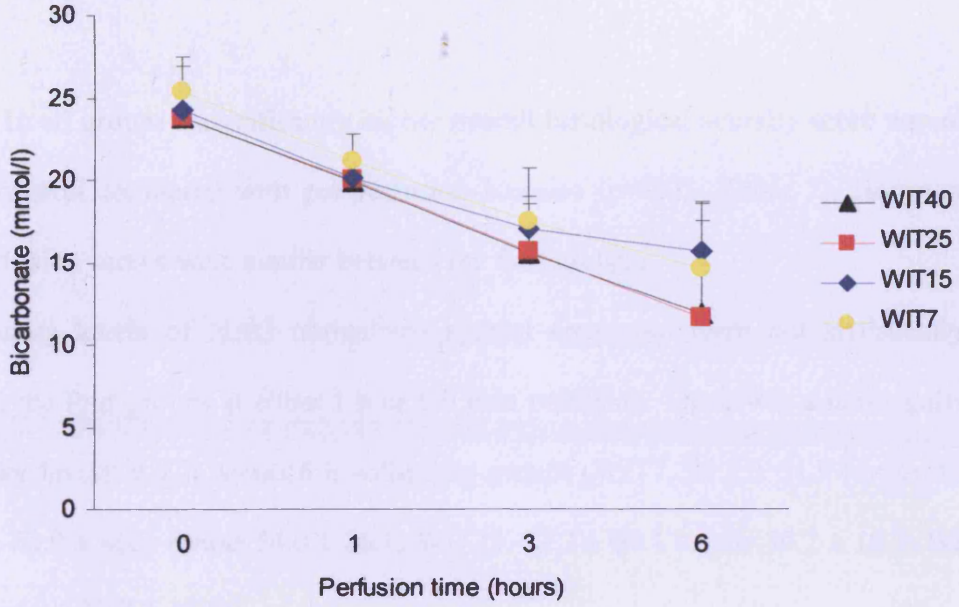


Figure 15: Serum bicarbonate levels over 6 h perfusion

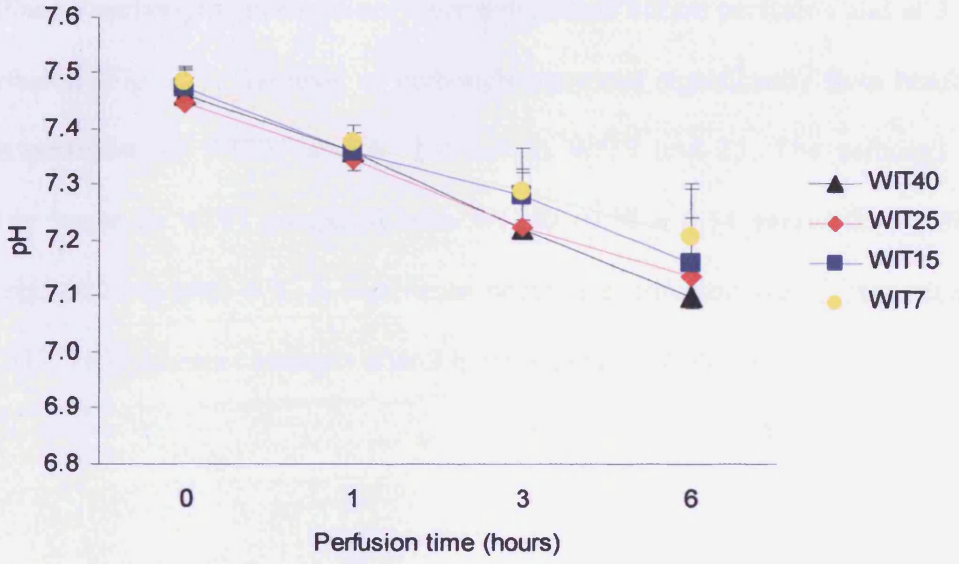


Figure 16: Serum pH over 6 h perfusion

Histology and urinary N-Acetyl-β-D glucosaminidase (NAG)

In all groups a significantly higher overall histological severity score was observed in post-perfusion compared with pre-perfusion biopsies ($p<0.05$; *Table 7*). However pre- and post perfusion scores were similar between the four groups.

The urinary levels of NAG normalised against creatinine were not statistically different between the four groups at either 1 h or 6 h post perfusion. There was a non-significant trend for higher levels at 1 h *versus* 6 h within the groups (WIT7, 54.2 ± 51.9 *versus* 40.7 ± 36.8; WIT15, 63.9 ± 40.3 *versus* 54.0 ± 26.1; WIT25, 72.2 ± 68.1 *versus* 30.2 ± 10.3; WIT40, 68.8 ± 18.3 *versus* 37.7 ± 48.0).

Protein oxidation products in plasma

Plasma carbonyl concentrations were determined before perfusion and at 3 h and 6 h post-perfusion (*Fig. 17*). The level of carbonyls increased significantly from baseline at 3 h and 6 h perfusion in WIT25 and 40 but not in WIT7 and 15. The carbonyl level was marginally lower in WIT7 compared with WIT40 (0.78 ± 0.14 *versus* 0.93 ± 0.11 ng/ml repectively; $p=0.06$) after 6 h. A significant negative correlation was shown between urine output (AUC) and plasma carbonyls after 3 h perfusion ($r = -0.415$, $p<0.05$; *Fig. 18*).

	WIT7	WIT15	WIT25	WIT40
Pre-perfusion	5.4 ± 1.1	4.2 ± 1.6	6.2 ± 1.4	4.2 ± 1.6
Post-perfusion	10.2 ± 2.3	9.0 ± 1.9	9.2 ± 1.8	8.4 ± 1.5

Table 7: Histology severity scores pre- and post perfusion

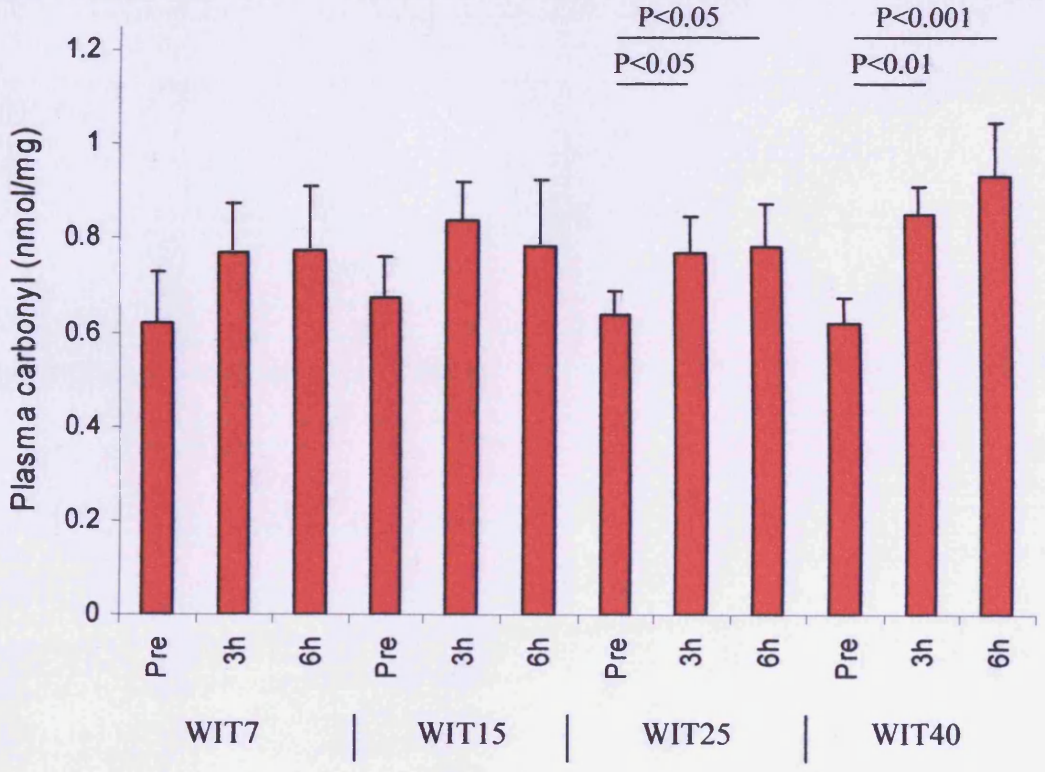


Figure 17: Plasma carbonyl pre and post reperfusion

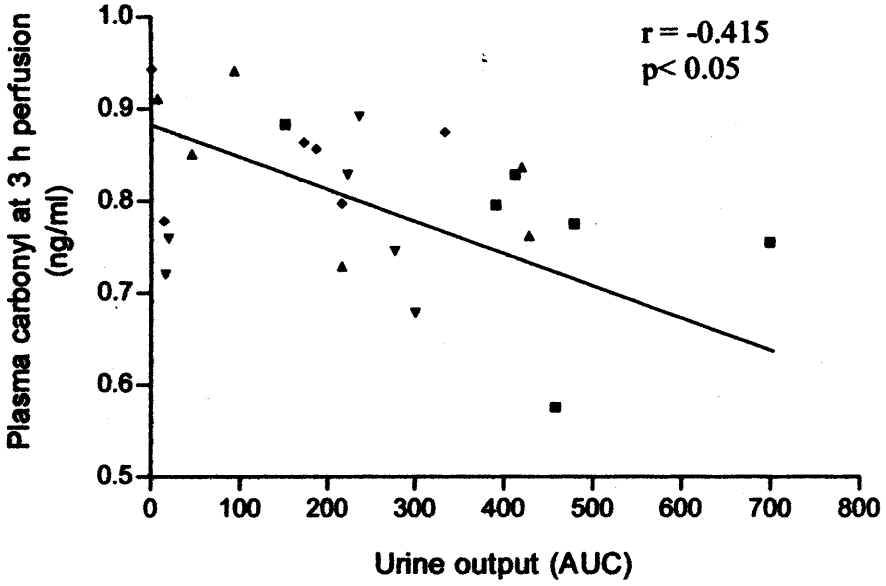


Figure 18: Correlation between plasma carbonyl at 3 h post reperfusion and AUC urine output. WIT7 = ■, WIT15 = ▲, WIT25 = ▼and WIT40 = ♦.

Protein oxidation in tissue

The results of carbonyl determination in biopsy tissue before perfusion and 30 min and 6 h after perfusion are shown in Figure 19. There were no significant differences between the groups or within the groups at different time points. No correlations between functional perfusion parameters and tissue carbonyl levels were identified when analysing all the groups. However within WIT7 alone tissue carbonyl levels at 30 min perfusion positively correlated strongly with renal blood flow ($r = 0.93$, $p<0.01$; *Fig. 20*).

Lipid peroxidation index in plasma

The levels of 8-iso-$PGF_2\alpha$ in plasma were significantly higher after 6 h perfusion compared to pre-perfusion in WIT40 (0.39 ± 0.18 *versus* 2.51 ± 1.61; $p<0.005$), a difference not found with the other 3 groups. (*Fig. 21*) The level of 8-iso-$PGF_2\alpha$ in WIT40 was also significantly higher than the other groups at 6 h post-perfusion ($p<0.05$). A significant negative correlation was found between 8-iso-$PGF_2\alpha$ levels at 6 h and AUC for creatinine clearance ($r = -0.649$, $p<0.005$; *Fig. 22*) and also creatinine clearance at 6 h ($r = -0.589$, $p<0.005$). A trend towards a positive correlation between 8-iso-$PGF_2\alpha$ levels and AUC serum creatinine was also found ($r = 0.427$, $p<0.053$).

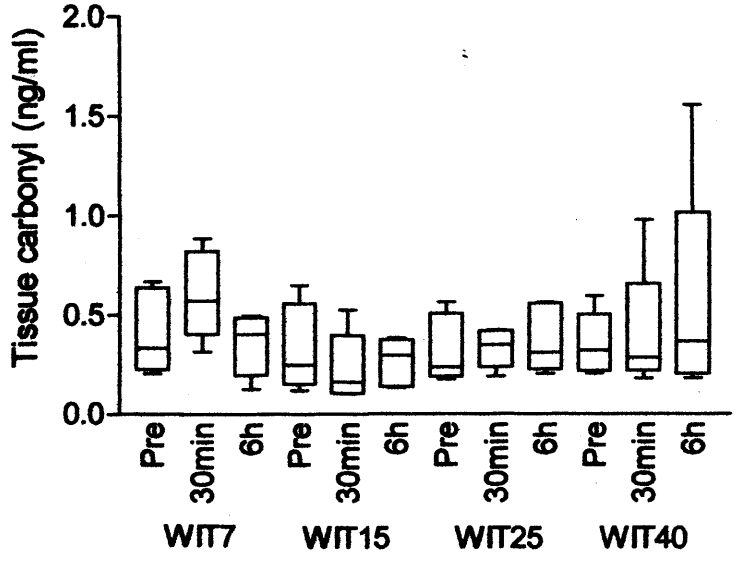


Figure 19: Tissue carbonyl pre and post perfusion. Values are median and range.

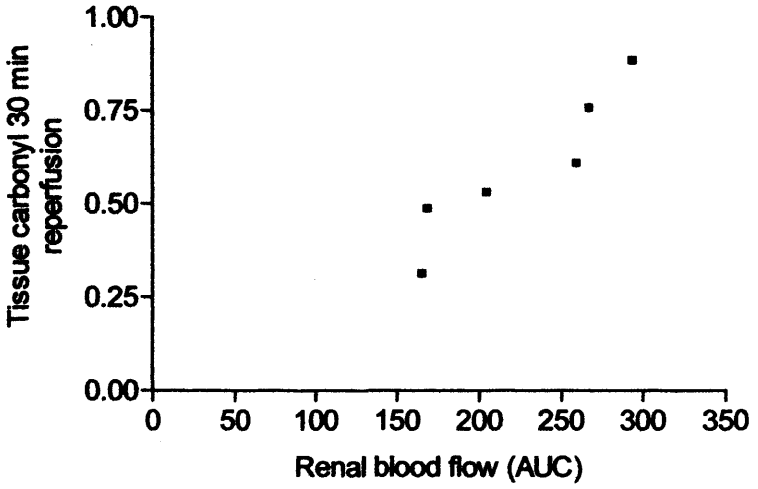


Figure 20: Correlation between tissue carbonyl at 30 min perfusion and AUC renal blood flow in WIT7 group (■).

DNA oxidation products in urine

Urinary 8-oxo-dG normalised to creatinine at 1h and 6 h reperfusion were 5.9 (3.5 - 10.2) and 5.1 (3.9 - 20.2) for WIT7, 4.9 (3.3 – 12.7) and 7.2 (5.4 – 12.6) for WIT15, 8.5 (4.9 – 22.9) and 3.6 (2.6 – 15.5) for WIT25, 9.6 (7.4 - 9.7) and 4.8 (3.2 – 10.9) for WIT40. Higher levels of urinary 8-oxo-dG were generally found at 1 h compared with 6 h but no significant difference was found within any group. There was a trend for increasing 8-oxo-dG levels at 1 h with increasing WIT but again differences were not statistically significant.

Caspase-3 activity in tissue

Caspase-3 activity (pmol AMC liberated/min/μg protein) after 6 h perfusion was higher in WIT7 (0.021 ± 0.004) compared to the other groups and this was statistically significant for WIT15 and 40 (0.01 ± 0.001 and 0.01 ± 0.006, $p<0.05$; *Fig. 23*). There was a trend towards increased caspase-3 activity post-perfusion compared with pre-perfusion within each group. Caspase-3 activity correlated negatively with AUC serum creatinine ($r = -0.614$, $p>0.005$; *Fig. 24*) and positively with AUC creatinine clearance ($r = 0.556$, $p<0.005$), AUC urine output ($r = 0.441$, $p<0.05$) and AUC renal blood flow ($r = 0.555$, $p<0.005$).

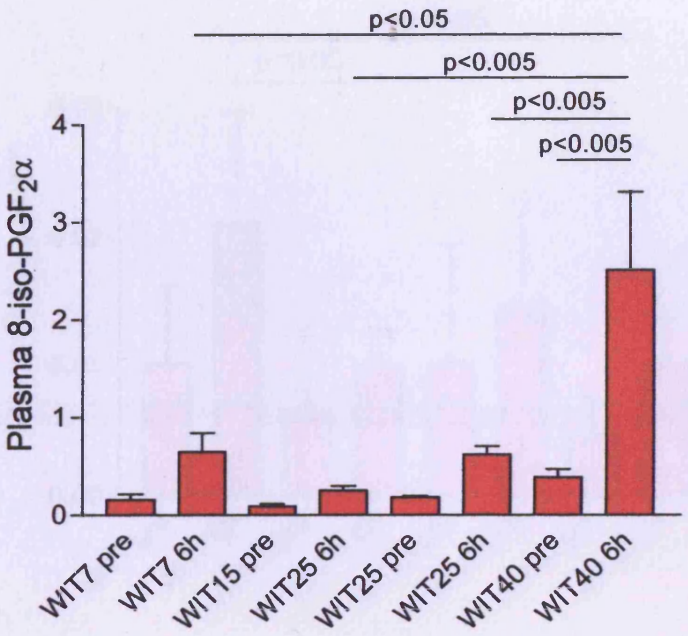


Figure 21: Plasma 8-iso-$PGF_{2\alpha}$ pre and post perfusion

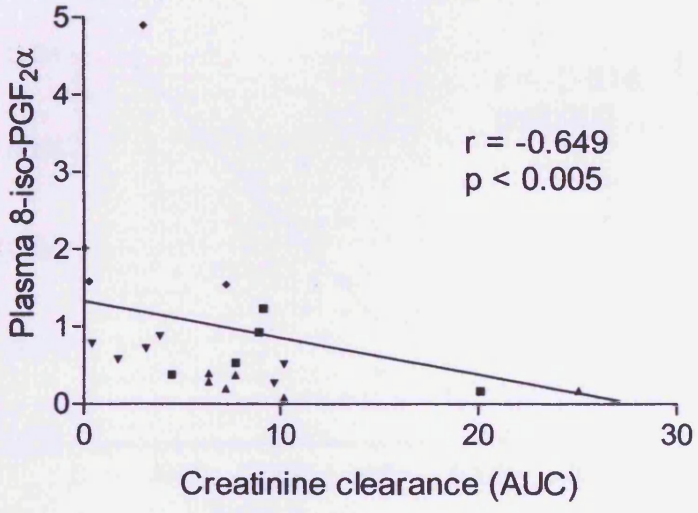


Figure 22: Correlation between plasma 8-iso-$PGF_{2\alpha}$ at 6 h post reperfusion and AUC creatinine clearance. WIT7 = ■, WIT15 = ▲, WIT25 = ▼and WIT40 = ♦.

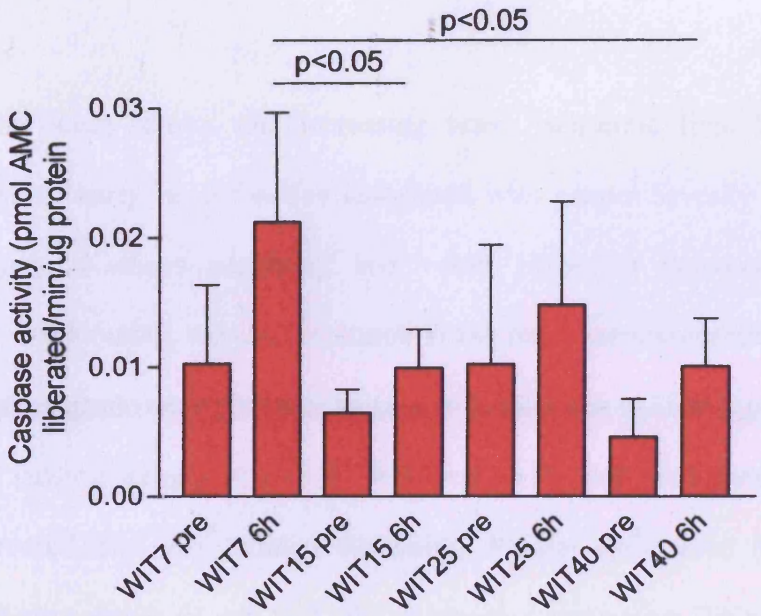


Figure 23: Caspase 3 activity pre and post perfusion

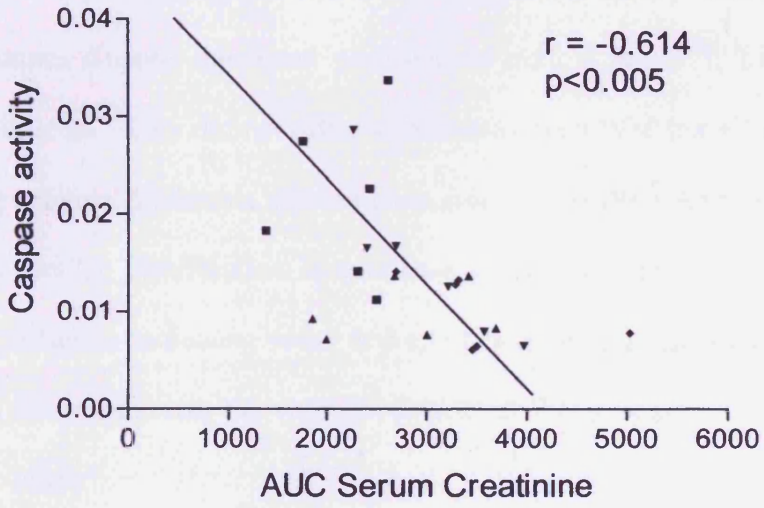


Figure 24: Correlation between caspase activity at 6 h post perfusion and AUC serum creatinine. WIT7 = ■, WIT15 = ▲, WIT25 = ▼and WIT40 = ♦.

Discussion

This study shows that increasing warm ischaemic time leads to a proportional impairment of early renal function associated with greater severity of underlying oxidative tissue injury. Kidneys sustaining less warm ischaemia demonstrated better creatinine clearance, urine output, oxygen consumption and renal haemodynamics. In parallel to superior function these grafts were shown to have significantly less oxidative protein and lipid damage.

In general terms a sequential deterioration in functional parameters was observed as WIT increased but only kidneys sustaining 40 min WIT were statistically significantly different from controls (7 min WIT) for all recorded parameters. This suggests that reasonable and comparable overall graft function can be maintained for up to at least 25 min WIT. However, it is important to note that despite showing significant impairment, the 40 minute WIT group still maintained demonstrable function throughout the 6 h perfusion period. This is in contrast to a previous similar haemoperfused porcine kidney model in which a mean WIT of 17 minutes limited functional perfusion to only 2 hours[216]. Interestingly, histological evidence of renal injury did not differ significantly with WIT but all kidneys, including those sustaining minimal ischaemia showed clear evidence of IRI after 6 h reperfusion. All groups showed a similar deterioration in acid-base homeostasis and similar urinary NAG levels during reperfusion indicating some degree of renal tubular injury regardless of WIT. This finding is consistent with the well recognised relative propensity of the proximal tubule to ischaemic injury[45].

Plasma protein carbonyls were significantly increased from baseline following reperfusion in kidneys sustaining longer WIT (25 and 40 min) but not in those sustaining

shorter periods (8 and 15 min). This finding, in addition to higher levels in the WIT40 compared with WIT7 kidneys at 6 h perfusion, indicates that prolonged warm ischaemic time is associated with increased severity of oxidative protein injury on reperfusion. The difference between the WIT7 and 15 groups compared with WIT25 and 40groups is suggestive of a threshold of WIT after which IRI becomes significantly greater, in this case a time period of between 15 and 25 min.

Carbonyl levels in tissue were not significantly different over perfusion time or between different groups. The reason for this finding, in contrast to plasma carbonyl levels, is not clear. It is recognised that moderately oxidised proteins are selectively degraded by the proteosome and macromolecular damage may upregulate phagocytosis by macrophages[248, 249]. The rapid removal of carbonyls in this way may lead to diminished carbonyl levels in tissue that do not reflect the amount of carbonyls generated and released into plasma. The correlation between renal blood flow and carbonyl levels at 30 min perfusion in the WIT7 group suggests that the increased oxygen delivery results in enhanced generation of ROS. Carbonyl formation on hepatic proteins in rats exposed to high oxygen concentrations has been demonstrated previously[250]. In this context, poorer renal blood flow in kidneys sustaining longer WIT may lead to less protein oxidation despite more severe underlying ischaemic injury.

Post-perfusion plasma 8-iso-$PGF_2\alpha$ was significantly higher in kidneys sustaining 40 min WIT when compared to all other kidneys suggesting that prolonged WIT is associated with increased renal parenchymal lipid peroxidation during reperfusion. These findings lend further support to an ischaemia threshold for more severe oxidative injury, although a longer period than protein carbonyl data may indicate. This longer critical WIT of 25 to 40 min is more in keeping with the functional data. Furthermore, it has been suggested that the

conversion of xanthine dehydrogenase to oxidase in the ischaemic kidney occurs over a period of about 30 min and thus after such a period of ischaemia much more capacity for ROS production is generated[53]. In the clinical setting, it has been reported that NHBD allografts sustaining over 30 min WIT had a significantly higher risk of PNF[39].

Plasma 8-iso-$PGF_2\alpha$ correlated negatively with renal function supporting an association between lipid oxidative injury and post-ischaemia renal impairment. This correlation, and that between protein oxidation and urine output, suggests that 8-iso-$PGF_2\alpha$ and carbonyls could represent useful markers of renal IRI severity and predictors of allograft function. The reliable detection of these markers in plasma makes them more clinically applicable than tissue samples (requiring renal biopsies) and urine samples (requiring urine production). One potential problem with 8-iso-$PGF_2\alpha$ as a marker of lipid peroxidation identified in some studies, is cyclo-oxygenase dependent production of 8-iso-$PGF_2\alpha$ during the ischaemic phase[251]. The numerical rise of 8-iso-$PGF_2\alpha$ in all groups post-perfusion in this study support ROS related formation following reoxygenation.

8-oxo-dG was detected in the urine where it is thought to represent a DNA repair product and an indirect marker of DNA damage, and is a simpler measurement than that from tissue which is more likely to induce artefactual damage[252]. Although there was no difference in the levels of 8-oxo-dG detected in this study, the sample size was limited, as some kidneys in the 40-minute group did not produce urine. However, it clearly demonstrated the assay could be used on porcine samples and that the levels of 8-oxo-dG were within the detectable range of the assay.

Caspase-3 activity was greatest with shorter WIT and was positively correlated with renal function. The lower levels of caspase activity following prolonged WIT is likely to

represent a higher ratio of necrotic to apoptotic cell death in the context of poorer energy supply for active programmed cell death[116]. However some caution must be taken with this conclusion as it has been suggested that Caspase-3 may also contribute to necrotic death signaling[253]. In addition, Caspase-3 activity may be associated with better graft function due to the beneficial role of apoptosis in deletion of damaged renal and inflammatory cells and remodeling of hypertrophic kidneys[165]. Caspase-3 levels may represent a potential marker of IRI severity and predictor of graft recovery and subsequent function. In this study, a numerical increase in caspase-3 activity was seen from pre- to post-perfusion within all groups but differences were not statistically significant. Among other reports it remains contentious whether significant initiation of apoptosis occurs during the ischaemic phase or solely on reperfusion and further investigation will be required to clarify this question[113, 114].

The IOPS provided a reliable model of early reperfusion with the major advantage of being able to manipulate any chosen variable in order to investigate factors influencing graft function and injury. The system allows for serial sampling of blood, tissue and urine for analysis not often possible in other models, in particular *in vivo* transplantation studies. However the *ex-vivo* system has some limitations. For practical reasons relating to the use of Schedule 1 pigs, the shortest period of warm ischaemia possible was 7 minutes. Perfusion of kidneys sustaining no WIT would require heart-beating organ retrieval from terminally anaesthetised pigs but would provide useful additional controls. The period of cold ischaemia was deliberately limited to 2 h but this is clearly very short in comparison to the average period of hypothermic preservation required in clinical renal transplantation. Further studies will need to be performed in order to dissect the effects of different warm and cold ischaemic times.

The perfusion period was limited to 6 h, as although longer haemoperfusion periods have been successful for livers[215], previous renal studies have shown that beyond this time it is difficult to maintain renal viability with a gradual deterioration of blood flow and urine output[216]. The centrifugal blood pump used in this model provided non-pulsatile flow and the mean perfusion pressure of 50-60 mmHg was lower than the normal physiological range for porcine and human kidneys. This arterial perfusion pressure was chosen after preliminary studies showed that higher pressures lead to the development of marked haematuria, possibly due to a loss of afferent vascular autoregulation following isolation from neuronal and humoral control. It is certainly possible that the use of sub-physiological perfusion pressures may limit the perfusion period and blunt potential differences between experimental groups. The investigation of the effect of arterial pressure on isolated haemoperfused kidneys would be a crucial part of the development of this model prior to applying it effectively to the investigation of interventions aimed at ameliorating renal allograft IRI.

In conclusion, the IOPS demonstrated a clear association between increasing warm ischaemic time and both greater severity of IRI and deterioration in renal function. In addition, serum markers of protein and lipid oxidative injury correlated well with WIT-dependent variation in renal function and as such may represent a clinically applicable tool for assessing graft viability. In this context, graft assessment and selection may be particularly useful in view of findings in this study suggestive of a warm ischaemia threshold beyond which graft function is significantly compromised.

CHAPTER 5

The Effects Of Arterial Pressure On Isolated Haemoperfused Porcine Kidneys

Introduction

The optimal conditions under which to perfuse kidneys normothermically still remain undetermined. In particular, the effects of different arterial perfusion pressures are unknown. Sub-physiological pressures used in previous experiments were based on significant perfusate haemolysis and haematuria observed at higher pressures during early development of the IOPS model. Perfusion at lower pressures was used successfully to investigate factors influencing renal function, such as WIT, during haemoperfusion. However, recorded glomerular filtration rates were universally well below the normal physiological range as a result of loss of autoregulation at low perfusion pressures. Higher pressures may be required to provide a more accurate model of allograft reperfusion and allow for prolonged, clinically applicable normothermic graft preservation. Isolated organ perfusion at near physiological pressures may however be limited by a propensity to cause endothelial injury[227]. The aim of this study was to test the effect of arterial pressure on renal function and preservation in an isolated blood perfused porcine kidney model.

Methods

Retrieval and Perfusion

Kidneys from large white pigs were retrieved and haemoperfused for 6 h as previously described in Chapter 3.

Experimental protocol

The kidneys were perfused on the isolated circuit for 6 h at a set mean arterial pressure of either 95, 75 or 55 mmHg (n = 6). Renal blood flow (RBF), mean arterial pressure (MAP) and intrarenal vascular resistance were recorded as previously described. Serum and urine samples were taken hourly for biochemical analysis and whole blood was sent for haematology. Creatinine clearance (U_{cr} x U volume / P_{cr}) and fractional excretion of sodium (U_t x U flow) / (GFR x P_t) x 100 were calculated and serum creatinine levels recorded hourly. Blood gases were measured for the calculation of oxygen consumption and acid-base homeostasis was measured.

Needle core biopsies were taken pre and post perfusion and histological severity scores determined as previously described. Blood samples were taken before, and after 6 h perfusion. The plasma was separated and stored at -80°C. Von Willebrand factor (vWF) was detected by enzyme-linked immunosorbent assay (Technoclone Ltd Dorking, Surrey).

Statistical analysis

Values are presented as mean ± s.d. Continuous variables such as serum creatinine were plotted against time and the area under the curve for individual perfusion experiments calculated using Excel software (Microsoft, Reading, UK). Mean AUC were then compared using Kruskal-Wallis with Dunn's post test, Welch's corrected t-test between pre- and post-samples within the same groups and non-parametric ANOVA between groups for AUC and for comparison of values between all three groups. $P \leq 0.05$ was taken as significant.

Results

Perfusion parameters

The warm ischaemic time was kept between 6 and 7 minutes, and cold time to 2 h in all three groups. There was some variation in the individual weight of the kidneys prior to perfusion (237 ± 6.1 *versus* 251 ± 15.4 *versus* 216 ± 35.2 grams in the 95-, 75- and 55-mmHg groups respectively; P = 0.049). The blood perfusion temperature was also statistically significantly lower in the 55-mmHg group 37.3 ± 0.5 °C compared to 37.9 ± 0.3 and 37.9 ± 0.2 °C in the 95- and 75-mmHg groups (P = 0.025). Despite the significance of these parameters they were still within a comparative range.

Haematology

Pre-perfusion levels of haematocrit, haemogloblin and red blood cells were all approximately half of normal physiological levels. There was a significant fall in the haemoglobin levels in the 55-mmHg group after 6 h (P = 0.007) and a significant fall in the red blood cell count in both the 75- and 55-mmHg groups (P = 0.06 and 0.01 respectively) after 6 h (*Table 8*).

Haemodynamics

RBF sharply increased during the first hour in all three groups. After this the flow remained almost constant in 55-mmHg group. In contrast to this RBF continued to increase gradually in the 95- and 75-mmHg groups, reaching flow rates of 132 ± 32 and 83 ± 17 ml/min/100g respectively at 6 hrs compared to 50 ± 10 ml/min/100g in the 55-mmHg group (P = 0.001) (*Fig 25*).

Intra-renal resistance rapidly decreased during the first hour but remained fairly constant thereafter in all three groups (*Fig 26*). The final level of intra-renal resistance was significantly lower in the 95- and 75-mmHg group kidneys (P = 0.015).

Renal function

There was a statistically significant difference in oxygen consumption over the 6 h perfusion period, with consumption reaching a peak of 70 ± 16 ml/min/g in the 95-mmHg pressure group at 6 h and only 37 ± 4 and 31 ± 6 ml/min/g at 6 h in the 75- and 55-mmHg groups (P = 0.002).

The 95- and 75-mmHg group kidneys all maintained normal physiological pH levels. In the 55-mmHg group the pH steadily declined to a significantly lower level (P = 0.001) (*Fig 27*). This loss of acid-base homeostasis was due to a fall in bicarbonate levels (29.5 ± 2.6 and 24.9 ± 4.6mmol/L in the 95- and 75-mmHg groups respectively compared to 14.6 ± 2.9mmol/L in the 55-mmHg by 6 h; P = 0.002). Serum creatinine fell rapidly in the 95- and 75-mmHg pressure groups (97 and 95% falls respectively at 3 hr *versus* 69% in 55-mmHg

group; P = 0.002). The mean AUC for creatinine was significantly lower in the 95- and 75-mmHg group than in the 55-mmHg group (938 ± 140 *versus* 1290 ± 394 *versus* 2404 ± 595 respectively; P = 0.003).

Creatinine clearance peaked at 2 h in all groups with clearance levels of 33.5 ± 10.9, 20.4 ± 11.6 and 4.6 ± 12 ml/min/100g in the 95-, 75- and 55-mmHg groups respectively (P = 0.022), before falling to comparatively low levels in all groups (*Fig 28*). Total urine production was higher in the 95- *versus* the 55-mmHg group (P = 0.002) (*Table 9*).

Fractional excretion of sodium was low in all three groups during the first 3 h but thereafter rose progressively in the 95- and 75-mmHg groups (91 ± 45 *versus* 22 ± 12% for the 95- and 55-mmHg groups after 6 hrs respectively; P <0.05).

All three groups gained a similar amount of weight during the 6 h perfusion period (21 ± 7.8 *versus* 21 ± 4.1 *versus* 21 ± 7.7 % for the 95-, 75- and 55-mmHg groups respectively; P = 0.994)

Parameters	95mmHg		75mmHg		55mmHg	
	Pre	Post	Pre	Post	Pre	Post
Hcrit(l/L)	0.21 ± 0.01	0.23 ± 0.03	0.20 ± 0.01	0.18 ± 0.01	0.22 ± 0.01	0.19 ± 0.01
RBC(x10 ^12/l)	3.48 ± 0.24	3.48 ± 0.59	3.1 ± 0.25	2.7 ± 0.42*	3.5 ± 0.5	2.6 ± 0.49*
Hb(g/dl)	6.2 ± 0.36	6.13 ± 0.98	5.4 ± 0.47**	4.6 ± 0.79	6.1 ± 0.43	4.6 ± 0.61*

Table 8: Haematology, pre and post perfusion.

* P <0.05 *versus* pre level at same pressure.

** P <0.05 *versus* 95- and 55-mmHg groups.

Group	1hr	2hr	3hr	4hr	5hr	6hr	Total
95mmHg	440±62	310±93	280±150	362±227	485±306	493±243	2370±832*
75mmHg	319±128	204±42	200±72	228±101	228±157	189±173	1368±502
55mmHg	153±73	122±46	84±28	62±37	60±33	63±33	536±221

Table 9: Urine output (mean ± SD; ml/hr) and total urine output over 6 h.

* P=0.002 *versus* 55-mmHg group.

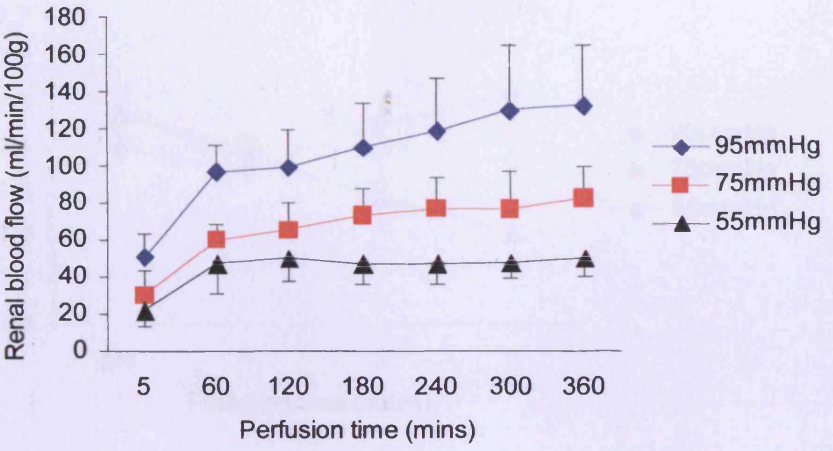


Figure 25: Renal blood flow over the perfusion period (mean ± SD). The mean AUC for the overall flow was significantly higher, in the 95-mmHg and 75-mmHg groups compared to the 55-mmHg group (647 ± 115 *versus* 411 ± 70 *versus* 275 ± 63; P = 0.001).

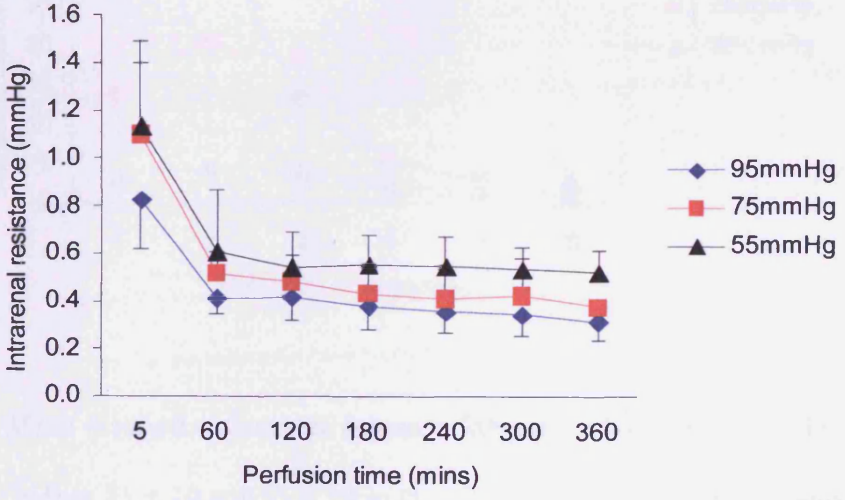


Figure 26: Intra-renal resistance over 6 h (mean ± SD).

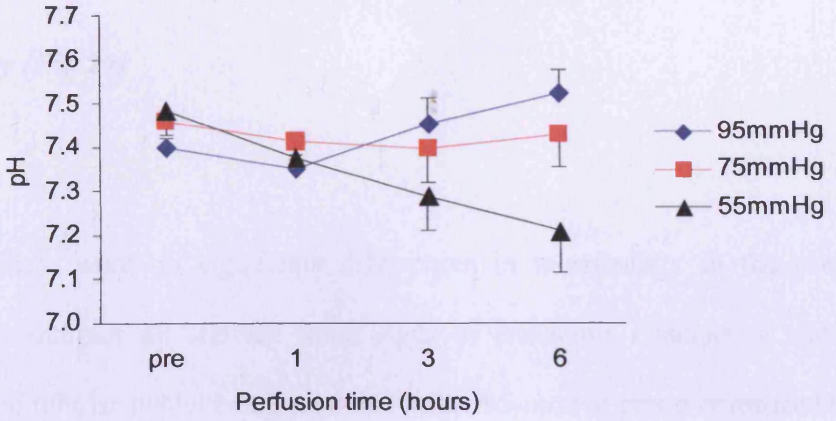


Figure 27: Arterial pH during the perfusion period (mean ± SD).

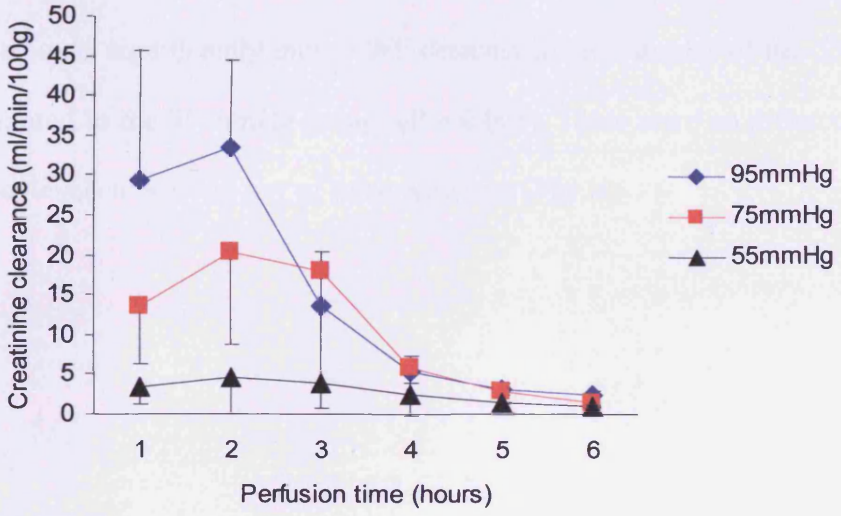


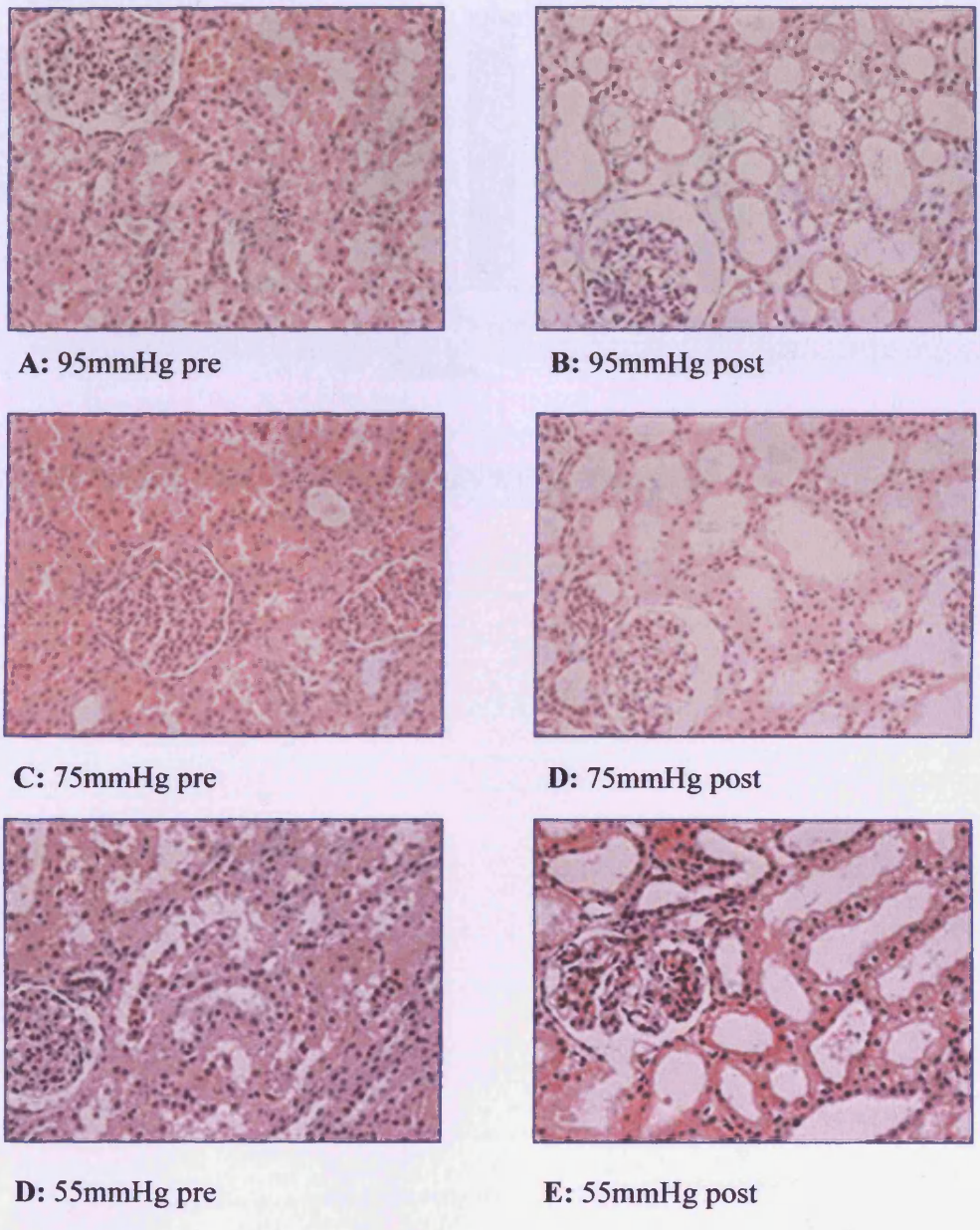
Figure 28: Mean creatinine clearance (mean ± SD) over 6 h of perfusion. The AUC was significantly higher 71 ± 19 and 55 ± 30 in the 95- and 75-mmHg group compared to 14 ± 12 in the 55-mmHg group (P = 0.002).

Histology (Fig 29)

There were no significant differences in morphology in the pre groups. The post perfusion samples all showed some signs of ischaemic changes, a significant increase in condensed tubular nuclei being recorded in the 95-mmHg group compared to the pre perfusion samples (P = 0.05). However, there were no significant differences between the post perfusion groups. The 6 h biopsies show acute ischaemic changes with tubular and vascular dilatation.

Endothelial damage

There was significantly more vWF detected in pre samples of the 55- and 75-mmHg group compared to the 95-mmHg groups (P = 0.003). There were no differences between pre and 6 h post levels of vWF at any of three pressures (*Fig 30*).



A: 95mmHg pre

B: 95mmHg post

C: 75mmHg pre

D: 75mmHg post

D: 55mmHg pre

E: 55mmHg post

Figure 29: Representative histology from pre and 6 h post reperfusion biopsies (H&E x20).

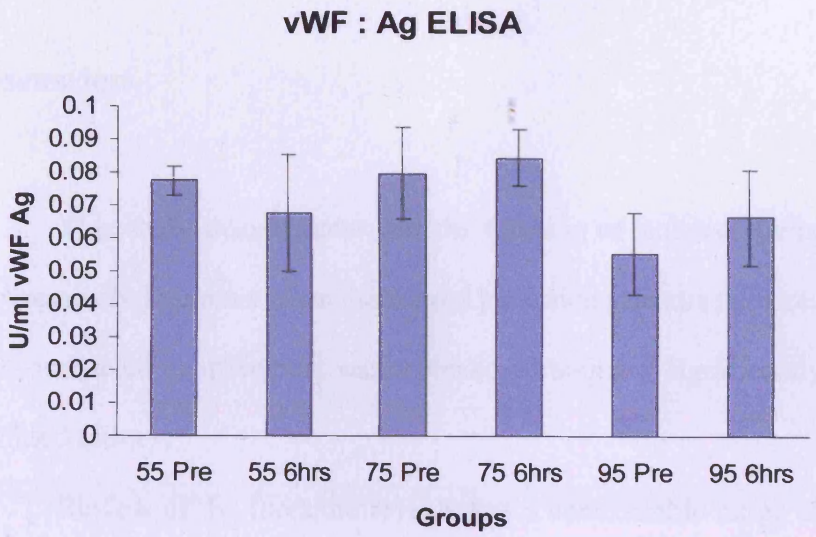


Figure 30: Pre and post perfusion results vWF (mean ± SD).

Discussion

This study demonstrates that the function of isolated haemoperfused porcine kidneys progressively improves when the arterial perfusion pressure is increased from 55 to 95 mmHg. This graduated improvement was achieved without any significantly deleterious effects on the perfused kidney.

Review of the literature reveals that a considerable range of perfusion pressures have been used in a number of disparate animal models. Lower pressures have been chosen because of fears that preservation at physiological pressures could fail to maintain organ integrity and induce endothelial damage[206, 226, 227]. Higher, more physiological pressures have been used in other models in order to achieve better renal function but at the expense of limiting the length of the perfusion period to a matter of a few hours[216, 226, 228, 229]. Most previous investigators have not justified their choice of perfusion pressure or presented data relating to the effects of different pressures in the same model. This study presents new information evaluating the effects of three different pressures in the porcine kidney.

Pig kidneys were chosen as they are recognised to have anatomical and physiological characteristics that mirror the human kidney[254]. In addition, the pig kidneys used here were of similar size to the adult human kidney. The lower level of 55 mmHg was chosen as a sub-physiological pressure that was less likely to cause mechanical endothelial damage. The higher pressure level of 75 mmHg was chosen as a more physiological pressure at the lower end of the range in which renal autoregulation of blood flow would be occurring and 95 mmHg representative of normal physiological pressure in both pig and man[255].

The IOPS machine used in these experiments maintained organ viability under normothermic conditions. Dilution of the blood with a crystalloid solution reduces viscosity and has been demonstrated to improve renal blood flow and kidney function[256]. At a perfusion pressure of 95 mmHg, renal blood flow was relatively high and the recorded levels of oxygen consumption were consistent with a high level of tubular metabolic function. At 75 mmHg renal blood flow was adequate and again demonstrated a high level of metabolic function. Creatinine clearances in the first 3 h were within the physiological range expected for human kidneys in both the 75- and 95-mmHg groups and the fall in clearance after this time was a reflection of the fact that most of the added creatinine had been removed to the urine rather than representing a true tailing off of renal function.

The higher pressure groups maintained normal acid-base homeostasis over the 6 h perfusion period. In contrast, although kidneys perfused at 55 mmHg kidneys remained viable with a reasonable level of oxygen consumption, renal function was much poorer than the higher pressure groups. The creatinine clearance values were always $\leq$ 5 ml/min/100g, confirming a very low glomerular filtration rate. This was presumed to be due to a loss of renal autoregulation at a perfusion pressure of 55 mmHg; in this group the renal blood flow at 6 h was approximately half the level in the 75-mmHg group and almost a third of that in the 95-mmHg group. Kidneys perfused at the lower pressure demonstrated a progressive and eventually severe acidosis, showing a failure to maintain acid-base homeostasis.

In all three groups there were some acute signs of ischaemic damage with cellular and interstitial swelling. This was also confirmed by the increase in weight gained by kidneys in all groups. A degree of tubular damage demonstrated by the supra-physiological urine output and excessive excretion of sodium was more evident in the 95-mmHg group. This group also

showed some changes in morphology with an increase in the incidence of condensed tubular nuclei. This may be representative of accelerated apoptotic changes.

The vWF data suggests that there was no difference in endothelial cell damage in any of the groups after perfusion. Haematological parameters were relatively stable in all groups. The declines in haemoglobin levels and red blood cell counts in the 75- and 55-mmHg groups were probably reflective of haemodilution throughout perfusion rather than a loss of red cells. The relative lack of damage to these kidneys may be attributed to a combination of features including the use of modern cardio-pulmonary bypass technology, careful use of physiological perfusate and protective effects of the parental nutrition to maintain endothelial cell function.

Successful *ex vivo* warm perfusion of canine kidneys for 18 h has been described using an acellular perfusate supplemented with bovine haemoglobin at a pressure of 50/30 mmHg[206]. A previous system reported by our group also successfully maintained kidneys for a period of 14 h at mean pressures of 50 mmHg with a blood perfusate. However, in this model conditions were continually adjusted during perfusion to meet physiological parameters[204]. The tested pressures in the present study ranged from less than normal physiological level, to a level just within the autoregulation range and to a normal physiological level.

The warm and cold ischaemic insults used in these experiments were relatively limited in comparison to those seen in clinical renal transplantation. Future studies will need to address the effects of more prolonged ischaemic injury in the context of near physiological perfusion pressures. Whilst the perfusion period was limited to 6 h the results from the 95- and 75-mmHg group suggest that porcine kidneys might be capable of functioning for much more extended periods. This would open up the possibility of developing the technique as a method of normothermic transplant organ preservation and resuscitation. The addition of higher levels

of creatinine or infusion of inulin into the perfusate may facilitate a more accurate measurement of creatinine clearance throughout the perfusion period, which would be relevant for viability assessment.

In conclusion, better renal function was established in this *ex-vivo* normothermic haemoperfused isolated kidney preservation system by increasing the arterial perfusion pressure. Mean arterial pressures of both 95 and 75 mmHg were capable of sustaining renal function over 6 h. However, the 95-mmHg group demonstrated overall superior renal function. The *ex-vivo* model described has potential as a viability assessment tool device and in the investigation of renal ischaemia-reperfusion injury syndrome.

CONCLUSIONS

Conclusions

The IOPS represents a novel, highly versatile and reliable model of early renal allograft reperfusion. The system is capable of extended physiological perfusion and accurate and detailed evaluation of renal function and injury not previously observed in isolated kidney perfusion models. The haemoperfused organs function in a manner closely comparable to the situation in the early post-transplant period and respond appropriately to conditions influencing IRI. The IOPS offers distinct advantages over other models of IRI, particularly *in vivo* transplantation models. The ability of the IOPS to effectively reproduce conditions akin to *in vivo* transplantation may allow for minimisation or even replacement of live animal experimentation in this context and could have significant benefits in terms of animal welfare and research costs. Furthermore, the system has the major advantage of allowing for continuous physiological monitoring and direct access to serial blood, urine and tissue sampling. The controlled nature of the ex-vivo system allows for precise manipulation of chosen variables under investigation. In this context it is possible to accurately investigate the effects of key influential factors on graft injury and function. In particular, the ability to precisely control warm and cold ischaemic time generates clinically applicable modelling of NHBD kidney transplantation. This crucial element in the IOPS model has not been previously applied effectively in large animal haemoperfusion systems where the focus has generally been limited to organ preservation.

Factors influencing renal allograft IRI are numerous but leucocytic activity and WIT must be considered key elements. A role for leucocytes in IRI is well accepted but the extent of their influence on renal allograft injury and function remains contentious. This study lends

support to an early and significant role of white cells affecting the severity of reperfusion injury and subsequent compromise in early graft function. On this basis, leucocyte-free normothermic perfusion prior to implantation may offer significant benefit in reducing early graft injury. Furthermore, the general consistency of the pathophysiological response of the organs to different perfusion conditions (ie. leucocyte depletion) indicates that the IOPS represents a sensitive tool for investigation of many aspects of renal allograft IRI.

Warm ischaemic time is likely to be the crucial variable influencing outcome from NHBD organs. However the relationship between WIT and subsequent graft function is poorly understood and is difficult to investigate meaningfully in the clinical setting. The IOPS, using porcine kidneys shown to be closely matched to human organs in anatomy and physiology, provides a model to investigate this important question. The results of this study suggest that increasing WIT is generally mirrored by micro- and macroscopic evidence of IRI and this in turn significantly influences function. However cellular and physiological evidence lends support to acceptable graft function being maintained up to a threshold of warm ischemic time, although the precise period of this remains unclear. Ongoing investigation incorporating longer and more clinically applicable cold ischaemic times in addition to the WIT are required to elucidate this question further.

Products arising from oxidative tissue injury represent potentially valuable markers of such injury. The IOPS model demonstrated that markers of lipid and protein IRI measured in plasma correlated well with the severity of early graft dysfunction and the extent of ischaemic insult sustained. In contrast, elevated caspase 3 activity was associated with better organ function, possibly indicating a predominance of apoptosis over necrosis in less severe IRI. In clinical transplantation, these markers could potentially be used for assessing organ viability

during organ perfusion preservation and also for monitoring graft injury following transplantation.

The optimal conditions for isolated normothermic kidney perfusion and preservation remain unknown and a crucial component of such conditions is perfusion pressure. Pressures used in previous experimental and clinical perfusion systems have tended to be significantly sub-physiological due to concern over endothelial injury and haemolysis. In this study higher physiological pressures were associated with better overall renal function without evidence of increased endovascular damage. Importantly, the use of physiological perfusion pressure allows for longer non-injurious perfusion periods required for clinically applicable organ preservation.

The kidney perfusion system described here has some important further potential clinical applications. First, it will allow the evaluation of new organ preservation strategies and interventions to reduce the adverse effects of ischaemia-reperfusion injury. Auto-transplant models have traditionally been used for these purposes but are prohibitively expensive if a clinically relevant large animal model is used. The IOPS should allow considerable reduction in the need for such experiments and may prove to be a useful screening model for translational research. Secondly, the IOPS may allow the development of a pre-transplant viability test for kidneys from NHBD and other marginal donors. Normothermic haemoperfusion of organs may also be used to minimize graft ischaemia and resuscitate organs in an environment in which factors influencing IRI can be manipulated in order to improve outcome following transplantation.

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